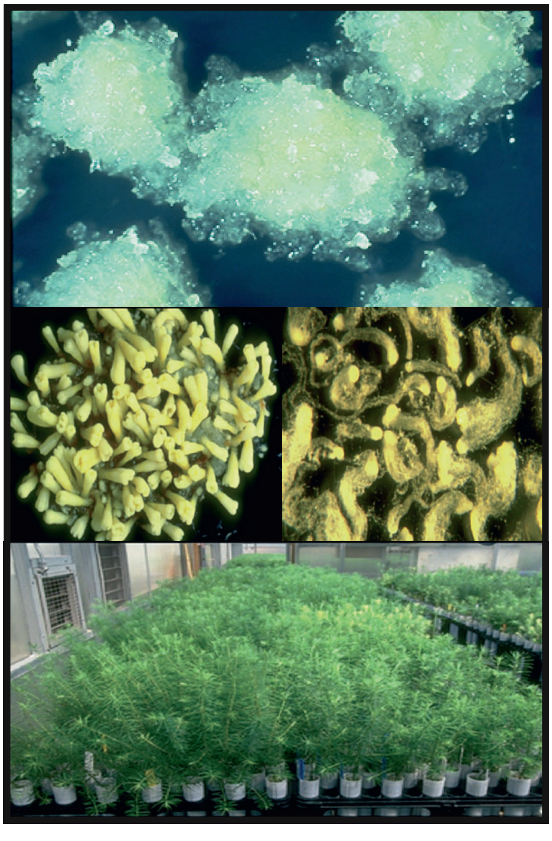
FORESTRY SCIENCES

Protocol for Somatic Embryogenesis in Woody Plants

S. Mohan Jain and Pramod K. Gupta
editors



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PROTOCOL FOR SOMATIC EMBRYOGENESIS IN WOODY PLANTS

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edited by

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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.)

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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.

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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.

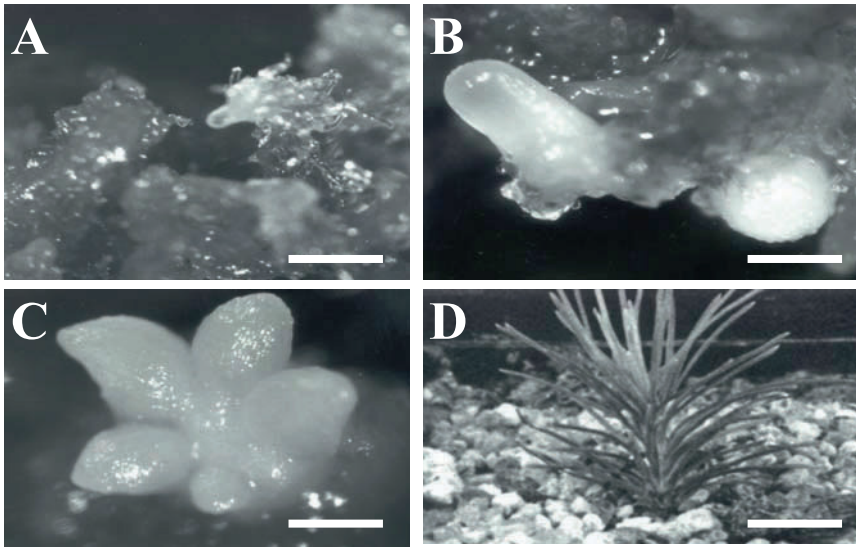

Figure 1 Plant regeneration via somatic embryogenesis in slash pine.
(A) Embryogenic callus obtained from zygotic embryo (bar = 0.1 cm); (B) Globular somatic embryo (bar = 0.5 cm); (C) Cotyledonary somatic embryos (bar = 0.5 cm); (D) Regenerated plantlet established in soil (bar = 0.8 cm)

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).

REFERENCES

Attree, S.M.; Budimir, S.; Fowke, L.C. Somatic embryogenesis and plantlet regeneration from cultured shoots and cotyledons of seedlings germinated from stored seeds of black and white spruce (*Picea mariana* and *Picea glauca*). Can. J. Bot. 68:30-34;1990.

Attree, S.M.; Fowke, L.C. Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tiss. Org. Cult. 35: 1-35; 1993.

Becwar, M.R.; Pullman, G.S. Somatic embryogenesis in loblolly pine (*Pinus taeda* L). In: Jain, S.M.; Gupta, P.K.; Newton, R.J. (eds), Somatic embryogenesis in woody plants, vol.3, Kluwer Academic Publishers, the Netherlands, pp 287-301; 1995.

Chalupa, V. Somatic embryogenesis and plantlet regeneration from immature and mature embryos of *Picea abies* (L.) Karst. Comm. Inst. For. 14:57-63; 1985.

Gupta, P.K.; Durzan, D.J. Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Technology 4:643-645; 1986.

Gupta, P.K.; Durzan, D.J. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/Technology 5:147-151; 1987.

Gupta, P.K.; Dandekar, A.M.; Durzan, D.J. Somatic proembryo formation and transient expression of a luciferase gene in Douglas fir and loblolly pine protoplasts. Plant Sci. 58: 85-92; 1988.

Gupta, P.K.; Durzan, D.J. Loblolly pine (*Pinus taeda* L.). In: Bajaj, Y.P.S. (ed), Biotechnology in agriculture and forestry, vol 16, Trees III, Springer-Verlag, Berlin, pp 383-407; 1991.

Gupta, P.K.; Pullman, G.; Timmis, R.; Kreitinger, M.; Carlson, W.C.; Grob. J.; Welty, E. Forestry in the 21st Century: The biotechnology of somatic embryogenesis. Bio/Technology 11:454-459; 1993.

Hakman, I.; von Arnold, S. Plant regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). J. Plant Physiol. 121:49-158; 1985.

Hakman, I.; Fowke, L.C. Somatic embryogenesis in *Picea glauca* (white spruce) and *Picea mariana* (black spruce). Can. J. Bot. 65: 656-659; 1987.

Handley, L.W. Method for regeneration of coniferous plants by somatic embryogenesis. U.S. Patent No. 5,731,203. Issued March 24, 1998.

Jain, S.M., Dong, N., Newton, R.J. Somatic embryogenesis in slash pine (*Pinus elliottii*) from immature embryos cultured *in vitro*. Plant Sci. 65: 233-241, 1989

Jain, S.M,.Gupta, P.K., Newton, R.J. Somatic embryogenesis in woody plants, vol 3, Gymnosperms, Kluwer Academic Publishers, 1995.

Nagmani, R.; Bonga, J.M. Embryogenesis in subcultured callus of *Larix decidua*. Can. J. For. Res. 15:1088-1091; 1985.

Newton, R.J., Marek-Swize, K.A., Magallanes-Cedeno, M.E., Dong, N., Sen, S., Jain, S.M. Somatic embryogenesis in slash pine (*Pinus elliottii* Engelm). In Jain, S.M,.Gupta, P.K., Newton, R.J. Somatic embryogenesis in woody plants, vol 3, Gymnosperms, Kluwer Academic Publishers, 1995. pp183-195

Tang, W. Peroxidase activity of desiccation-tolerant loblolly pine somatic embryos. In Vitro Cell. Dev.-Plant 36: 488-49; 2000.

Tang, W., Ouyang, F., Guo, Z.C. Plantlet regeneration via somatic embryogenesis in slash pine. J. Plant Res. Environ. 6(2):8-11, 1997a

Tang, W., Ouyang, F., Guo, Z.C. Somatic embryogenesis and plantlet regeneration in callus cultures derived from zygotic embryos of slash pine. J. For. Res. 8(2):83-86, 1997b

Tautorus, T.E.; Fowke, L.C.; Funstan, D.I. Somatic embryogenesis in conifers. Can. J. Bot. 69:1873-1899; 1991.

von Arnold, S. Eriksson, T. Bud induction on isolated needles of Norway spruce (*Picea abies* L. Kast.) grown in vitro. Plant Sci. Lett. 15:363-372; 1979.

Wenck, A.R.; Quinn, M.; Whetten, R.W.; Pullman, G.; Sederoff, R. High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*) Plant Mol. Biol. 39: 407-416; 1999.

SOMATIC EMBRYOGENESIS AND GENETIC TRANSFORMATION IN *PINUS RADIATA*

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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

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 11–24.

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

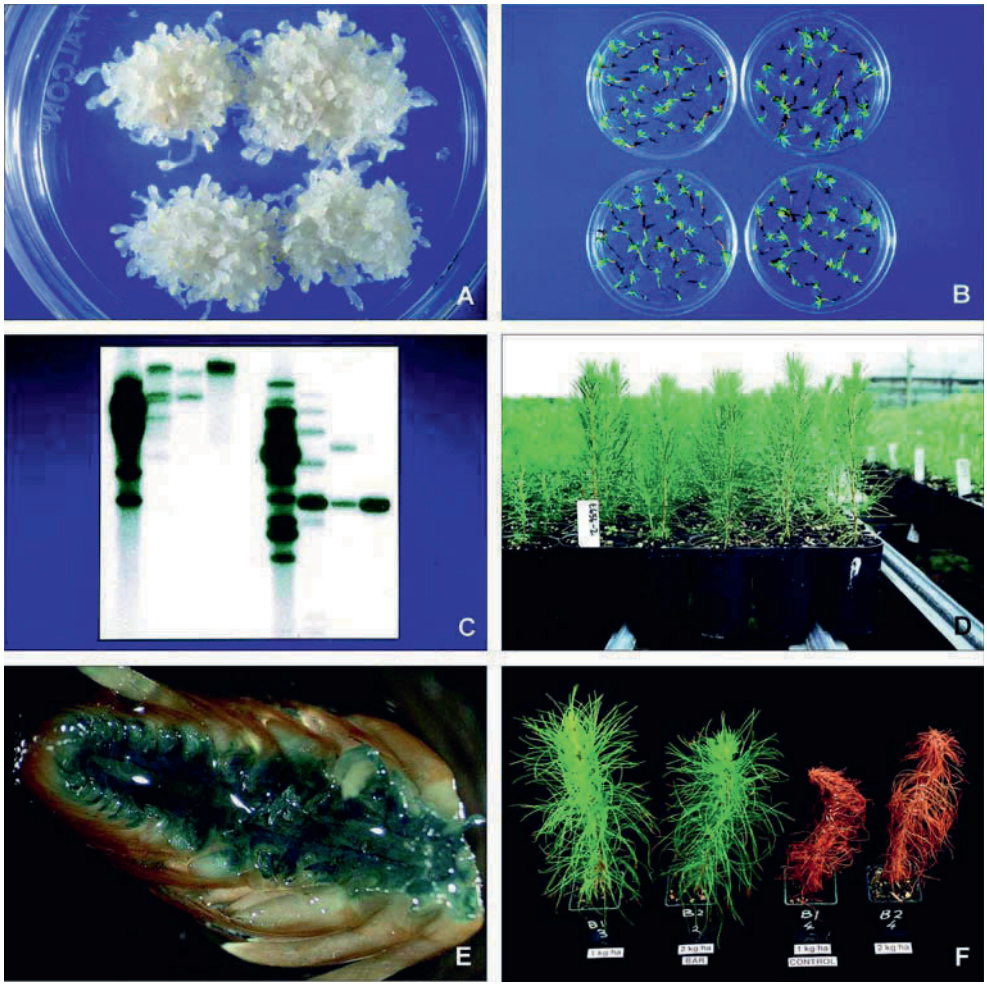

Figure 1: *Pinus radiata* embryogenesis and transformation. A: Embryogenic tissue grown on Maturation Medium; B: Embryos regenerating and germinating. C: Southern hybridisation analysis of transgenic *P radiata* trees; D: Trees grown from somatic seedlings of *P radiata*; E: Cross section through a vegetative 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

is very low. All reagents and equipment required to carry out this test, can be obtained from Agdia (Agdia Patho-Screen test, http://www.agdia.com/).

A similar ELISA technique (also available from Agdia) can be used to confirm the expression of the *Bacillus thuringiensis* toxin gene (*cry*1Ac) in *P. radiata* embryogenic tissue. The protocol is as described in the manufacturers instructions.

3.5.1. PCR analysis

PCR is the fastest way to confirm or otherwise, the transgenic nature of a new line selected on antibiotic media, even when very small amounts of tissue are available for testing. DNA is isolated from embryogenic tissue or needles as described above. Alternatively, for many PCR applications, a DNA FastPrep protocol is sufficient to yield enough DNA of sufficient quality for the reaction (http://www.qbiogene.com/index.shtml).

PCR reactions should be carried out in 20µL 1 x Taq PCR buffer, 1U Taq Polymerase (Boehringer Mannheim, Germany), 250µMol dNTPs, 80 pMol primer, 1.5mM $MgCl_2$, and 100ng of genomic DNA. A standard reaction consists of 35 cycles with 25 sec denaturation at 94˚C, 30 sec annealing at the appropriate temperature (i.e. 60˚C for *npt*II primers;) and 30sec elongation at 72˚C. The last cycle should be terminated with a final 3min extension step at 72˚C. The primers that we use routinely in our laboratory for *npt*II are: 5'AATCTCGTGATGGCAGGTTG3' and 5'GAGGCTATTCGGCTATGACT3'. For amplification of the *uid*A gene, we routinely use the following primers: 5'CATTACGCTGCGATGGATCCC3' and 5'CTGTAGAAACCCCAACCCGTG3' PCR reaction results can be made visible using gel electrophoresis, under standard running conditions (Sambrook et al, 1989).

3.5.2. Histochemical GUS staining

Histochemical assays for *uid*A expression (Fig. 1e) can be performed by incubating tissue in 1mL of filter-sterilised GUS-staining solution (preparation see below) for 24-48h at 37˚C. Stained tissue can be analysed under any Stereomicroscope for presence of blue stain in embryogenic cells. The blue staining can also be observed with the naked eye, even in transiently transformed embryogenic tissue. Stably transformed tissue usually shows intensive blue staining, often with the exception of tissue and plants of *P. radiata*, which appears to inhibit histochemical GUS staining at other than early embryogenic stages. However, *uid*A expression can readily be observed in radiata pine embryogenic tissue and sometimes in mature tissue as well (Fig. 1e).

Standard GUS staining solution for conifer tissue contains: 10mM KHPO4, 10mM KH_2PO4, 4mM potassium ferrocyanide, 4mM potassium ferricyanide, 1mM Xgluc (sodium salt), 0.1% Triton X-100. Filter sterilise the solution and keep in the freezer in small aliquots.

3.5.3. Fluorometric uidA expression analysis:

For fluorometric analysis, total protein is extracted from tissue using a technique described by Gallagher (1991). A fluorometer (e.g.TK100, Hoefer, San Francisco) is used for quantitative measurements. For a simple and reliable protocol that works well with conifers see Jefferson (1987).

3.5.4. Northern hybridisation analysis

RNA isolation from conifer tissue requires extra precautions to avoid contamination with RNAses during the isolation process, which would lead to rapid degradation of RNA. All labware and chemicals that are used should be treated for removal of RNAse and clean gloves should be worn during the experiment. RNA can be isolated from embryogenic tissue using either the RNeasy RNA Plant Miniprep Kit (QIAGEN, http://www.qiagen.com/) or a method described by Chang *et al.* (1993). For Northern hybridisation, five micrograms of total RNA are denatured and separated by glyoxal gel electrophoresis and transferred to a Hybond N^+ membrane (Amersham Life Sciences, U.K) by capillary blotting (Sambrook *et al*. 1989). DNA probes can be random-prime labeled (Rediprime DNA labeling system; Amersham Life Sciences, U.K) and hybridised (12 to 16h at 65˚C) to membranes in 1% (w/v) SDS, 1M NaCl, 10% (w/v) dextran sulfate, 2x Denhardts solution (Sigma, St. Louis, U.S.A) and 0.5mg sonicated salmon sperm DNA (Pharmacia Biotech, Upsala, Sweden). The membranes are subsequently washed twice in 2x SSC at room temperature for 5min and twice in 0.1x SSC with 1% (w/v) SDS at 65˚C for 30min. After exposure to X-ray film (Kodak X-Omat), the hybridised membranes can be stripped with a boiling solution of 0.5% (w/v) SDS and re-probed with a ^{32}P-labelled 28S rRNA probe that was previously PCR-amplified from 100ng *P. radiata* genomic DNA. Unequal loading of RNA can be corrected by normalising to the amount of 28S rRNA.

4. CONCLUDING COMMENTS

For somatic embryogenesis to be a realistic choice for future breeding and plant production, it is important that the method does not introduce strong and unwanted selection on the breeding population. For this reason the method must be applicable to most genotypes. Our research aims at improving the steps of initiation and

maturation, because these steps at present impose the strongest genetic selection on the system.

The genetic transformation of conifers is now a routine technology in many laboratories. However, efficiencies are still not comparable to those observed in many agricultural plants and even more so when model plants such as tobacco or *Arabidopsis* are considered. Those who wish to establish conifer transformation will be required to adjust protocols to their conditions and may experience significant difficulties and often failure to produce any transgenic lines. In ideal conditions, and using the Biolistic technique described in this chapter, one should achieve a transformation efficiency of around 10% (transgenic lines per number of plates bombarded).

5. REFERENCES

Bishop-Hurley, S.L., Zabkievicz, R.J., Grace, L., Gardner, R.C., Wagner, A. and Walter, C. (2001). Conifer genetic engineering: transgenic *Pinus radiata* (D Don) and *Picea abies* (Karst) plants are resistant to the herbicide Buster. Plant Cell Reports 20: 235-243.

Battraw, M. and Hall, T. C. 1992. Expression of a chimaeric neomycin phosphotransferase II gene in first and second generation transgenic rice plants. Plant Science 86: 191-202.

Chang S, Puryear T, Caimey I (1993). A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Rep 11(2): 113-116.

Charest, P.J., Calero, N., Lachance, D., Datla, R.S.S., Duchesne, L.C., and Tsang, E.W.T. (1993) Microprojectile-DNA delivery in conifer species: factors affecting assessment of transient gene expression using the ß-glucuronidase reporter gene. Plant Cell Rep 12: 189-193.

Charity, J.A.; Holland, L.; Grace, L.J. and Walter, C. (in press). Consistent and stable expression of the *npt*II, *uid*A and *bar* genes in transgenic *Pinus radiata* after *Agrobacterium tumefaciens*-mediated transformation using nurse cultures. Plant Cell Reports.

Clapham, D., Demel, P., Elfstrand, M., Koop, H-U., Sabala, I., and van Arnold, S. (2000) Gene transfer by particle bombardment of embryogenic cultures of *Picea abies* and the production of transgenic plantlets. Scand J Forest Res 15: 151-160.

D'Halluin, K., Bonne, E., Bossut, M., De Beuckeleer, M., and Leemans, J. (1992) Transgenic maize plants by tissue electroporation. Plant Cell 4: 1495-1505.

Doyle, J. (1990). Isolation of plant DNA from fresh tissue. Focus 12: 13-15.

Ellis, D.D., McCabe, D.E., McInnis, S., Ramachandran, R., Russell, D.R., Wallace, K.M. Martinell, B.J., Roberts, D.R., Raffa, K.F., and McCown, B.H. (1993) Stable transformation of *Picea glauca* by particle acceleration. Nat Biotechnol 11: 84-89.

Fladung, M. (1999) Gene stability in transgenic aspen (Populus). I. Flanking DNA sequences and T-DNA structure. Mol Gen Genet 260: 574-581.

Gallagher, S.R. (1991). Quantitation of *uid*A activity by fluorometry. In: *uid*A protocols: using the *uid*A gene as a reporter for gene expression. Academic Press, New York.

Hargreaves, C. and Smith, D. (1994a). The effects of short-term and long-term cryopreservation on embryo maturation potential of *Pinus radiata* tissue. Cryobiology 31, 577.

Hargreaves, C. and Smith, D. (1994b). Techniques used for cryopreservation of *Pinus radiata* embryogenic tissue. Cryobiology 31, 578.

Huang, Y., Diner, A.M., and Karnosky, D.F. (1991) *Agrobacterium rhizogenes* mediated genetic transformation and regeneration of a conifer: *Larix decidua*. In Vitro Cell Dev Biol 27: 201-207.

Iida, A., Yamashita, T., Yamada, Y., and Morikawa, H. (1991) Efficiency of particle-bombardment-mediated transformation is influenced by cell stage in synchronised cultured cells of tobacco. Plant Physiology 97: 1585-1587.

Jefferson, R.A. (1987). Assaying chimaeric genes in plants: the *uid*A gene fusion system. Plant Molecular Biology Reporter 5:387-405.

Klein, T.M., Wolf. E,D,. Wu, R., and Sanford, J.C. (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. Nature 327: 70-73.

Klimaszewska, K., Lachance, D., Pelletier, G., Lelu, A.M., and Seguin, A. (2001) Regeneration of transgenic *Picea glauca*, *P. mariana* and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. In Vitro Cell Dev Biol 37:748-755.

Krogstrup, P., Nielsen E.N., Møller J.D. and Roulund H. (1988) Somatic embryogenesis in sitka spruce (*Picea sitchensis* (Bong.) Carr.), Plant Cell Reports 7: 594-597.

Kumar, S and Fladung, M. (2001). Gene stability in transgenic aspen (Populus). II. Molecular characterisation of variable expression of transgene in wild and hybrid aspen. Planta 213 (5), 731-740.

Li, L.; Zhou, Y.; Cheng, X.; Sun, J.; Marita, J.M.; Ralph, J.; and Chiang, V.L. (2003). Combinatorial modification of multiple lignin traits in trees through multigene co-transformation. PNAS 100 (18), 4939-4944.

Menzies, M.I., and Aimers-Halliday, J. (in press). Propagation options for clonal forestry with conifers. In "Plantation Forest Biotechnology for the 21st Century (Ed. C. Walter and M.J. Carson). Research Signpost, Trivandrum, Kerala, India.

Sanford, J.C., Smith, F.D., Russell, J.A. (1993). Optimising the biolistic process for different biological applications. Methods in Enzymology 217: 483-509.

Sangwan, R.S., Bourgeois, Y., Brown, S., Vasseur, G., and Sangwan-Norrell, B. (1992) Characterisation of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana.* Planta 188: 439-456.

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. 2nd Edition. Cold Spring Harbor, N. Y: Cold Spring Harbor Laboratory Press.

Smith D. (1996). Growth medium US Patent number: 5,565,355

Tang, W. and Tian, Y (2003). Transgenic loblolly pine (*Pinus taeda* L.) plants expressing a modified delta-endotoxin gene from *Bacillus thuringiensis* with enhanced resistance to *Dendrolimus punctatus* Walker and *Crypyothelea formosicola* Staud. Journal of Experimental Botany 54 (383), 835-844.

Wagner, A., Moody, J., Grace, L.J., and Walter, C. (1997) Stable transformation of *Pinus radiata* based on selection with Hygromycin B. New Zealand J Forestry Sci 27: 280-288.

Walter, C., Smith, D.R., Connett, M.B., Grace, L.J., White, D.W.R. (1994). A biolistic approach for the transfer and expression of a *uid*A reporter gene in embryogenic cultures of *Pinus radiata*. Plant Cell Reports 14: 69-74.

Walter, C., Grace, L.J., Wagner, A., Walden, A.R., White, D.W.R., Donaldson, S.S., Hinton, H.H., Gardner, R.C., and Smith, D.R. (1998) Stable transformation and regeneration of transgenic plants of *Pinus radiata* D. Don. *Plant Cell Rep* 17: 460-468.

Walter, C., Grace, L.J., Donaldson, S.S., Moody, J., Gemmell, J.E., van der Maas, S., Kwaalen, H., and Loenneborg, A., (1999). An efficient Biolistic transformation protocol for *Picea abies* (L) Karst embryogenic tissue and regeneration of transgenic plants. *Can J Forest Res* 29: 1539-1546.

Walter, C., Charity, J., Grace, L., Hoefig, K., Moeller, R. and Wagner, A. (2002). Gene technologies in *Pinus radiata* and *Picea abies*: tools for conifer biotechnology in the 21st century. Plant Cell, Tissue and Organ Culture 70: 3-12.

Waterhouse, P.M.; Helliwell, C.A. (2003). Exploring plant genomes by RNA-induced gene silencing. Nat Rev Genet 4(1):29-38.

White, T.L. and Carson, M.J. (in press). Breeding programs of conifers. In "Plantation Forest Biotechnology for the 21st Century (Ed. C. Walter and M.J. Carson). Research Signpost, Trivandrum, Kerala, India

DOUGLAS - FIR (*PSEUDOTSUGA MENZIESII*)

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1. INTRODUCTION

Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco), one of the most important timber species in the world, is a member of the family Pinaceae. It is the principal timber species in the Pacific Northwest of America, where it reaches up to 126 m in height and 5 m in diameter (Hermann and Lavender 1990) and a standing volume up to 5,460-m^3/ h in the old growth stands. The forest industry is an important contributor to the base economy and employment in western North America, especially in the western parts of Oregon, Washington, and British Columbia, where Douglas-fir is the predominant species. It is also planted in New Zealand, Central and Western Europe (including the British Isles), Argentina and Chile.

The strong, straight-grained Douglas-fir wood is ideal for structural uses. It is utilized for construction lumber, plywood, particleboard, pulp, poles, pilings etc. Its wood is also used in products such as laminated construction beams, flooring, and decorative beams. Douglas-fir is also grown as a Christmas tree on rotation ranging from 4-7 years.

Douglas-fir grows under a wide variety of climatic conditions. The coastal region of the Pacific Northwest has a maritime climate characterized by mild, wet winters and cool, relatively dry summers, a long frost free season, and narrow diurnal fluctuations of temperature. Precipitation, mostly as rain, is concentrated in the winter months. Climate in the Cascade and Sierra Nevada ranges tends to be more severe. The species reaches its best growth on well-aerated, deep soil with a pH range from 5-6. It will not thrive on poorly drained or compacted soils (Hermann and Lavender 1990).

Douglas-fir is monoecious; trees commonly begin to produce cones at 10 to 15 years of age, although observations of younger seedlings bearing cones have been reported. Cone ripening varies from late July to mid-August at the lower elevations (about 850 m) and occurs as late as mid- September at higher

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elevations (about 1700 m). Seed production is erratic because of environmental factors. Abundant seed crops have occurred in a 2- to 11-year cycle. Variable seed production complicates operational reforestation and tree improvement efforts.

Douglas-fir is subject to serious damage from a variety of agents, the most damaging and widespread is *Phellinus pini*, which results in redring rot. The most damaging stem disease of Douglas-fir is caused by *Arceuthobium douglasii*. This dwarf mistletoe occurs throughout most of the range of Douglas-fir. Over 60 species of insects are indigenous to Douglas-fir cones, but only a few species damage a significant proportion of the seed crop. The most destructive insects include: a) the Douglas-fir seed chalcid (*Megastigmus spermotrophus*), which matures in developing seed without any external sign of its presence; b) the Douglas-fir cone moth (*Barbara colfaxiana*) and fir cone worm (*Dioryctria abietivorella*); c) the Douglas-fir cone gall midge (*Contariana oregonensis*), which destroys some seed but prevents harvest of many more by causing a gall that makes normal opening of cones impossible. The Douglas-fir beetle (*Dendroctonus pseudotsugae*) is a destructive insect pest in old growth stands of coastal and interior Douglas-fir.

Douglas-fir propagates naturally from seed. Genetic improvement and propagation using seeds are slowed by its long life cycle. Asexual methods of propagation of proven superior genotypes have not been very successful due to poor rooting and plagiotropic growth of cuttings. Success has been achieved with asexual propagation of juvenile material by cuttings from 1-2 year old seedlings (Ritchie 1991) and also by organogenesis in-vitro (Goldfarb and Zaerr 1989). This method has not been commercialized due to high costs and poor performance in the field. Success has also been achieved with somatic embryogenesis in Douglas-fir (Durzan and Gupta 1987, Gupta and Pullman 1990, 1991, Gupta et al. 1995). In this chapter, we will describe the protocol of plantlet regeneration via somatic embryogenesis from immature seeds.

2. MATERIALS

1. H_2O_2, Liquinox and Tween-20 detergents, sterile water
2. Flow hood, petri plates, pipettes, forceps, and scalpel
3. Immature female cones
4. Dissecting microscope
5. Media (see Tables 1 and 2)

TABLE 1: DOUGLAS-FIR BASIC CULTURE MEDIA (BM)

Constituent	mg/L	Constituent	mg/L
Basal Salts		$CoCl_2.6H_2O$	0.025
KNO_3	varies	KI	1.00
$CaCl_2.6H_2O$	200.0	$AlCl_3$	0.02
$Ca(No_3)_2.2H_2O$	varies		
KH_2PO_4	340.0	Organic Additives	
$MgSO_4.7H_2O$	400.0	Myo-Inositol	varies
$MnSO_4.H_2O$	20.8	Thiamine.HCl	1.00
$ZnSO_4.7H_2O$	8.0	Nicotinic acid	0.50
$CuSO_4.5H_2O$	0.024	Pyridoxine.HCl	0.50
$FeSO_4.7H_2O$	27.85	Glycine	2.00
Na_2EDTA	37.25	L-Glutamine	varies
H_3BO_3	5.0	Casamino acids	500.0
$NaMoO_4.2H_2O$	0.20	Sucrose	varies
		pH	5.7

TABLE 2: FORMULATIONS OF DOUGLAS-FIR MEDIA

	BM-1	BM-2	BM-3	BM-4	BM-5
	Stage I Initiation	Stage II Maintenance	Stage III Singulation	Stage IV Development	Stage V Germination
KNO_3	1250(1)	1250	1050	2500	1170
$Ca(NO_3)_2..2H_2O$	—	—	200	—	220
Myo-Inositol	100	5000	100	100	100
L-Glutamine	450	1000	1000	750	—
Amino acid mixture(2)	—	—	—	290	—
Sucrose	15,000	30,000	20,000	60,000	20,000
PEG 8000	—	—	—	190,000	—
2.4-D	110	1.1	—	—	—
N6-Benzyladenine	45	0.22	—	—	—
Kinetin	43	0.22	—	—	—
Abscisic acid	—	—	5/5	10	—
Activated charcoal	2500	—	—	1000	2500
Agar	—	5000	—	—	8000(4)
Gelrite	1800	—	—	—	—

(1)All units are in mg/L (or ppm)
(2)L-Proline – 100, L-Asparagine – 100, L-Arginine – 50, L-Alanine – 20, L-Serine – 20
(3)Not used for liquid media
(4)Tissue culture agar
The pH of all media are adjusted to 5.7

Basic medium composition is listed in Table 1. Required modifications for each culture stage are listed in Table 2. Mix together all ingredients, with the exception of ABA, and bring the media to volume prior to autoclaving (15 minutes at 121^{o}C, 15psi). Filter ABA to sterilize and add to sterile media aseptically. Add gelrite to make solid BM-1 plates. Pour 10 ml/plate in 60x15mm plates, or 20ml/plate in 100x25mm plates.

3. METHOD

The regeneration method can be divided into four main steps: initiation of embryogenic cultures from explants, multiplication of embryogenic cultures, maturation of somatic embryos, and regeneration of plants from the somatic embryos.

Initiation of Embryogenic Cultures

Use immature embryos at pre-cotyledonary stage for initiation of embryogenic cultures. Collect female cones in the first week of July (about 4-6 weeks after fertilization) until the first appearance of cotyledon primordia (middle of July). The optimal embryo stage for initiation is when the apical dome begins to develop.

1. Remove the seeds *(Figure 1)* from the cones.
2. Treat the seeds with 10% Liquinox plus 2 droppers of Tween-20 detergent and agitate for 5 min.
3. Wash the seeds 3 – 4 times with distilled water.
4. Sterilize the seeds with 20% (v/v) H_2O_2 for 15 min.
5. Wash the seeds 6 – 8 times with sterile water in the laminar-flow hood.
6. Transfer the sterile seeds in a petri plate.
7. Remove the seed coat with scalpel and forceps and excise the embryos so that they remain attached to the female gametophyte (see *Figure 2*). Work under a dissecting microscope.
8. Put the excised embryos and attached female gametophyte on to BM-1 induction medium. Make sure that embryos are touching the medium.
9. Wrap plates with a double layer of parafilm and incubate the cultures in the dark at 23 ^{o}C.

Figure 1. Douglas-fir seeds.

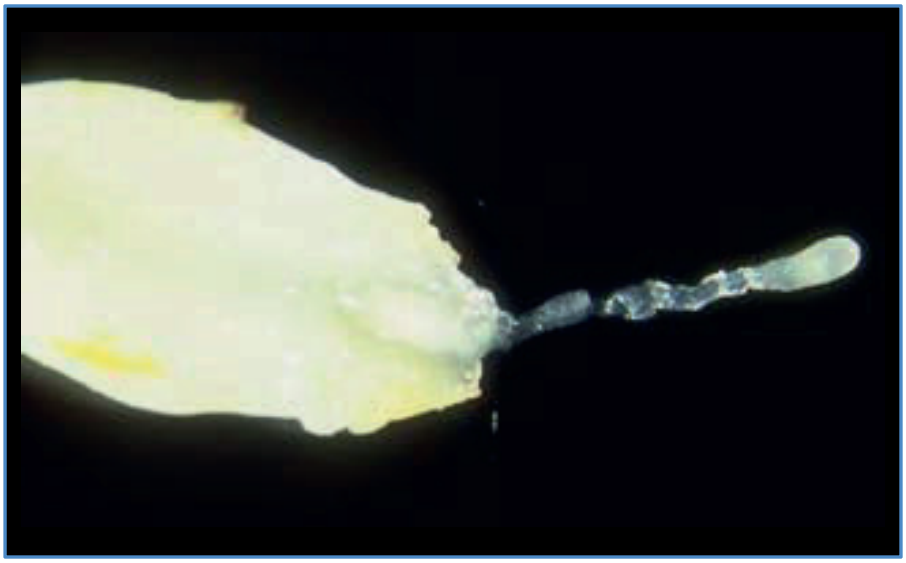

Figure 2. *Douglas-fir female gametophyte with excised attached embryo on BM-1.*

Maintenance of Embryonal Suspensor Mass

After 4 – 6 weeks, a mucilaginous, translucent-white mass develops (0.5 – 10 mm) around the heads of the immature embryos. This is called an embryonal suspensor mass (ESM). An embryonal suspensor mass consists of embryos at varying early stages of development, each containing an embryonal head and suspensor system. This can be confirmed by double staining of ESM as described in chapter # in this book. At this stage, separate the ESM from the original explants and transfer onto maintenance medium (BM-2). ESM cultures multiply by natural conifer-type cleavage polyembryony. Maintain ESM cultures by subculture every two weeks onto fresh medium *(Figure 3)* and incubate in the dark at 23 ^{o}C.

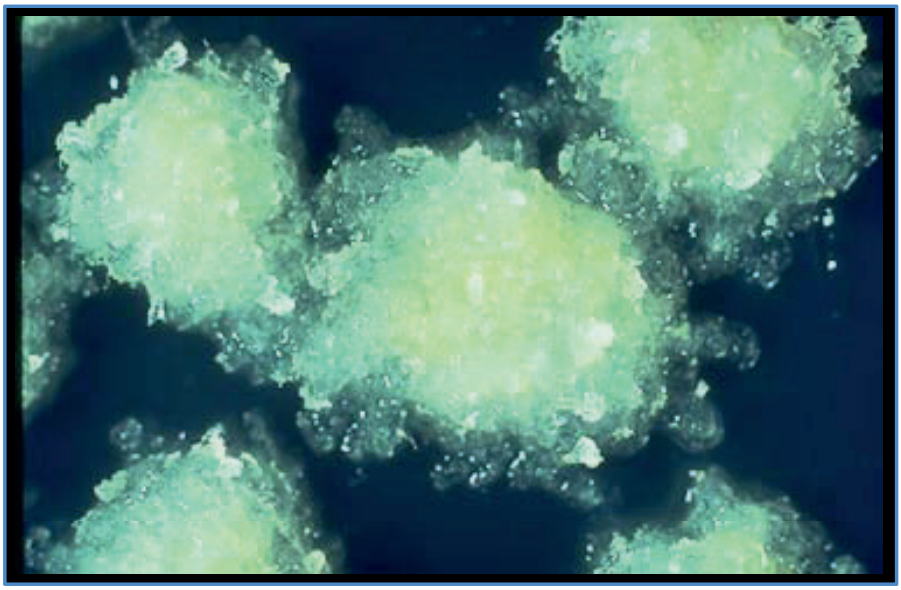

Figure 3. Douglas-fir ESM (Embryonal Suspensor Masses) multiplying on BM-2.

Establishing Suspension Cultures

1. Transfer 1-2 grams (fresh weight) ESM into a 250 ml Erlenmeyer flask containing 25 ml of BM-2 liquid medium.
2. Place the flask on a rotary shaker (90-110 rpm) in darkness at 23 ^{o}C. After one week add 20 ml of the same medium into the flask. After suspension culture establishment (3- 4 weeks), pour the ESM liquid culture into a 50-ml measuring cylinder and allow to settle for 30 minutes. Discard the supernatant (spent medium) and measure the settled ESM. Subculture the settled ESM into fresh liquid medium at a ratio of 1:9 (v/v). Transfer 5 ml

of settled ESM into a 250 ml flask containing 45 ml of fresh BM-2 liquid medium. Maintain ESM liquid cultures by regular weekly subculture.

Embryo Development

Abscisic acid (ABA) is important for cotyledonary embryo development. It inhibits the cleavage polyembryony and allows embryo singulation and further embryo development *(Figure 4)*.

1. Transfer ESM suspension cultures into BM-3 liquid medium containing 5.0 mg/L ABA. After one week, subculture again into same medium.
2. Get polyester padding materials from a fabric store (about 4.0-mm thickness) and cut into 40-mm x 40-mm squares.
3. Soak the polyester pads with development BM-4 liquid medium and place them into 120-mm petri dishes. Place a filter paper on the top of the pad.
4. After two weeks of ABA treatment, settle the ESM cultures in a measuring cylinder for 30 minutes. Discard the supernatant and transfer 1.0-ml settled ESM on the top of the filter paper placed on polyester pads soaked with medium for cotyledonary embryo development. Incubate the culture plates in dark at 23 ^{o}C.
5. After 7-8 weeks ESM cultures develop cotyledonary embryos *(Figure 5)*.

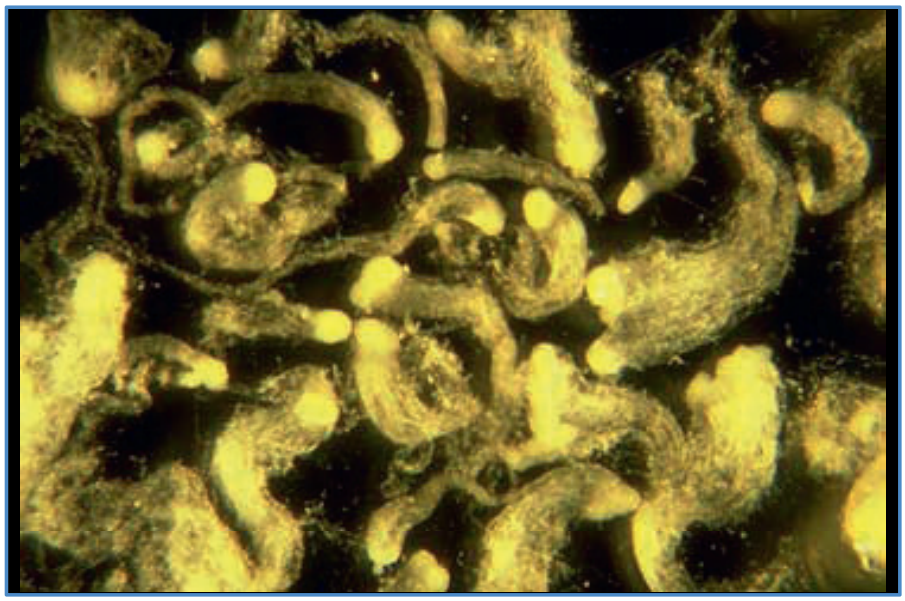

Figure 4. Singulation of Douglas-fir embryos on exposure to ABA in BM-3.

Figure 5. Douglas-fir cotyledonary embryos at the end of development on BM-4.

Germination and Conversion to Plantlets

1. Using a stereo microscope, select zygotic-like cotyledonary embryos from the development medium using forceps. Transfer the selected embryos to semi-solid BM-5 germination medium.
2. Incubate the cultures in dark for 5-7 days and then transfer to light (2000 Lux) with a 16-hour photoperiod.
3. After 7-9 weeks, embryos germinate and develop somatic seedlings *(Figure 6)*.
4. Transplant somatic seedlings with epicotyls into 20 cubic inch supercell pots containing a mixture of peat, vermiculite and perlite (2:1:1 ratio).
5. Grow the somatic seedlings in pots for 6 months in the greenhouse *(Figure 7)* and then transplant into the field *(Figure 8)*.

Cryostorage of Embryonal Suspensor Mass

Cryostore the ESM cultures of Douglas-fir as described elsewhere in this book.

Figure 6. Douglas-fir somatic seedlings after germination on BM-5.

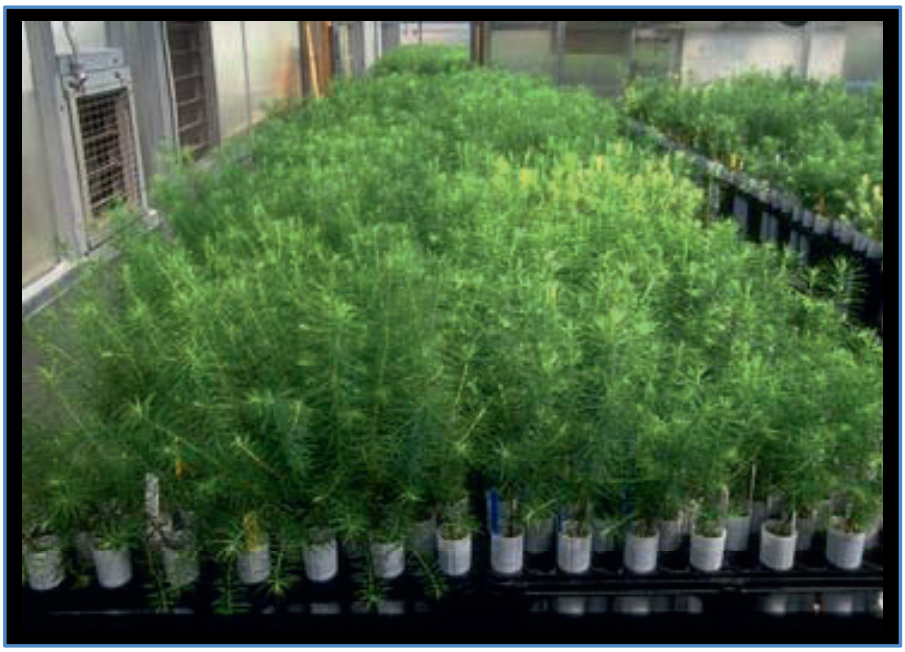

Figure 7. Douglas fir somatic seedlings in greenhouse culture.

Figure 8. Douglas fir clones in the field.

REFERENCES

1. Durzan, D.J. and P.K. Gupta (1987). Somatic embryogenesis and polyembryogenesis in Douglas-fir cell suspension cultures. Pl. Science. 52: 229 - 235
2. Galerne, Marc, Joelle Bercetche and Jean Dereuddre. (1992). Cryoconservation de cals embryogenes d'Epicea [*Picea abies* (L.) Karst.]: Effets de differents facteurs sur la reactivation des cals et la production d'embryons puis de plantules. Bull. Soc. bot. fr., 139 Lettres bot. (4/5), p. 331-344.
4. Goldfarb B. & J.B. Zaerr (1989) Douglas-fir. In Biotechnology in Agriculture and Forestry. Vol. 5, Bajaj Y.P.S. (ed.) Springer-Verlag Berlin Heidelberg. pp. 526 - 548.
5. Gupta P.K., A.M. Dandekar & D.J. Durzan (1988) Somatic proembryo formation and Transient Expression of a Luciferase gene in Douglas-fir and Loblolly pine Protoplast. Pl. Science. 58: 85 - 92.
6. Gupta, P.K. and G.S. Pullman (1990). Methods for reproducing coniferous plants by somatic embryogenesis. U.S. Patent No. 4,957,866.
7. Gupta, P.K. and G.S. Pullman (1991). Method for reproducing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. U.S. Patent No. 5,036,007.
8. Hermann R.K. & D. Lavender (1990) *Pseudotsuga menziesii* (Mirb.) Franco Douglas-fir. In Silvics of North America Vol. 1, Conifers. USDA Forest Service (Publ.) pp. 527-540.
10. Nagmani R., M.A. Johnson & R.J. Dinus (1991) Effect of explant and media on initiation, maintenance, and maturation of somatic embryos in *Pseudotsuga menziesii* (Douglas-fir). In Woody Plant Biotechnology. M.R. Ahuja (ed.), Plenum Press, New York. pp. 170 - 178.
11. Pullman, G.S. and P.K. Gupta (1991). Method for reproducing coniferous plants by somatic embryogenesis using absorbent materials in the development stage media. U.S. Patent No. 5,034,326.

OMORIKA SPRUCE (*Picea omorika*)

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1. INTRODUCTION

The omorika spruce (*Picea omorika* (Panč.) Purk.) is a Balkan endemic conifer species, taxonomically close to the common European spruce *Picea abies* (L.) Karst. Actual natural populations of *P. omorika* are restricted to rare sites along the middle course of the Drina River in South-West Serbia and South-East Bosnia, sites that represent refuges for the vegetation during the last glacial age. However, *P. omorika* is cultivated throughout Europe as a highly appreciated ornamental tree. In addition to its highly decorative appearance this species can also grow in a wide range of climatic and soil conditions as well as withstand the impact of atmospheric pollution.

The trees of this species can reach up to 50 m in height. *Picea omorika* propagates naturally from seeds. Cone ripening occurs from October to November, at altitudes around 1000 m. Seed germination is about 45 %. Vegetative propagation by rooting of the lowest branches has been observed in nature. Grafting is possible but infrequently used.

As the natural sources of this relict species are drastically reduced, a program for conservation and propagation of the remaining germplasm has become necessary. The *in vitro* regeneration capacity of *P. omorika* was, thus, investigated (Kolevska-Pletikapić and Buturović-Đerić 1995). Furthermore, somatic embryogenesis in *P. omorika* has been accomplished by using shoot explants (Budimir and Vujičić 1992) and, in our laboratory, in cultures of mature zygotic embryos (Kolevska-Pletikapić et al. 1995). This chapter describes the protocol of plantlets regeneration via somatic embryogenesis from mature seeds.

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2. EMBRYOGENIC CULTURE INITIATION

2.1. Explant-type and Sterilization

Materials and equipment required: sterile distilled water**,** 1% w/v commercial bleach, 6% w/v H_2O_2, stereo microscope, flow hood, sterilized filter paper, Petri dishes, forceps and scalpel, culture media (see Table 1).

Collect mature seeds at the end of the growing season and store them in the dark at 4-8 °C until use. In order to isolate mature zygotic embryos select healthy seeds 2.5-3.8 mm in size.

1. Wash the seeds under running tap water for 10 min.
2. Place the seeds in a 100 ml beaker, cover them with 50 ml 1% w/v commercial bleach containing 1-2 drops of Tween-20 detergent and agitate for 20 min.
3. Rinse the seeds four times with sterile distilled water in a flow hood.
4. Leave the seeds on moist filter paper in a covered Petri dish for two days, at 4°C. Wrap dishes with Parafilm.
5. Treat the seeds with 6% w/v H_2O_2 for 15 min.
6. Wash the seeds three times with sterile distilled water.
7. Isolate zygotic embryos under a stereomicroscope. Remove the seed coat with scalpel and forceps and isolate the embryo from the endosperm.
8. Place about 20 embryo-explants horizontally into M1 induction medium in a 100 mm Petri dish.
9. Wrap dishes with Parafilm and incubate the cultures in the dark at 25±1°C, for three weeks.

2.2. Culture Medium Composition

The composition of the media employed is shown in Table 1. Add all hormones except abscisic acid (ABA) before autoclaving. Adjust media to pH 6.0-6.2 before autoclaving and solidify media with 0.6% w/v tissue culture agar. Sterilize media by autoclaving for 20 min at 110 kPa, 121 °C. Sterilize the ABA solution by filter sterilization.

To increase the embryogenic suspensor mass (ESM) formation, L-glutamine (250 mg l^{-1}) and/or casein hydrolysate (500 mg l^{-1}) can be added into M1 induction medium.

Table 1: Composition of culture media for *Picea omorika*: modifications of the LP basal medium (von Arnold and Eriksson, 1981) minus the amino acids (Jain *et al.*, 1988). Concentrations in mg l^{-1}.

Constituent	Induction Medium **M1**	Maintaining Medium **M2**	Maturation Medium **M3**	Germination Medium **M4**
Inorganic macroelements				
KH_2PO_4	340	170	170	85
KNO_3	1900	800	800	475
NH_4NO_3	1200	600	600	300
$MgSO_4 \cdot 7H_2O$	370	185	185	92.5
$CaCl_2 \cdot H_2O$	180	90	90	45
Inorganic microelements				
$MnSO_4 \cdot H_2O$	1.69	1.69	1.69	0.42
H_3BO_4	0.63	0.63	0.63	0.15
$FeSO_4 \cdot 7H_2O$	14.00	14.00	14.00	3.5
Na_2EDTA	19.00	19.00	19.00	4.75
KI	0.75	0.75	0.75	0.18
$Na_2MoO_4 \cdot 2H_2$	0.025	0.025	0.025	$6.25 \cdot 10^{-3}$
O	0.0025	0.0025	0.0025	$6.25 \cdot 10^{-4}$
$CuSO_4 \cdot 5H_2O$	0.0025	0.0025	0.0025	$6.25 \cdot 10^{-4}$
$CoCl_2 \cdot 6H_2O$				
Vitamins				
Nicotinic acid	2.0	2.0	2.0	2.0
Pyridoxine-	1.0	1.0	1.0	1.0
HCl	5.0	5.0	5.0	5.0
Thiamine-HCl	100	100	100	100
myo-inositol				
Sucrose	10 000	10 000	30 000	10 000
Agar	6 000	6 000	6 000	8 000
Hormones (µM)				
2,4-D	10	10	-	-
BA	5	5	-	-
ABA	-	-	20-40	-

2.3. Initiation

Callus proliferation from mature zygotic embryos occurred within three weeks of culture on M1 induction medium, mostly in the hypocotyl area of embryo explants. The average frequency of embryogenic tissue formation in *P. omorika* is 38 %.

1. After three weeks of growth in the dark, expose cultures to light (a 16-hour photoperiod) for two weeks. Green, compact non-embryogenic and white, translucent embryogenic tissues will proliferate overgrowing the whole embryo explants (Fig. 1a).
2. Transfer cultures into fresh medium after 5 weeks; re-establish them in the dark and subculture in three-week intervals.

2.4. Culture Maintenance

Two-month-old embryogenic tissue shows morphology typical for the embryonal-suspensor mass (ESM) in *Picea* sp. The proembryogenic structure consists of intensely acetocarmine-stained small cells (Fig. 1b). The staining procedure is described in part 2.5.1.

1. Separate the white, translucent, embryogenic tissue from the green non-embryogenic callus.
2. Subculture the embryogenic tissues from individual seeds separately into M2 maintaining medium. Culture ESMs in pieces of at least 200 mg (70 mm in diameter).
3. Keep the stock culture lines in the dark at 25±1°C, with 3-week subcultures.

Determine the growth increase (g.i.) of the lines as the increase of fresh weight within two-wk intervals [g.i. = (final weight minus initial weight) divided by initial weight].

On the basis of their tissue color, morphogenic and histogenic characteristics, the two tissue types can be classified as white (**W**) and brown (**B**) lines. In the **W** lines clusters were mostly composed of 5 cells, rarely of 20 and more cells, while in the **B** lines clusters of more than 20 cells were most frequent. The **B** lines grow more slowly (g.i. 0.5) than the **W** lines (g.i. 0.8). Also, the two phenotypes of *P. omorika* embryogenic tissue have different capacities of maturation (Tramišak-Milaković et al. 1999) and may be compared with *Picea abies* embryogenic cell lines A and B (Jalonen and von Arnold 1991).

2.4.1. Staining To Confirm Embryogenic Nature

Material and equipment required: formalin, glacial acetic acid, EtOH, glass slides, light microscopy

1. Take a tissue specimen (50 mg) from proliferating ESM.
2. Fix tissues in formalin, glacial acetic acid and 70% ethyl alcohol (90:5:5, FAA) and store at 4°C until use.
3. Chop fresh or fixed tissue with a scalpel, place on a glass slide and stain with 2% acetocarmine as described by Gupta and Durzan (1987).
4. Observe and count proembryos (Stage 1, von Arnold and Hakman, 1988) using a light microscope.

Determine the embryogenic potential (e.p.) as the number of Stage 1 embryos per gram of fresh tissue.

2.5. Embryo Maturation

1. Pre-treat ESMs on hormone/free maintaining medium supplemented with 1% activated charcoal for one week (optionally). This step can benefit maturation by reducing the level of endogenous hormones.
2. Transfer the ESMs (about 500 mg) containing Stage 1 embryos into maturation medium (M3). Sucrose at concentration of 30 g l^{-1} may be replaced by glucose. Incubate the cultures for 4-6 weeks in the dark, at 25±1 °C (Tramišak-Milaković et al. 1999).

Determine the maturation capacity of the lines as the number of mature embryos per gram of tissue fresh weight.

The maximum production of Stage 3 (cotyledonary) embryos is usually achieved during the sixth week of culture, but varies depending on the cell line (Fig. 1c). The maturation capacity of the **B** lines is about two times higher than that of the **W** lines (97 and 27 cotyledonary embryos per gram of tissue, respectively) (Tramišak-Milaković et al. 1999).

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2.6. Embryo Development

During maturation, the somatic embryos of *P. omorika* accumulate starch, lipids and storage proteins (Fig. 1d) (Salopek et al. 1997). Plantlets from somatic embryos that contain higher levels of storage proteins are more

vigorous. Storage proteins have then been proposed as markers of embryo quality (Redenbaugh et al. 1986).

Material and equipment required: glutaraldehyde, cacodylate buffer, OsO_4, Epon resin (or a convenient embedding resin), uranyl acetate, citrate, microtome, electron microscope, toluidin blue, 2% sodium tetraborate solution, light microscope.

Electron microscopy

1. Fix tissues with 1% glutaraldehyde in cacodylate buffer pH 7.2.
2. Postfix with 1% OsO_4 in the same buffer.
3. Dehydration through a series of EtOH (20, 40, 60, 80, 100%, at least one hour in each concentration of EtOH).
4. Embed tissue samples in an epoxy resin (Epon).
5. Prepare thin sections (50-70 nm) using an ultra microtome.
6. Stain sections with uranyl acetate and lead citrate.
7. Examine sections under a transmission electron microscope.

Light microscopy

1. Prepare semi-thin sections using a microtome (0.9-1µM).
2. Stain sections with toluidine blue (2%, w/v in 2% sodium tetraborate solution).
3. Examine sections under a light microscope.

2.7. Embryo Germination

1. Collect cotyledonary somatic embryos (Stage 3) from embryogenic tissue grown on maturation medium (M3).
2. Leave embryos to germinate on M4 medium without growth regulators. Incubate embryos in the light at 25°C. In well developed somatic seedlings, 6 to 8 cotyledons can be observed (Fig. 1e).

3. APPLICATIONS

3.1. Genetic Stability of Long-Term Embryogenic Tissue Cultures

Under tissue culture conditions, somaclonal variations could appear due to the growth regulators used during subculture and preservation of long-term-cultured embryogenic tissue. Long-term embryogenic cultures should

be analyzed for chromosomal variations to monitor genetic stability of plants regenerated from the embryogenic culture.

P. omorika has a diploid chromosome complement, 2n=24, consisting of ten metacentric and two submetacentric pairs (Šiljak-Yakovlev et al. 2002). Comparative analysis of proembryogenic structures and somatic embryo cells from 2-year-old embryogenic tissue, line BB19, showed neither mitotic disturbances nor changes in chromosome number, morphology and heterochromatin distribution (Besendorfer et al. 1999). High stability of the chromosomal number was also noticed in the 6-year-old embryogenic line BB19.

For karyotype analysis we use differential staining with CMA_3 and DAPI (Hizume et al. 1989).

Material and equipment required: 0.05% colchicine, EtOH, acetic acid, pectinase, cellulase, 0.1M citrate buffer, 45% acetic acid, dry ice ($C0_2$), Chromomycin A_3 and DAPI.

1. Pre-treat proembryonal cells and somatic embryos with 0.05% colchicine for 20 hours at room temperature.
2. Fix tissues in formaldehyde:ethanol:acetic acid solution (3:1 w/v, FAA) for at least one hour. Store fixed samples in EtOH at -20°C until use.
3. Macerate tissue sample enzymatically in a mixture of 20% pectinase (Sigma) and 2% cellulase (Calbiochem) dissolved in 0.1M citrate buffer for 30 min at 37°C.
4. After the enzyme treatment, hydrolyze cells in 45% acetic acid for one hour at 37°C.
5. Observe samples under a phase-contrast microscope.
6. Remove the cover slip by the dry-ice method.
7. Let slides air-dry overnight.
8. Stain slides with 0.1 mg ml^{-1} Chromomycin A_3 (CMA_3) (Sigma) for 10 min and incubate them for 2-3 days at 37°C (Schweizer and Ambros 1994).
9. The same slide can be re-stained with 0.2µg ml^{-1} DAPI (Sigma). Before additional staining with DAPI, samples should be re-fixed with FAA and series of EtOH (70, 90, 96%, 5 min each).

3.2. Genetic transformation

Cotyledonary somatic embryos represent good target tissue for genetic transformation because they are receptive to the introduction of foreign

DNA and capable of re-inducing embryogenic tissues for subsequent regeneration. In a transformation procedure for *P. omorika* somatic embryos, we used *A. tumefaciens* strain LBA 4404 bearing the binary plasmid pBI121 (Clontech) that contains a beta-glucuronidase gene (*gusA*) under the transcriptional control of the CaMV 35S promoter, and the *nptII* gene for kanamycin selection (Mihaljević et al. submitted).

Material and equipment required: bacterial YEB liquid medium (Petit et al. 1983), culture induction medium, cefotaxime, neomycin, GUS-staining buffer (Jefferson et al. 1987) and stereo microscope.

1. Grow an over-night bacterial culture in liquid YEB medium supplemented with 50 µg ml^{-1} kanamycin and 100 µg ml^{-1} rifampicin at 28 °C, to reach an optical density of 1.0 (λ=600 nm).
2. Collect mature somatic embryos (cotyledonary stage) from M3 maturation medium using a stereo microscope.
3. Before the treatment, place collected embryos on 0.8% water-agar.
4. Centrifuge the bacteria culture (5000 rpm, 5 min, at 4°C).
5. Re-suspend bacteria pellet in M1 induction medium to reach an optical density of 0.6 (λ=600 nm).
6. Immerse somatic embryos in bacterial suspension for 10 min without agitation.
7. Transfer embryos without rinsing into solidified M1 medium without antibiotics. Group embryos in clumps of 6 embryos.
8. Co-cultivate embryos and bacteria in darkness at 28°C for two days.
9. Wash cultures with M1 medium containing 500 mg l^{-1} cefotaxime to kill the *Agrobacterium* (two times for 15 min).
10. Blot embryos on sterile filter paper.
11. Transfer embryos into M1 medium containing 500 mg l^{-1} cefotaxime for 14 to 21-day pre-selection (until embryogenic tissue re-induction start).
12. Transfer cultures into M1 medium containing 250 mg l^{-1} cefotaxime and 50 mg l^{-1} neomycin for selecting transformed cells.
13. Evaluate transformation efficiency using a histochemical staining procedure (Fig. 1f) and DNA analysis.

P. omorika proved to be very sensitive to selective antibiotics such as kanamycin, paromomycin, and hygromycin (Mihaljević et al. 2001). The higher tolerance of embryogenic tissue to neomycin indicated that neomycin may be more useful for selection of *npt*II-transformed embryogenic tissue of *Picea omorika* than other commonly used antibiotics.

According to our experiments, there is no significant difference in transient GUS expression when embryos and *Agrobacterium* are co-cultivated in the presence of 100μM acetosyringone, or when embryos are pre-incubated (two days) on induction medium and then used for co-cultivation (Mihaljević et al. submitted). However, wounding of somatic embryos prior the co-cultivation declines the percent of GUS-positive embryos.

4. REFERENCES

Besendorfer V., Šiljak-Yakovlev S., Mihaljević S., Jelenić S., Jelaska S., Papeš D. Karyotype analysis of *Picea omorika* (Panč.) Purk. *in vivo* and *in vitro* with a special emphasis on nucleolar organizer regions (NORs). Proceedings of the second IUFRO Cytogenetic Working Party S2.04.08 Symposium, Cytogenetic Studies of Forest Trees and Shrubs - Review, Present Status, and Outlook on the Future, H. Guttenberger et al. (eds.), 1998 September 6-12, Special Issue of Forest Genetics, 2000, pp. 27-31.

Budimir S., Vujičić, R. Benzyladenine induction of buds and somatic embryogenesis in *Picea omorika* (Panč.) Purk. Plant Cell Tissue Organ Cult 1992; 31:89-94

Gupta P. K., Durzan D. J. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Biotechnology 1987; 5:147-151

Hizume M., Ohgiku A., Tanaka A. Chromosome banding in the genus *Pinus.* II. Interspecific variation of fluorescent banding pattern in *P. densiflora* and *P. thunbergii*. Bot Mag Tokyo 1989; 102:31-42

Jain S.M., Newton R.J., Soltes E.J. Enhancement of somatic embryogenesis in Norway spruce *(Picea abies* L.). Theor Appl Genet 1988; 76:501-506

Jalonen P., von Arnold S. Characterization of embryogenic cell lines of *Picea abies* in relation to their competence for maturation. Plant Cell Rep 1991; 10:384-387

Jefferson R.A. Assaying chimeric genes in plants: The GUS gene fusion system. Plant Mol Biol Rep 1987; 5:387-405

Kolevska-Pletikapić B., Buturović-Derić Z. Regeneration of *Picea omorika* via organogenesis. Plant Cell Tiss Org Cult 1995; 41:189-192

Kolevska-Pletikapić B., Krsnik-Rasol M., Lorković Z., Besendorfer V., Tramišak T., Jelaska S. Somatic embryogenesis in *Picea omorika* (Panč.) Purk. Acta Pharm 1995; 45:267-271

Mihaljević S, Leljak-Levanić D, Jelaska S. Factors affecting *Agrobacterium*-mediated transformation of *Picea omorika* (Panč.) Purk somatic embryos. Submitted 2003

Mihaljević S., Perić M., Jelaska S. The sensitivity of *Picea omorika* embryogenic culture to antibiotics. Plant Cell Tissue Organ Cult 2001; 67:287-293

Petit A., David C., Dahl G., Ellis J.G., Guyon P., Casse-Delbart F., Tempé J. Further extension of the opine concept: Plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. Mol Gen Genet 1983; 190: 204-214

Redenbaugh K., Paasch B.D., Nichol J.W., Kosser M.E., Viss P.R., Walker K.A. Somatic seeds: Encapsulation of asexual plant embryos. BioTech 1986; 4:797-801

Salopek B., Tramišak-Milaković T., Mihaljević S., Jelaska S. Storage product accumulation during the maturation of *Picea omorika* (Panč.) Purk. somatic embryos. Period Biol 1997; 99:117-124

Siljak-Yakovlev S., Cerbah M., Coulaud J., Stoian V., Brown S.C., Zoldoš V., Jelenić S, Papeš D. Nuclear DNA content, base composition, heterochromatin and rDNA in *Picea omorika* and *Picea abies*. Theor Appl Genet 2002; 104:505-512

Schweizer D., Ambros P.F. ″Chromosome banding.″ In *Methods in Molecular Biology Vol. 29: Chromosome Analysis Protocols* J.R. Gosder, ed. Totowa, New York: Human Press Inc. 1994.

Tramišak-Milaković T., Mihaljević S., Jelaska S. Effects of abscisic acid and carbohydrates on the maturation of *Picea omorika* (Panč.) Purk. somatic embryos. Acta Bot Croat 1999; 58:87-97

von Arnold, S., Eriksson, T. In vitro studies of adventitious shoot formation in *Pinus contorta*. Can J Bot 1981; 59: 870-874

von Arnold S., Hakman I. Organogenesis and embryogenesis in mature zygotic embryos of *Picea sitchensis*. Tree Physiol 1988; 4:291-300

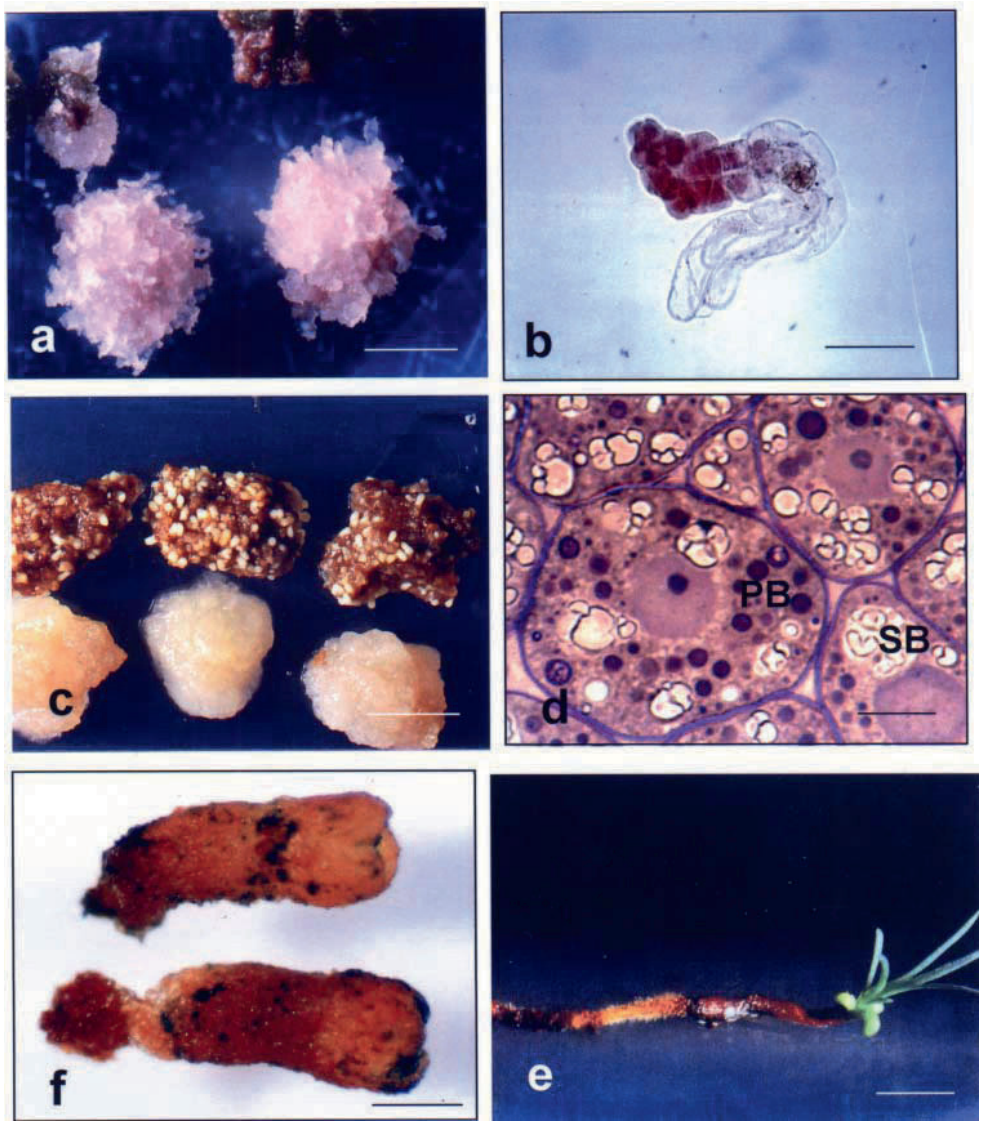

Figure1a-f. Embryogenic callus of *Picea omorika* obtained from mature zygotic embryos grown on M1 medium (a). Somatic embryo Stage 1 developed from proliferating ESMs (b). Maturation of somatic embryos of a B line (upper) and a W line (lower) after 5 weeks on M3 medium(c). Light micrograph of a torpedo stage 2 somatic embryo showing starch (SB) and protein (PB) bodies (d). Geminated somatic seedling on M4 medium (e). GUS expression in a somatic embryo infected with *A. tumefaciens* LBA4404 (pBI121) and stained with x-gluc after 14 days of growth on selection medium (f). (a-Bar 5 mm, b-Bar 50 µm, c-Bar 8 mm, d-Bar 10µm, e-Bar 2.6 mm, f-Bar 1 mm).

SOMATIC EMBRYOGENESIS IN *Picea glauca*

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1. INTRODUCTION

White spruce (*Picea glauca*) is one of the most widely distributed conifers in North America. It represents an ecologically valuable species, that is also extensively utilized by the forestry industry for wood and lumber production (Hosie, 1979). For this reason, in the last few years there has been an increasing interest towards the development of *in vitro* procedures, that would allow for rapid multiplication of this species and a large-scale production of superior materials for reforestation. Although white spruce has been regenerated by both organogenesis and somatic embryogenesis (see Thorpe and Harry, 2000), it is the latter method that has gained the most attention, as it represents the lowest cost method for producing uniform plants.

White spruce was one of the first conifers to be cultured and propagated in vitro. Durzan (1980) observed the development of embryo-like structures from cultured white spruce cells. Such cell aggregates, however, were not able to undergo further maturation. Generation of mature somatic embryos of white spruce, characterized by well defined shoot and root poles and expanded cotyledons, was reported independently seven years later by Lu and Thorpe (1987) and Hakman and Fowke (1987). The process of somatic embryogenesis in white spruce, as is true for conifers in general, involves a series of well-defined steps each of which requires proper execution for success. The process can be broken down into three major steps namely: (1) the induction, maintenance and proliferation of embryogenic tissue, (2) maturation (both morphological and physiological) of somatic embryos, and (3) germination and conversion of the somatic embryos (Thorpe and Harry, 2000). Continued empirical research on various factors, including culture conditions, choice of explant, type and level of growth regulators, osmolarity, have enhanced the process, and it is now recognized that optimization of each stage of the process is required for maximum conversion of somatic embryos.

Somatic embryogenetic tissue of white spruce is commonly initiated from zygotic embryos cultured in the presence of 2,4-dichlorophenoxyacetic acid

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 47–58.

and N^6-benzyladenine and maintained in solid or liquid cultures. Induction of somatic embryo development requires the presence of abscisic acid (ABA). To increase the number and optimize the development of embryos, further treatment with a non-plasmolysing agent (PEG 4000 or dextran) or with a partial drying treatment (PDT) is required. In this chapter, a more "traditional" protocol is presented, i.e. using solid medium, without the addition of osmotica but with a PDT. For protocols involving the use of liquid suspension cultures and PEG, methods detailed by Attree and Fowke (1995) should be consulted.

2. EMBRYOGENIC CULTURE INITIATION

2.1. Explant – type and sterilization

Immature and mature seeds are the usual explants for the generation of embryogenic tissue. Immature cones are harvested from trees. Although different ages can be used, cones with embryos after the initiation of cotyledons are more suitable as embryos have completed the process of histogenesis. Care should be used in selecting healthy cones. Since the cone scales are still tightly pressed against one another, a harsh surface sterilization procedure should be used to ensure that all surface contaminations are destroyed. The cones are sterilized in 20% Javex bleach with a few drops of Tween acting as a surfactant for 30 minutes. The cones should be stirred slowly using a magnetic stirrer for about 30 minutes. Rinse 3 times with sterile distilled water, 10 minutes each. To ensure complete surface sterilization, the cones can be subjected to a 5-minute treatment of 70% ethanol followed by rinsing in sterile distilled water. The seeds are first removed from the cone scales and the embryo is dissected out under a stereomicroscope on a clean bench. The dissected embryos are placed directly onto the induction medium.

Mature seeds are also suitable for the generation of embryogenic tissues (Tremblay, 1990). Since the seeds are small, about 1x 1.5 mm, it is better to place the seeds onto a small piece of cheesecloth. The cloth is tied into a small bag using a small string. The seeds are sterilized with 10% Javex bleach with a few drops of Tween as a surfactant. The bag is again placed into a beaker with the sterilizing solution and stirred gently with a magnetic stirrer for 30 minutes. The small bag is then rinsed with sterile distilled water 3 times with continuous stirring on a clean bench. The bag is then placed inside a petri dish with a few ml of sterile distilled water and the seeds are allowed to imbibe overnight at 4 °C. The purpose is to allow the seeds to swell so that they are easier to dissect. The following day, the seeds are again sterilized using the same disinfectant solution for 15 minutes and undergo a sterile distilled water rinse 3 times at about 10 minutes each. The small cheese cloth bag is cut open and the seeds are removed to a sterile

petri dish with water (10 ml of water in a 15 cm petri dish). Seeds that float should be discarded as they have not imbibed properly and the air trapped inside may harbour contaminants. During dissection, the scalpel and forceps should be flamed often to avoid cross contamination. If indeed too many seeds appear to be "bad", it is better to discard them and check alternate batches of seeds.

2.2. Culture medium

White spruce embryogenic tissues have been successfully obtained using a slightly modified AE medium (von Arnold and Eriksson, 1981; Joy, 1991) and the LV medium (Litvay et al., 1985) as reported by Joy et al. (1991) and Tremblay (1990) respectively. Table 1 details the components of both media and Table 2 indicates additional components needed for the induction, maintenance, maturation and germination media.

2.3. Inoculation

The embryos are placed on the induction medium and kept in the dark. Approximately 10-15 embryos can be placed in the same petri-dish. The dishes are sealed with Parafilm™. The cultures should be examined carefully for contamination after 2-3 days. If indeed there are contaminated embryos, the non-contaminated ones can be transferred to a new induction medium.

2.4. Initiation

The induction medium causes the cells of the embryo to proliferate. Different tissue morphologies can be found. Developing embryos tend to respond faster than embryos from dry mature seeds. In general, two weeks after culture, tissues begin to proliferate from cultured embryos. It ranges from compact nodular masses to friable tissues with filamentous structures (Fig. 1). The colour varies from pale brown to whitish cell masses. The initial growth of tissues is usually slow. Once the tissues reach the size of approximately 5-6 mm, the whitish, friable tissues should be carefully dissected away from the nodular masses and transferred to the maintenance medium. The friable tissues usually have a higher embryogenic potential. A code number should be assigned to each line or genotype cultured.

2.5. Culture maintenance

Each cell line is maintained separately in the maintenance medium in the dark at room temperature. Different growth rates are observed among different tissue pieces. The tissues are regularly transferred to a fresh maintenance medium. The frequency of transfer is approximately at weekly intervals. Once enough tissue has been accumulated, a small amount is

transferred to maturation medium to test for the embryo forming capacity. Cell lines with good embryo forming capacities should be maintained, others may be discarded or maintained for different experimental studies. If cryogenic equipment is available, the cell line with good embryogenic characteristics should be stored for future use as the embryogenic property usually declines over time under continuous culture. A detailed protocol of cryogenic preservation of embryogenic tissues can be found in a publication by Park et al. (1994).

2.6. Making cell suspension

To generate cell suspension cultures, the stock maintenance cultures can be transferred to a liquid maintenance medium (AE or LV medium without agar) and sub-cultured weekly. The cultures should be maintained in the dark at room temperature under continuous agitation using a gyratory shaker with a speed of approximately 100 rpm. See Attree and Fowke (1995) for further details.

2.7. Morphology of embryogenic tissues

The embryogenic tissues can take on different appearances. In general, the embryogenic tissue is white and mucilaginous in texture (Fig. 2). Among the highly vacuolated cells, single or small groups of densely cytoplasmic cells are present. In some cell lines, more prominent immature embryos consisting of a small group of apical, densely cytoplasmic cells forming the embryogenic head are present (Fig. 3). These cells are subtended by vacuolated, elongated suspensor cells. The apical cytoplasmic-rich cells of the embryo consist of a large nucleus, a complement of organelles, and plastids, which are usually devoid of starch.

Although many cell lines take on a similar morphology, the embryo forming capacity varies between lines. The best way to determine the embryogenicity of the line is to place small amounts of tissues on the embryo maturation medium to determine their ability to form somatic embryos, as well as the quality and quantity formed. After preliminary testing, if a cell line is deemed suitable as an experimental material, i.e. with high embryo formation capability, the cell line can be maintained on the maintenance medium for further studies.

2.8. Embryo development and maturation

Further embryo development and maturation is initiated by transferring embryogenic tissues from the maintenance medium onto the abscisic acid

(ABA) containing maturation medium (Joy et al. 1991). Alternatively, the embryogenic mass first can be transferred to the maturation medium devoid of growth regulators for 1 week to reduce the auxin content.

A small amount of embrygenic tissue is removed from the edge of the maintenance culture and is gently teased to form a thin layer (1 mm thick) on the maturation medium. A thin layer of culture allows better contact with the medium and promotes better embryo development.

Embryo maturation can also be initiated from a liquid suspension. This is achieved by plating 1 ml of settled cell aggregates onto a Whatman no. 2 (5.5 cm diameter) sterile filter paper placed onto the maturation medium. The whole filter paper can be transferred onto fresh medium after 2 weeks in culture if deemed necessary. This technique enables the removal of excessive carry over of the liquid maintenance medium and eases the transfer of maturing embryos onto fresh maturation medium.

After 1 week in the presence of ABA-containing medium, increased meristematic activity within the embryonal mass results in a small, light yellow nodular embryo surrounded by a layer of translucent, vacuolated cells. The embryo at this time consists of a group of densely plasmatic cells, an intermediate region of partially vacuolated cells, and the highly vacuolated suspensor cells.

In week 2, continued mitotic activity, especially in the basal end of the embryo adjacent to the suspensor, results in the elevation of the embryo above the tissue mass. The cells in this region become part of the future root cap. Further development leads to the embryo taking on a glossy dome shape. The embryo continues to expand both in length and girth, and the suspensor system remains attached, as the suspensor is an integral part of the developing embryo.

In week 3, further structural differentiation can be observed with the appearance of the apical meristem, procambium and cotyledon primordia. Usually about 4-6 meristematic ridges appear, marking the formation of the cotyledons. The embryo axis expands rapidly in length and starts to turn a creamy-yellow (Fig. 4).

In week 4, the embryos continue to grow and expand, and developing cotyledons are evident (Fig. 5). The embryos turn completely yellow during this time. The suspensor system at this point generally develops a necrotic area between itself and the embryo proper, and can be separated easily from the embryo.

As for the storage products, mostly polysaccharide was produced during early stages (1 to 2 weeks on ABA), whereas during later stages, polysaccharides, lipids, and proteins accumulate (Joy et al., 1991).

2.9. Embryo germination and conversion

Germination, i.e. radicle emergence or growth from base of the embryo, is not always followed by growth of the plantlet; thus the concept of conversion leading to plantlet growth is a better assessment of embryo quality.

In order for the embryo to germinate and undergo proper conversion, a partial drying treatment (PDT) has to be carried out. This treatment was introduced by Roberts et al. (1990) and it greatly enhances embryo conversion.

Mature embryos are placed into half of the wells of a 24-well tissue culture plate (Falcon 3847, Frankling Lakes, New Jersey, U.S.A.). The other 12 wells are three-quarters full of sterile distilled water. In order to prevent the sticking of embryos to the surface of the well after drying, the embryos are placed on sterile filter paper disks (Whatman no. 1), 6mm in diameter (Fig. 6). The sterile filter paper disks are moistened by placing them on the surface of the maturation medium. Approximately, 6-7 embryos are placed on each disk and 3 disks can be housed in a single well. The plates are sealed with Parafilm and incubated in the dark at room temperature (22 °C) for 10 days. Under these conditions, the embryos lose approximately 10% of their fresh weight.

After the 10 day PDT, embryos are ready for germination. The germination medium is made of the half-strength AE or LV medium, supplemented with 1% sucrose and solidified with 0.8% agar, pH 5.8. The filter disks with the embryos are placed on the germination medium allowing the embryos to imbibe in the dark or under dim light for one to two days. Individual embryos are then placed vertically with the root pole embedded in the agar medium. Germinating cultures are kept in light (80 $\mu Em^{-2}.s^{-1}$) under a 16-h photoperiod. Shoot and root emergence occurs approximately 2 weeks later.

For cell lines with a low conversion number, the addition of ascorbic acid can improve the conversion frequency (Stasolla and Yeung, 1999). The germination medium can be supplemented with 100 µM of ascorbic acid. The ascorbate solution is added to an autoclaved germination medium before the plates are poured. The plates should be used by the following day.

In treatment where PEG is used in conjunction with ABA, complete desiccation is required for proper germination. For protocol, see Attree and Fowke (1995) for details.

2.10. Acclimatization and field transfer

After 1 month on the germination medium, plantlets are placed inside sterile glass jars (Pyrex 100 x 80, No. 3250) containing sterile peat pellets saturated with ¼ strength LV medium without sucrose. Plantlets are maintained in these closed, unsealed vessels under a 16-h photoperiod (80 $\mu Em^{-2}s^{-1}$) for 3-4 weeks and then transferred to the greenhouse.

3. IDENTIFICATION OF STEPS REQUIRED FOR PROTOCOL MODIFICATION

Since open pollinated seed embryos are used as explants, the concentrations of growth regulators used may only be suitable for a certain population of seeds. It is advisable to test different concentrations of BA and 2,4-D in the induction medium in order to induce embryogenic tissue formation.

Auxin is a key component for embryogenic tissue induction. However, high levels of 2,4-D is not a desirable component for embryo maturation. A reduction of the auxin level can be accomplished by transferring the embryogenic tissue to a hormone-free medium for a short period of time (3-7 days) prior to the ABA treatment. This treatment promotes somatic embryo development in both quality and quantity. This is especially true if the embryogenic tissue is maintained in a liquid maintenance medium.

Similarly, different embryogenic cell lines may have different requirements for ABA during the maturation process. Therefore, the concentration of ABA should be tested to determine the optimal levels needed for a particular cell line. Since the developing embryos require approximately 40 days to mature, the effective concentration of ABA in the maturation is reduced over the entire culture period. Hence, transferring the cultures to a fresh ABA maturation treatment at the mid-point of the culture (20 days in maturation medium) will ensure proper embryo development and maturation.

For cultures on a solid medium, good quality agar is used. However, besides agar, Phytagel and other gelling agent can also serve as suitable alternatives. A 0.4% Phytagel solution is a suitable substitute for agar in the maturation medium.

Additional compounds can be added to the maturation medium. For example, ethylene has been shown to be produced by the embryogenic tissues and negatively effects the maturation process (El Meskaoui et al., 2000). The addition of silver

nitrate and aminoethoxyvinylglycine can improve the quality of the somatic embryos by leading to better-organized shoot apical meristems (Kong and Yeung, 1994; 1995).

4. REFERENCES

Attree, S. and Fowke, L.C. (1995) Conifer somatic embryogenesis, embryo development, maturation drying, and plant formation, *in* O.L. Gamborg and G.C. Phillips (eds.), *Plant Cell, Tissue and Organ Culture – Fundamental Methods*, Springer, Berlin, pp.103-113.

Durzan, D.J. (1980) Progress and promise in forest genetics, *in* Proceedings of the *50th Anniversary Conference, Paper Science and Technology, The Cutting Edge*, pp. 31-60. The Institute of Paper Chemistry, Appleton, WI, U.S.A.

El Meskaoui, A., Desjardins, Y. and Tremblay, F.M. (2000) Kinetics of ethylene biosynthesis and its effects during maturation of white spruce somatic embryos. Physiol. Plant. **109**, 333-342.

Hakman, I. and Fowke, L.C. (1987) An embryogenic cell suspension culture of *Picea glauca* (white spruce). Plant Cell Rep. **6**, 20-22.

Hosie, R.C. (1979) Native Trees of Canada, 8th ed. Fitzhenry and Whiteside, Don Mills, Ontario, Canada.

Joy, R.W. IV (1991). Nitrogen metabolism during somatic embryogenesis in *Picea glauca* and *Daucus carota*: A NMR study. Ph. D. Thesis, University of Calgary, Calgary, Alberta, Canda.

Joy, R.W. IV, Yeung, E.C., Kong L. and Thorpe T.A. (1991) Development of white spruce somatic embryos: I. Storage product deposition. In Vitro Cell. Dev. Biol. – Plant **27**, 32-41.

Kong, L. and Yeung, E.C. (1994) Effects of ethylene and ethylene inhibitors on white spruce somatic embryo maturation. Plant Sci. **104**, 71-80.

Kong, L. and Yeung, E.C. (1995) Effects of silver nitrate and polyethylene glycol on white spruce (*Picea glauca*) somatic embryo development: enhancing cotyledonary embryo formation and endogenous ABA content. Physiol. Plant. **93,** 298-304.

Litvay, J.D., Verma, D.C. and Johnson, M.A. (1985) Influence of a loblolly pine (Pinus taeda L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (Daucus carota L.). Plant Cell Rep. 4, 325-328.

Lu, C.-Y. and Thorpe, T.A. (1987) Somatic embryogenesis and plantlet regeneration in cultured immature embryos of Picea glauca. J. Plant Physiol. 128, 297-302.

Park, Y.S., Pond, S.E. and Bonga, J.M. (1994) Somatic embryogenesis in white spruce (Picea glauca): genetic control in somatic embryos exposed to storage, maturation treatments, germination, and cryopreservation. Theor. Appl. Genet. 89, 742-750.

Roberts, D.R., Stutton, B.C. and Flinn, B.S. (1990) Synchronous and high frequency germination of interior spruce somatic embryos following partial drying at a high relative humidity. Can. J. Bot. 68, 1086-1090.

Stasolla, C. and Yeung, E.C. (1999) Ascorbic acid improves conversion of white spruce somatic embryos. In Vitro Cell. Dev. Biol. – Plant 35, 316-319.

Thorpe, T.A. and Harry, I.S. (2000) Micropropagation of Canadian spruces (Picea spp.). In C. Kubota and C. Chun (eds.), Transplant production in the 21st Century. Kluwer Academic Publishers, Dordrecht, pp. 197-203.

Tremblay, F.M. (1990) Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of Picea glauca. Can. J. Bot. 68, 236-242.

Von Arnold, S. and Eriksson, T. (1981) In vitro studies on adventitious shoot formation in Pinus contorta. Can. J. Bot. 59, 870-874.

Table 1. Composition of the basal AE[1] and the full-strength LV[2] media

Component	AE medium	LV medium
Macronutrients	mg l^{-1}	mg l^{-1}
NH_4NO_3	1200	1650
KNO_3	1900	1900
$MgSO_4.7H_2O$	370	1850
KH_2PO_4	340	340
$CaCl_2.2H_2O$	180	22
Micronutrients		
KI	0.75	4.15
H_3BO_3	0.63	31
$MnSO_4.4H_2O$	2.2	27.7
$ZnSO_4.7H_2O$	4.05	43
$Na_2MoO_4.2H_2O$	0.025	1.25
$CuSO_4.5H_2O$	0.0025	0.5
$CoCl_2.6H_2O$	0.0025	0.125
$FeSO_4.7H_2O$	5.57	27.8
Na_2.EDTA	12.85	37.3
Vitamins and organics		
Thiamine HCl	5	0.1
Pyridoxine HCl	1	0.1
Nicotinic acid	2	0.5
myo-Inositol	500	100

For preparing the half-strength LV medium, use half dilution of the macronutrients, micronutrients, vitamins and organics. In addition, for both media, 400 mgl^{-1} glutamine and 500 mgl^{-1} casein hydrolysate are added to the complete basal medium.

[1]AE, von Arnold and Eriksson, 1981. [2] LV, Litvay et al., 1985.

Table 2. Additional components needed for preparing the induction, maintenance, maturation, and germination media.

Medium	AE[1]	LV[2] (half-strength)
Induction	5 μM BA, 10 μM 2,4-D, 5% sucrose, pH 5.8 0.8% agar	5 μM BA, 10 μM 2,4-D, 5% sucrose, pH 5.8 0.8% agar
Maintenance	2 μM BA, 10 μM 2,4-D, 3% sucrose, pH 5.8 0.8% agar	2 μM BA and 10 μM 2,4D 3% sucrose, pH 5.8 0.8% agar
Maturation	50 μM ABA 5% sucrose, pH 5.8 0.8% agar	50 μM ABA 5% sucrose, pH 5.8 0.8% agar
Germination	Half-strength AE 1% sucrose, pH 5.8 0.8% agar	1% sucrose, pH 5.8 0.8% agar

Notes:

[1] AE, von Arnold and Eriksson, 1981. [2] LV, Litvay et al., 1985.

All chemicals used can be obtained from Sigma (Missouri, U.S.A.).

Good quality water should be used to prepare culture media. Poor water quality results in rapid decline in the embryo forming capacity of the embryogenic tissues.

The purified agar used is obtained from Becton Dickinson, Sparks, MD, U.S.A. (Cat. No. 211853). Quality agar should always be used.

A 0.4% Phytagel (Sigma P 8196) can be used instead of agar for the maturation medium.

Freshly prepared growth regulator stock solutions are preferred, especially for the ABA solution. The BA and 2,4-D stock solution are prepared by dissolving a suitable amount of each in a minimum volume of a 1 M sodium hydroxide solution. These growth regulators can be added to the medium prior to autoclaving. The ABA stock solution is prepared by dissolving it in a minimum amount of a 1 M sodium hydroxide solution. Using a pH meter, the pH of the solution is brought to 5.8 using a 1 N hydrochloric acid solution. The volume of the solution is adjusted to bring it to the desired concentration and it is then filter-sterilized. The ABA solution is added to warm maturation medium just prior to the pouring of plates.

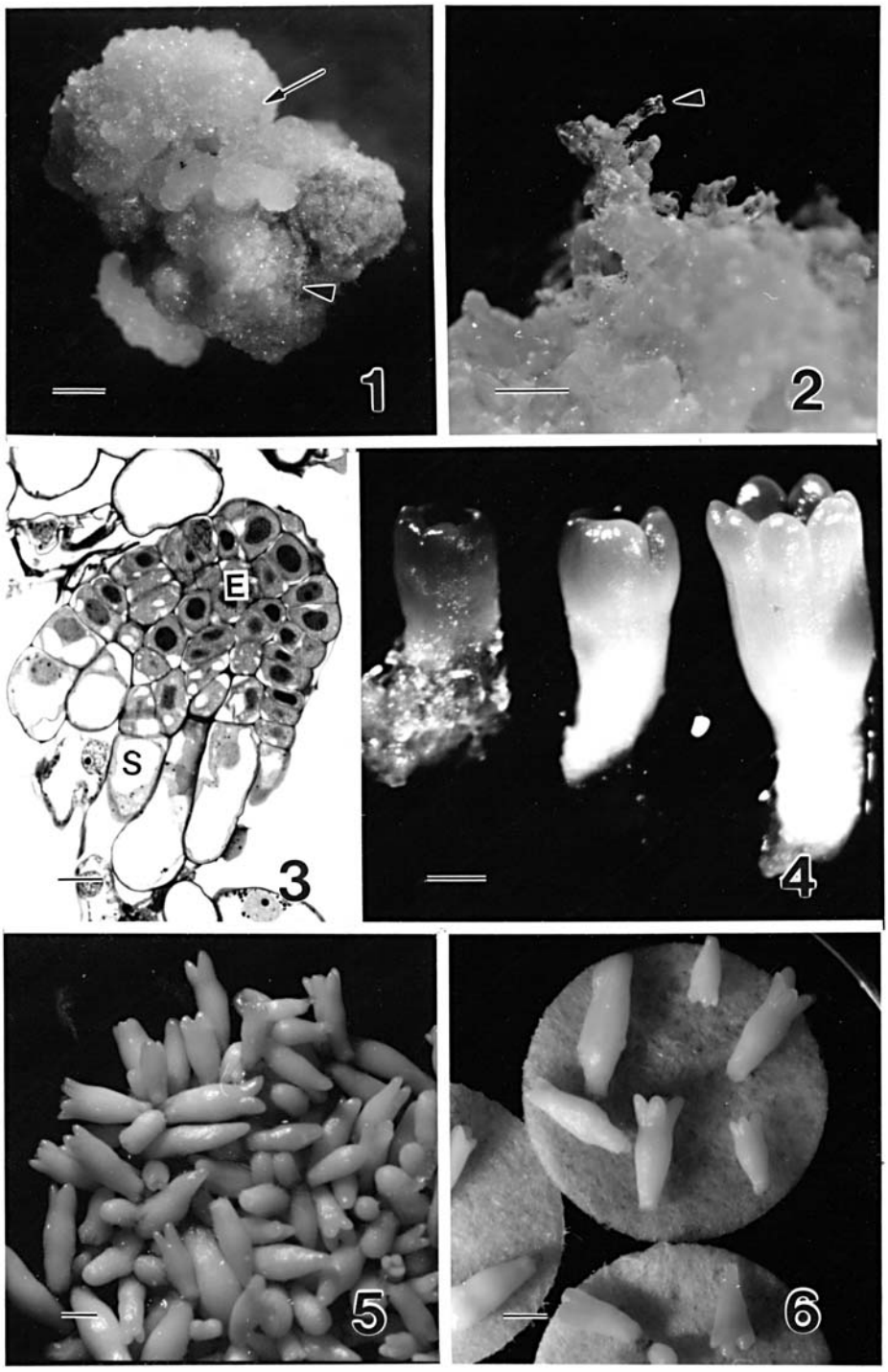
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Figure 1. A photograph showing the successful induction of embryogenic tissue from an explant placed on the induction medium. Often, both embryogenic and non-embryogenic tissues are present. The non-embryogenic tissue appears as brown nodular masses (arrowhead) and the embryogenic tissue is represented by white fluffy cell masses (arrow). Scale bar = 1 mm.

Figure 2. Upon transferring the embryogenic tissues onto the maintenance medium, embryogenic tissue continues to proliferate. Filamentous embryogenic structures (arrowheads) are common especially at the edge of the culture. Scale bar = 0.5 mm

Figure 3. A light micrograph showing the structural organization of a proembryogenic cell mass. The developing embryo proper (E) consists of cells with dense cytoplasm and prominent nuclei. These cells are subtended by vacuolated, elongated suspensor cells (S). Scale bar = 20 μm

Figure 4. Upon transferring to the maturation medium, embryo development continues with the formation of a well-defined embryonic axis and cotyledons. Scale bar = 0.5 mm

Figure 5. At the end of the fourth week, numerous somatic embryos are formed. Scale bar = 1 mm

Figure 6. Good quality embryos, i.e. with four or more cotyledons are selected for the partial drying treatment. Approximately six embryos are placed on a sterile filter paper disk and this in turn is placed inside a well of a multi-well plate for slow drying. Three disks can be housed within the same well. Scale bar = 1 mm

PROTOCOL OF SOMATIC EMBRYOGENESIS: BLACK SPRUCE (*PICEA MARIANA* (MILL.) B.S.P.)

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1. INTRODUCTION

The contribution of forest products to Canada positive trade balance in 2001 represented 37.5 billion Can $ and supported 352 800 direct jobs. Black spruce [*Picea mariana* (Mill.) B.S.P.] is one of the most widely distributed conifers in the North American boreal forest. In spite of its slow growth, the high quality of its fibres maintains black spruce as a species of choice in the Canadian pulp and paper as well as in the sawmill industries. Black spruce is mostly present in the eastern part of Canada, especially in Quebec, Ontario and Newfoundland provinces. It is on the public lands of Quebec that we find the largest black spruce forest in the world, adding to its economical role an important ecological aspect.

The vast majority of the black spruce forests is from natural origin, arising from natural regeneration after harvesting, fires, or insect epidemics. This natural process results however in a very low productivity. For example, in Quebec province, the average productivity of the natural black spruce forest is estimated at 1.4 m^3/ha/yr. Although reforestation programmes exist, only 3% of the planting stock comes from the black spruce improvement programme through vegetative propagation by cuttings of selected families. However, genetic tests have shown that utilization of selected clonal planting stocks would generate the maximal productivity gains, reaching 4-5 m^3/ha/yr. Although this might seem low when compared with other forest species from various countries, the climatic limitations encountered in eastern Canada constitute an important limitation to forest growth. In such conditions, black spruce remains a very well adapted species to Canadian northern climate. As in other coniferous species, cuttings do not allow clonal selection because of the aging of the ortets, making somatic embryogenesis the only way to maximize genetic gains in black spruce.

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 59–68.

2. INDUCTION OF EMBRYOGENIC TISSUE

Induction of black spruce embryogenic tissue can be obtained from immature (Hakman and Fowke 1987) or mature zygotic embryos (Tautorus et al. 1990) but also from needles collected on 3-year-old plants (Tremblay, unpublished). In general, in our laboratory, we work with mature zygotic embryos dissected from stored seeds because they allow induction all year round; furthermore, with this type of explant, we can use controlled crosses performed in previous years in the improvement programmes. Induction of black spruce embryogenic tissue from mature seeds is as described for white spruce in Tremblay (1990).

Essentially, seeds are surface-sterilized for 15 min in 1% (w/v) sodium hypochlorite solution prepared from commercial bleach to which 1-2 drops of Tween 20 are added. After rinsing three times in sterile distilled water, they are left to imbibe for 4 h before dissection of the zygotic embryos. Embryos are placed onto the induction medium (HLM-1: Table 1) containing half-strength Litvay's salts (Litvay et al. 1985), full strength vitamins, 500 mg l^{-1} glutamine, 1 g/l casein hydrolysate, 1% (w/v) sucrose, 10µM 2,4-dichlorophenoxyacetic acid (2,4-D), 5µM benzylaminopurine (BA), and 0.7% (w/v) Difco Bacto-agar. Iron is supplied as Sequestrene 330Fe at 28 mg l^{-1}. The pH is adjusted to 5.7 with 1 N NaOH before autoclaving at 121°C. Glutamine is filter-sterilized and added to the cooled medium (~30˚C), which is then poured at the rate of 25 ml per 100 x 15 mm plastic Petri dishes. Ten zygotic embryos per Petri dish are optimal for induction, which is realized at 25°C in the dark. All through the induction period and even before embryogenic tissue can be observed, cultures are transferred onto the fresh medium at every 2-week interval since this increases the frequency of induction as compared to cultures without subculture. Embryogenic tissues develop after 3-8 weeks on the induction medium. When they reach 0.5-1.0 cm in diameter, are transferred under maintenance conditions.

3. MAINTENANCE OF EMBRYOGENIC TISSUE

Maintenance conditions are set at 23°C with a 16-h photoperiod under dim light (3-5µmol m^{-2} s^{-1}). Cultures are maintained on the same medium as for induction except that Difco Bacto-agar is raised at 1% (w/v). Like other coniferous species, black spruce embryogenic tissue can be maintained either on solid (Fig. 1a) or in liquid medium by serial subcultures to fresh medium every 14 or 7 days, respectively. When compared with other species that we worked with, black spruce is extremely sensitive to the size of the tissue used for the subculture. For most genotypes, transfer of tissue portions bigger than 25-30 mg results in rapid degenerating and eventually death of the cultures. At the time of subculture, it is also important to keep a maximum of 6 tissue

portions per Petri dish to avoid decline of the growth rate, due to the fact that sucrose in the medium is completely depleted 10 to 12 days after subculture (unpublished data). Cell suspension cultures are established with 2-3 g embryogenic tissues dispersed in 40 ml liquid maintenance medium in 250 ml Erlenmeyer flask. Flasks are placed on a rotary shaker (90-110 rpm) under the same incubation conditions. Black spruce suspension cultures can also be easily grown in a bioreactor for mass production. To avoid somaclonal variation (Tremblay et al 1999), tissues are actively maintained in the laboratory for a maximum of one year after which time they are replaced by thawed embryogenic tissues.

4. MATURATION

Black spruce somatic embryo development is carried out according to Tremblay and Tremblay (1995). The maturation medium (Table 1) is derived from the HLM-1 medium except that glutamine is raised at 1 g l^{-1}, growth regulators are replaced by 25-60µM (±) cis, trans-abscisic acid (ABA), sucrose is raised at 6% (w/v) and the medium is solidified with 0.6-0.8% (w/v) Gelrite™ gellan gum. Glutamine, ABA and sucrose are filter-sterilized. Mature somatic embryos can be easily obtained from different protocols; we present two of them, the choice depends on the number of embryos needed.

Protocol I: Four portions of 75-100 mg embryogenic tissues are taken 7 days after transfer onto the maintenance medium and placed in 100 x 15 mm Petri dishes containing 25 ml maturation medium. Tissues are left without transfer for the duration of the 5-weeks maturation (Fig. 1b).

Protocol II: Seven days after subculture, 1.5 g embryogenic tissues are dispersed in 40 ml liquid HLM-1 medium. After 5-7 days, 7 ml of the resulting cell suspension is poured over a Nylon mesh (60µm) placed on the Whatman filter in a Buchner funnel. After removing of the medium under negative pressure, the Nylon mesh supporting a thin layer of embryogenic tissue is placed on the maturation medium (Fig. 1c). For both protocols, Petri dishes are sealed with Parafilm™ and incubated at 23˚C with a 16-h photoperiod under a light intensity of 10-15µmol m^{-2} s^{-1} given by Vita-Lite Plus fluorescent lamps (Duro-Test, USA)

5. SOMATIC EMBRYO DESICCATION

The two maturation protocols produce somatic embryos that can be partly or totally

desiccated under relative humidity (RH) varying from 79% to 97% and generating 7 to 19% somatic embryo moisture content (MC) (Bomal and Tremblay 1999). The embryo MC can be lower when a combination of 48 h under a 97% RH is followed by a period of fast desiccation under 31% RH (Bomal et al. 2002). In the last case, the tolerance to fast desiccation is acquired during the first 48 h of slow desiccation, which induces starch depletion in the somatic embryos, sucrose accumulation and raffinose occurrence and appearance or increment of dehydrins. In the case of black spruce, somatic embryo desiccation does not improve germination or plant conversion rate but root emergence is better synchronized from 7.1 days in the control to 5.6 days after desiccation under RH 97% to a 16 % MC (Bomal and Tremblay 1999). The improvement obtained is, however, not sufficient to justify the addition of a desiccation step to produce plants. Therefore, we use this technique only for storage either on a short term to synchronize the plant production from various genotypes for clonal testing or on a long term as the equivalent of a "seed" bank. Bomal and Tremblay (1999, 2000) described an efficient and reproducible protocol for desiccation of black spruce somatic embryos. Essentially samples of 80 mg mature somatic embryos (or 40 mg embryos + 40 mg water) are dispersed onto a 60-µm-nylon mesh screen (B&SH Thompson, Montreal, Canada). After blotting of any residual liquid over sterile paper, screens are placed in 60 x 15 mm Petri dishes. Embryo desiccation is performed by placing unsealed Petri dishes in homemade Plexiglas™ desiccation chambers (56 cm long, 29 cm wide, 46 cm high) equipped with a fan operating for 30 min every six hours to avoid any RH stratification. Different salt solutions can be used to generate different levels of RH; we give here an example for desiccation under 97% RH. One litre of saturated Na_2HPO_4 solution (Rockland 1960) is placed in a polypropylene tray (48 cm long, 27 cm wide, 14 cm high, Nalgene) supporting a plastic grid on which the Petri dishes are placed. Embryos are left in the desiccating chamber for seven days at 23°C in the dark. Desiccated embryos are removed from the nylon screen and directly germinated or treated for cryopreservation as described in section 7.2. Before utilisation of the desiccated embryos, they are first hydrated to prevent imbibitional damage (Anandarajah and McKersie 1990). To do so, desiccated embryos are placed in unsealed Petri dishes (Fig. 1d) placed overnight at room temperature on a grid above water in a closed black germinating box (Spencer-Lemaire Industry Ltd, Alberta, Canada).

6. GERMINATION AND TRANSFER TO SOIL

Somatic embryos are germinated onto 30 x 16 mm Sorbarod™ plugs saturated with germination medium (5 ml medium per plug) composed of Campbell and Durzan's salts (Campbell and Durzan 1975) at half-strength, supplemented with 1.5% (w/v) sucrose at pH 5.7 adjusted before autoclaving at 121˚C. Germination is realized at

23˚C with a 16h photoperiod given by Vita-Lite Plus florescent lamps generating a light intensity of 70–90µmol m^{-2} s^{-1}. Germination can be done in test tubes or in larger containers. When somatic seedlings have developed epicotyls at least 1 cm long (Fig. 1e), the test tubes or containers are directly placed in the greenhouse for acclimatization, which is done prior transfer into soil. Somatic seedlings in the germination containers are submitted to acclimatization over a nine days period by letting increasingly greater air exchanges between the containers and the greenhouse. The greenhouse is operated with a day/night temperature of 25/18°C, a RH of 35-45% and an 18 h photoperiod. On day one, containers are left undisturbed. From day 2 to 5, the containers are half opened. From day 6 to 8, containers are completely opened and somatic seedlings are directly exposed to greenhouse conditions. On day 9, the acclimatized somatic seedlings are transplanted to soil as regular seedlings (Fig. 1f) in Styroblock (Beaver Plastics Ltd., Edmonton, Canada) containers (45 cavities per block, 340 cm^3 per cavity) filled with a moistened mixture of peat moss and vermiculite (3/1, v/v). The survival to acclimatization and soil transfer (%) is determined as the (number of surviving plants eight weeks after transfer into soil/number of plants transferred into soil) x 100. The conversion rate (%) of embryos into plants growing in soil is determined as the (number of surviving plants eight weeks after transfer into soil/number of embryos placed in germination) x 100. The acclimatization procedure described here allows survival rates higher than 90% without transplanting shock. The anatomical and physiological effects of this technique are described in more details in Lamhamedi et al. (in press).

7. CRYOPRESERVATION

Black spruce embryogenic tissue lines can be cryopreserved either as embryogenic tissues or as desiccated mature somatic embryos for short or long- term preservation.

7.1. Embryogenic tissues

To prepare nine cryovials of one genotype, two grams of embryogenic tissues grown on solid or in liquid maintenance medium are respectively taken 10 and 5 days after transfer and placed in a 50-ml Erlenmeyer flask containing 7 ml of HLM-1 medium supplemented with 0.4 M sorbitol. Flasks are placed for 24 h on a gyratory shaker at 90 rpm under the same environmental conditions as used in maintenance. After 24 h, flasks are placed on ice and 3 ml of the same solution but containing 17% DMSO are added to the flask at the rate of 0.5 ml every 5 min for a final concentration of 5% DMSO. Flasks are left on ice for an additional 30 min after which time they are gently stirred. One ml of the suspension corresponding to about 200-300 mg of cells is used per 1.2 ml cryovial (Corning, USA). Cryovials are placed in a Mr Frosty™ freezing

container (Nalgen Company) filled with 100% iso-propyl alcohol and pre-cooled to 4°C. The Mr Frosty container is then placed in freezer at -80°C for 70 min after which time cryovials are immediately placed into liquid nitrogen.

For thawing, cryovials are gently shaken under running water (set at 38˚C) during 2 min followed by 10 min in a laminar flow hood at room temperature. The supernatant is then discarded and the cells deposed onto a filter paper (Whatman™ No. 4) laid over solid maintenance medium. The filter paper supporting the cells is transferred twice at 24 h intervals onto fresh medium. The cultures are kept under the same conditions as described in maintenance.

7.2. Dried mature somatic embryos

Desiccated somatic embryos are cryopreserved without cryoprotectant by direct immersion of the embryo-containing 1.2 ml cryovials in liquid nitrogen. The number of embryos per cryovial can vary from 5 to >100 depending on the utilization planned for them. For thawing, cryovials are gently shaken for 5 min under running water set at 38˚C. Embryos are immediately placed in a Petri dish (60 x 15 mm) lined with a 60µm nylon mesh screen for rehydration in the dark under 100% RH for 12 h (Bomal and Tremblay 1999). Somatic embryos can then be used either in germination for plant production or as explants to induce development of embryogenic tissues.

8. CONCLUDING REMARKS

Among the species that we worked with (*Picea glauca*, *P. rubens*, *P. abies* and several hybrid larches), black spruce is the most difficult species when comes time to produce plants. To overcome the difficulties encountered in plant production, efforts were devoted in the last 10 years to understand different physiological aspects of this species in comparison with white spruce that we consider easy. In this way, we studied the microenvironment (El Meskaoui and Tremblay 1999, 2001; El Meskaoui et al. 2000) as well as the sugar dynamics in the medium in relation to carbohydrate metabolism in embryogenic tissues (Iraqi and Tremblay 2001a, b; Iraqi et al. in press). This approach was profitable as it helped to develop a very complete protocol, that is from induction of embryogenic tissues to plants in the field passing through short and long term preservation, desiccation of embryos, in vitro acclimatization, bio-reactor culture, etc. Furthermore, most of these conditions are directly applicable to other species. We are now waiting for the results from the field tests comparing several black spruce clones.

To date, most research work aimed at improving somatic embryogenesis protocols while few studies have focused on plant growth and development resulting from this process. It is therefore of great interest to intensify morphological and physiological quantification at the plant level of the various in vitro treatments. Indeed, independently of genetics, growth of the somatic seedlings is affected by the treatments used during the in vitro culture process (Högberg et al. 2001). As long as the effects of the different in vitro treatments have not been characterized at the plant level, it is hazardous to integrate the somatic embryogenesis into reforestation programmes.. Research in our laboratory is presently oriented toward this goal (Lamhamedi et al. 2000, in press).

REFERENCES

Anandarajah, K. & B.D. McKersie, 1990. Manipulating the desiccation tolerance and vigor of dry somatic embryos of *Medicago sativa* L. with sucrose, heat shock and abscisic acid. Plant Cell Rep. 9: 451-455.

Bomal, C., V.Q. Le & F.M. Tremblay, 2002. Induction of tolerance to fast desiccation in black spruce (*Picea mariana*) somatic embryos: relationship between partial water loss, sugars and dehydrins. Physiol. Plant. 115: 423-530.

Bomal, C. & F.M. Tremblay, 1999. Effect of desiccation to low moisture content on germination, synchronization of root emergence, and plantlet regeneration of black spruce somatic embryos. Plant Cell Tiss. Org. Cult. 56:193-200

Bomal, C. & F.M. Tremblay, 2000. Dried cryopreserved somatic embryos of two *Picea* species provide suitable material for direct plantlet regeneration and germplasm storage. Ann. Bot. 86: 177–183.

Campbell, R.A. & D.J. Durzan, 1975. Induction of multiple buds and needles in tissue cultures of *Picea glauca*. Can. J. Bot. 53: 1652-1657.

El Meskaoui, A., Y. Desjardins & F. M. Tremblay, 2000. Kinetics of ethylene biosynthesis and its effects during maturation of white spruce somatic embryos. Physiol. Plant. 109: 333-342.

El Meskaoui, A. & F.M. Tremblay, 1999. Effects of sealed and vented gaseous microenvironments on the maturation of somatic embryos of black spruce with a special emphasis on ethylene. Plant Cell Tiss. Org. Cult. 56: 201-209.

El Meskaoui, A. & F.M. Tremblay, 2001. Involvement of ethylene in the maturation of black spruce embryogenic cell lines with different maturation capacities. J. Exp. Bot. 52: 761-769.

Hakman, I. & L.C. Fowke, 1987. An embryogenic cell suspension culture of *Picea glauca* (White spruce). Plant Cell Rep. 6: 20-22.

Högberg, K.-A; Bozhkov, P.V., Grönroos, R. and S. Von Arnold.2001. Critical factors affecting ex vitro performance of somatic embryo plants of Picea abies. Scan. J. For. Res. 16: 295-304

Iraqi, D., V.Q. Le, S.M. Lamhamedi & F.M. Tremblay. Sucrose utilization during somatic embryo development in black spruce: Role of apoplastic invertase in the tissue and of extracellular invertase in the medium. J. Plant Physiol. (in press).

Iraqi, D. & F.M. Tremblay, 2001a. The role of sucrose during maturation of black spruce [*Picea mariana* (Mill.) BSP] and white spruce [*Picea glauca* (Moench) Voss] somatic embryos. Physiol. Plant.111: 381-388.

Iraqi, D. & F.M. Tremblay, 2001b. Analysis of carbohydrate metabolism enzymes and cellular levels of sugars and proteins during spruce somatic embryogenesis suggests a regulatory role of exogenous sucrose in embryo development. J. Exp. Bot. 52: 2301-2311.

Lamhamedi, M. S., Chamberland, H., Bernier, P. Y. et F. M.Tremblay, 2000. Clonal variation in morphology, growth, physiology, anatomy and ultrastructure of container-grown white spruce somatic seedlings. Tree Physiol. 20 :869-880;

Lamhamedi, M.S., H. Chamberland, & F.M. Tremblay. Epidermal transpiration, ultrastructural characteristics and net photosynthesis of white spruce somatic seedlings in response to in vitro acclimatization. Physiol. Plant. (In press).

Litvay J.D., D.C. Verma & M.A. Johnson, 1985. Influence of a loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.). Plant Cell Rep. 4: 325-328.

Rockland L.B., 1960. Saturated salt solutions for static control of relative humidity between 5° and 40°C. Anal. Chem. 32:1375-1376.

Tautorus, T.E. & S.M. Attree, 1990. Somatic embryogenesis from immature and mature zygotic embryos, and embryo regeneration from protoplasts in black spruce (*Picea mariana* Mill.). Plant Sci. 67: 115-124.

Tremblay, F.M., 1990. Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. Can. J. Bot. 68: 236-242.

Tremblay, L., C. Levasseur & F.M. Tremblay, 1999. Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability. Amer. J. Bot. 86: 1373-1381.

Tremblay, L. & F.M. Tremblay, 1995. Somatic embryogenesis in black spruce (*Picea mariana* (Mill.) B.S.P.) and red spruce (*P. rubens* Sarg.). In: Y.P.S. Bajaj (Ed). Biotechnology in agriculture and forestry, vol. 30, pp. 431-445. Somatic embryogenesis and synthetic seed I, Springer-Verlag. Berlin, Heidelberg.

Table 1. Composition of the different media used for the different stages of somatic embryogenesis in black spruce.

	Induction and maintenance medium (HLM-1)' (mg/l)	Maturation medium (mg/l)	Germination medium (mg/l)
	(except when otherwise stated)		
NH_4NO_3	825	825	400
KNO_3	950	950	170
KH_2PO_4	170	170	8.5
KCl	-	-	32.5
$MgSO_4.7H_2O$	925	925	185
$Ca(NO_3)_2.4H_2O$	-	-	490
$CaCl_2.2H_2O$	11	11	-
KI	2.075	2.075	0.415
H_3BO_3	15.5	15.5	3.1
$MnSO_4.H_2O$	10.5	10.5	8.4
$ZnSO_4.7H_2O$	21.5	21.5	4.3
$Na_2MoO_4.2H_2O$	0.625	0.625	0.125
$CuSO_4.5H_2O$	0.25	0.25	0.0125
$CoCl.6H_2O$	0.065	0.065	0.0125
Fe DTPA	40	40	20
Nicotinic Acid	0.5	0.5	-
Pyridoxine-HCL	0.1	0.10	-
Thiamine-HCl	0.1	0.10	2.5
Myo-inositol	100	100	250
Casamino Acids	1 000	1 000	-
Glutamine	500	1 000	-
Sucrose	1%	6%	1.5%
BA	5 □M	-	-
2-4,D	10 □M	-	-
ABA	-	25-60 □M	-
Difco Bacto-agar	0.7%[1] or 1%[2]	-	-
Gellan gum	-	0.6-0.8%	-
pH	5.7	5.7	5.7

[1]Induction medium; [2]Maintenance medium

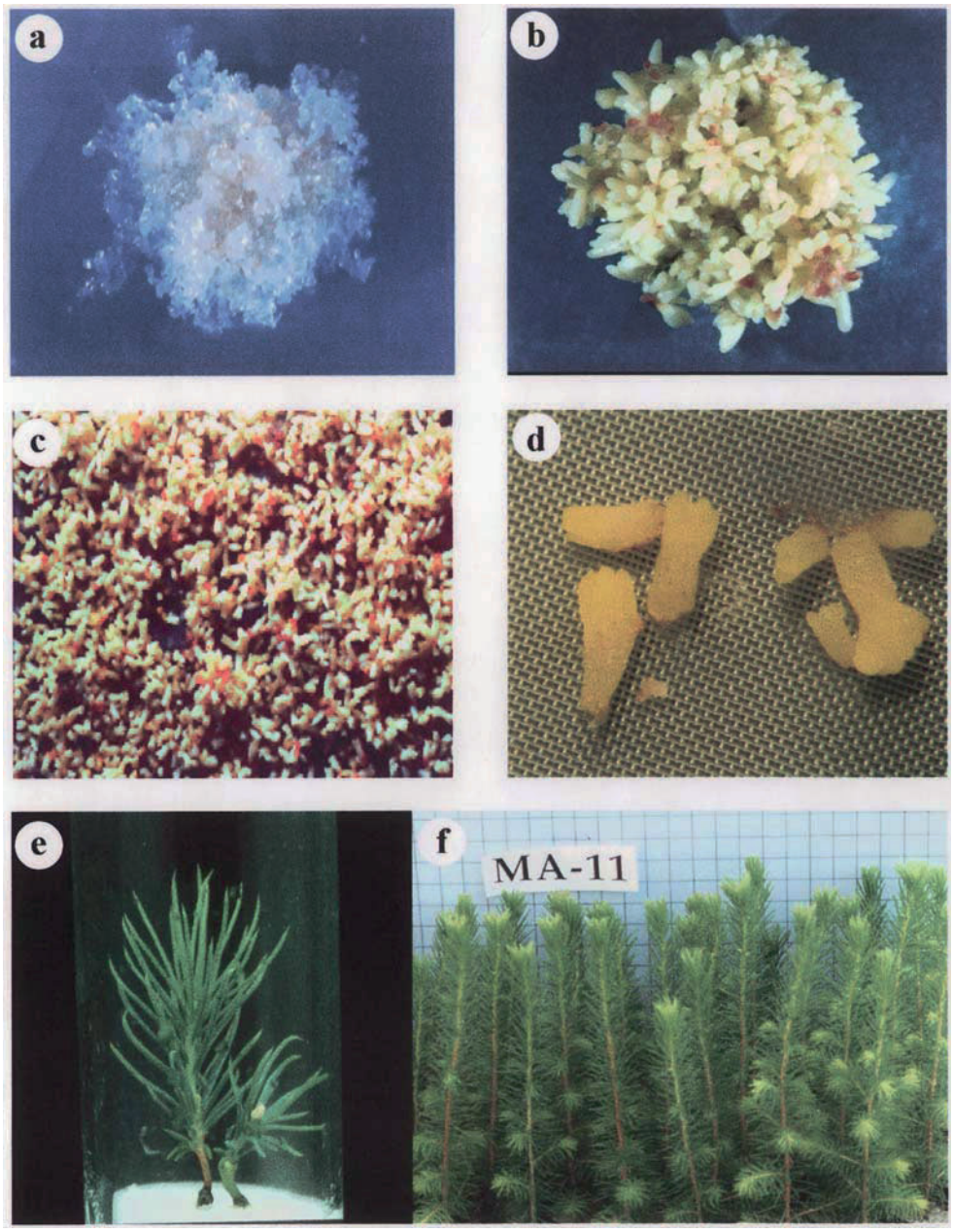

Figure 1. Somatic embryogenesis in black spruce: (a) Proliferation of embryogenic tissue on maintenance medium. Somatic embryo matured according to (b) protocol I and (c) protocol II. (d) Dried somatic embryo on a nylon screen. (e) Somatic embryo-derived plantlets under germination conditions. (f) Somatic embryo-derived plants growing under greenhouse conditions, 3 months after soil transfer.

SITKA SPRUCE (*PICEA SITCHENSIS*)

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1. INTRODUCTION

Sitka spruce is the largest of the world's spruces and is native to a thin narrow coastal strip along the Pacific Ocean from south-central Alaska (61 ^{O}N) to northern California (39 ^{O}N) or a distance of almost 3,000 km. It grows in elevation from sea level to 900 m but is more common below 300 m. Although Sitka spruce is not a major commercial timber species in its native species range (reasons discussed later) it is an important commercial species in Ireland and the United Kingdom where it thrives on the type of sites available for forestry. It has also been grown commercially in Denmark, northwestern France and southern Sweden.

Sitka spruce wood is used for pulp, paper, wood based panels and sawn timber. It is noted for its strength in relation to its lightness. Sitka spruce was important during World War I because it provided a strong, lightweight timber for aircraft frames. For pulp and paper the white wood and long fibers are very desirable. The clean white wood, that nails easily is well suited for sawn timber, including carcassing and structural uses.

Sitka spruce grows best in a maritime climate with abundant rainfall (1,000 to 3,000 mm/year) throughout the year, mild winters and cool summers and an absence of extreme heat or cold. It is the least frost tolerant of all the spruce species. It grows in association with western hemlock (*Tsuga heterophylla*) throughout its natural range and with western red cedar (*Thuja plicata*) and Douglas-fir (*Pseudotsuga menziesii*) in the southern part of its range. Stands of Sitka spruce tend to be very dense and high yielding due to the ability of the species to tolerate moderate shade. It is a vigorous and fast growing species that can rapidly overtop other species to become dominant. In Alaska mature trees at sea level can exceed 61m in height, while in Oregon trees between 66 to 87 m have been recorded. The average productivity of Sitka spruce across all sites in Ireland is 17 m^3/ha/year.

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Sitka spruce grows on a wide range of soils from sand dunes to glacial outwashes to alluvial soils to well drained better peats. It does best on soils derived from rock with a high calcium and magnesium content. It prefers a deep, moist, well aerated soil, usually with a high organic matter content. Good drainage is critical to success of the species. In spite of the fact that the species is also known as "Tidewater Spruce" it cannot tolerate long periods when the roots are under anaerobic conditions. As a coastal species it tolerates salt spray.

There are relatively few damaging agents that affect Sitka spruce. Windthrow is perhaps one of the most important non-biological agent. The Sitka spruce or white pine weevil (*Pissodes strobi*) is the most damaging insect pest in Oregon, Washington and southern British Columbia This insect attacks young trees 3 to 6 m tall making the tree commercially useless. The damage done by this insect has limited its planting as a commercial species in western North America. Recently some populations with weevil resistance have been identified which has led to an increased interest in planting Sitka spruce in southern British Columbia. Fortunately this insect does not occur in western Europe. The large pine weevil (*Hylobius abietis*) causes losses in recently planted stock of a range of species including Sitka spruce in European forests. The root rots caused by the fungi *Heterobasidion annosum* and *Armillaria mellea* can cause losses. The green spruce aphid (*Elatobium abietnium*) causes needle losses which reduces growth, but outbreaks are sporadic and usually short-lived. The spruce beetle *Dendroctonus rufipennis* can kill Sitka spruce.

Sitka spruce regenerates naturally by seed provided a suitable seed bed is available. It requires a mineral or mixed mineral and organic matter seed bed under light shade with adequate moisture, but also well drained. Flowering generally takes place in trees over 20 years-old but maximum seed production begins at age 30 to 40 years-old. In its natural habitat Sitka spruce produces a good cone crop every 3 to 4 years with light seed crops in between. In western Europe good seed crops occur once every 3 to 7 years and in some years no seed crop is produced. Flower stimulation techniques (combined water stress, warm air temperatures and injection with gibberellic acid GA 4/7) have been developed and make seed production more reliable. Pollen is released in mid-April to mid-May and pollination occurs in late April to late May. Cones ripen in mid-September to early October. The seed is small (460,000/kg). Sitka spruce is reported to naturally air-layer under very humid conditions. Grafting is possible with a 50 to 60% success rate. Rooted cuttings are possible from stock plants up to 10 years-old before the rooting percentage declines and plagiotropism increases to adversely affect the quality of the rooted cutting. Nevertheless large-scale commercial rooted cutting propagation facilities currently produce several million improved Sitka spruce cuttings in the UK and Ireland each year. In vitro organogenesis propagation

methods have been developed (Mac an tSaoir et al., 1992; Drake et al., 1997), but due to limited production rates and high labour costs these propagation methods have not be used commercially. Somatic embryogenesis, first reported in Sitka spruce by both von Arnold and Woodward and Krogstrup et al. in 1988, has been very successful. In fact white pine weevil resistant clones of Sitka spruce are currently commercially produce using somatic embryogenesis in Canada (El-Kassaby et al., 2001).

2. MATERIALS

2.1 Materials, Equipment and Supplies

1. Commercial bleach, Tween-20 wetting agent and sterile distilled water
2. Laminar air flow hood, petri plates, forceps, and scalpel
3. Immature female cones or mature seed
4. Stereo microscope

2.2 Media Preparation

The Initiation/Maintenance and Development/Maturation media are based on media used by Gupta and Pullman (1993) while the Conversion medium is based on that used by Krogstrop et al. (1988).

All growth regulators are dissolved in 1 N NaOH to produce stock solutions.

All solidified media has the pH adjusted to 5.7 prior to the addition of sucrose and agar and prior to autoclaving. Liquid media has the pH adjusted to 5.0 prior to the addition of sucrose and autoclaving.

Phytagel (Sigma) is used as the solidifying agent in the Initiation/Maintenance medium while Sigma agar is used in the Development/Maturation and Conversion media.

The activated charcoal used is acid washed, neutralized charcoal.

All components of the media are autoclaved together with the exception of ABA.

ABA is filter sterilized and added to the cooling autoclaved Development/Maturation medium (already containing the activated charcoal) in a water bath set at 40 ^{O}C and stirred for 10 minutes before pouring.

2.3 Culture Conditions

All cultures in the Initiation/Development, Development/Maturation and Partial Desiccation stages are maintained in the dark at 22 to 25 ^{O}C.

Matured somatic embryos in the Conversion stage are cultured in the dark at 22 to 25 ^{O}C for the first 5 to 7 days and are then transferred to the light (42 micromoles/second/m^2) under a 16 hour photoperiod at 22 to 25 ^{O}C.

Table 1 Media for the production of Somatic Embryos of Sitka spruce (in mg/l) .

		Initiation & Maintenance		Development & Maturation		Conversion
Macronutrients						
NH_4NO_3		206		206		360
KNO_3		2340		1170		506
$Ca(NO_3)_2$ 4 H_2O		----		----		709
KH_2PO_4		85		85		272
$CaCl_2$ 2H_2O		220		220		----
$MgSO_4$ 7H_2O		185		185		493
KCl		----		----		149
$FeSO_4$ 7H_2O		13.9		13.9		13.9
Na_2EDTA	18.6		18.6		18.6	
Micronutrients						
H_3BO_3		3.1		3.1		3.1
KI		0.4		0.4		0.4
Na_2MoO_4 2H_2O		0.125		0.125		0.125
$CoCl_2$ 6H_2O		0.125		0.125		0.125
$MnSO_4$ H_2O		8.45		8.45		8.45
$ZnSO_4$ 7H_2O		4.3		4.3		4.3
$CoSO_4$ 5H_2O		0.0125		0.0125		0.0125
Organics						
Myo-inositol		1,000		1,000		1,000
Casamino acids		500		500		----
L-glutamine		450		450		450
Thiamine	1.0		1.0		1.0	
Pyridoxine	0.5		0.5		0.5	
Nicotinic acid		0,5		0.5		0.5
Arginine		----		40		----
Asparagine		----		100		----
Growth Regulators						
2,4-D		1.1		----		----
BAP		0.45		----		----
Kinetin		0.45		----		----
ABA		----		50		----
Activated charcoal	----		1,250		----	
Other Additions						
Sucrose		30,000		30,000		30,000
Phytagel		2,000		----		----
Agar		----		7.000		7,000

3. METHODS

The production of emblings of Sitka spruce can be divided into 6 major steps: 1) initiation of the embryogenic cell lines, 2) maintenance of the Embryonal Suspensor Mass (ESM), 3) development and maturation of somatic embryos, 4) partial desiccation, 5) conversion of somatic embryos to emblings and 6) their transfer to soil and growth under ex vitro conditions.

3.1 Initiation of Embryogenic Cultures

Zygotic embryos from either immature cones or mature seed can be used to initiate embryogenic cultures. The frequency of success is much lower with mature seed (2 to 7%), but mature seed is more readily available.

Immature cones provide a higher success rate (30 to 60%), but are 1) available only at a specific time of the year, 2) the "window" for the optimum time of culture is narrow and variable and 3) immature cones may be difficult to collect or simply not available. Optimum time for culturing immature embryos presently appears to be about 8 weeks after pollination (Dr. Allan John, Forest Research, Forestry Commission, Roslin, Midlothian, Scotland, personal communication), but the "window" extends one to two weeks before and after this date. However, because zygotic embryos from different clones vary in their rate of development and variations in weather conditions occur from year to year during zygotic embryo development, bracketing of this 8 week post-pollination date is advisable. The optimum stage appears to be before cotyledons begin to develop on the immature zygotic embryos.

1. For immature zygotic embryos, remove the developing seed from the cone.
2. Surface sterilize the seeds with 10% commercial bleach for immature embryos and 20% bleach for mature seed together with several drops of Tween 20 for 10 minutes.
3. Rinse three times with sterile distilled water.
4. Place the seed in a sterile petri dish.
5. Use a scalpel and forceps to remove the seed coat and female gametophyte tissue to expose the zygotic embryo (best accomplished under a stereo microscope).
6. Place excised zygotic embryo on a petri dish containing the Initiation/Maintenance medium (4 to 5 embryos can be cultured per plate).
7. Wrap petri dishes with Parafilm and place in the dark at 22 to 25 ^{O}C.
8. Examine plates every 3 to 4 days for contamination and reculture if necessary.
9. Embryogenic Suspensor Mass (ESM) will begin to appear after 6 to 12 weeks.

3.2 Maintenance of Embryonal Suspensor Mass (ESM)

After 6 to 12 weeks a white, translucent, mucilaginous mass will develop from some of the cultured zygotic embryos. This is not a callus because it consists of organized embryonic heads and suspensor cells and is therefore referred to as an Embryonal Suspensor Mass (ESM). Once the ESM has reached 1 cm in diameter they can be separated from the original zygotic embryo and subcultured onto fresh Initiation/Maintenance media. In order to maintain these cell lines in good condition they are subcultured to fresh media every two weeks and are maintained in the dark at 22 to 25 ^{O}C. Up to 50% of the ESM cultures may fail to grow even when subcultured to fresh Initiation/Maintenance media. Those cell lines that do become established can be divided into quarters (50 to 75 mg pieces) every two weeks and 5 to 6 of these ESM masses placed on a fresh plate of Initiation/Maintenance media to produce tissue for the formation of somatic embryos.

3.3 Establishing Suspension Cultures

Suspension cultures can be used to grow up large amounts of ESM to regenerate emblings, but they are not essential for the production of emblings by somatic embryogenesis. While the cells in the suspension culture may be physiologically more uniform than ESM grown on solidified media, ESM grown on solidified media can produce embryos just as well as cell suspension cultures. Although establishing cell suspension cultures can be difficult, once established they are easy to maintain.

1. Transfer 1 gram (fresh weight) of ESM into a 125 ml Erlenmeyer flask (one with baffles in the base can help break up ESM masses) containing 25 ml of liquid Initiation/Maintenance media.
2. Place the flask on a rotary shaker (about 100 rpm) in the dark at 22 to 25 ^{O}C.
3. After 2 weeks remove as much liquid media as possible with a sterile pipette and replace with fresh liquid Initiation and Maintenance media.
4. Continue to replace the liquid media every 2 weeks until a good cell suspension culture is established.

3.4 Development and Maturation of Somatic Embryos

When sufficient amounts of ESM have been grown up either on solidified or liquid Initiation/Maintenance media, the next step is to develop and mature the somatic embryos. Up until this stage somatic embryos will not have been visible to the naked eye.

Treatment with a combination of Abscissic acid (ABA) and activated charcoal (AC) as proposed by Gupta and Pullman (1993) has been found to be the most effective method to induce somatic embryo development and maturation under our conditions. While some cell lines may produce well developed and matured somatic embryos on media containing only ABA (at lower concentrations), many more do well on the combination of ABA and AC and for this reason the combined ABA/AC treatment is routinely used in this laboratory.

1. Transfer either masses of ESM (200 to 300 mg pieces) or plate out ESM cells from suspension cultures (1 to 2 ml with a minimum of liquid culture media) onto a petri dish of Development/Maturation media and place in the dark at 22 to 25 ^{O}C for 8 weeks without re-culturing to fresh media.
2. After 8 weeks if well developed somatic embryos with cotyledons have formed, they can either be transferred to a Partial Desiccation treatment or to the Conversion medium. If the Somatic embryos have not developed distinct cotyledons, they can be left on the Development/Maturation media without subculture to fresh Development/Maturation media for further development.

3.5 Partial Desiccation of Somatic Embryos

Partial desiccation of somatic embryos of Sitka spruce, while adding an additional step and the resulting increased production costs, has nevertheless been reported to result in a higher quality somatic embryo and subsequently a better quality embling (Roberts et al., 1990). Results in this laboratory suggest that partial desiccation may be useful in improving the root growth in cell lines that have been in culture for extended periods of time, whereas recently initiated cell lines do not benefit as much from the partial desiccation treatment.

We have used two methods of partial desiccation including A) a slight desiccation involving culture in a reduced relative humidity environment and B) culture on the Development and Maturation medium which contains a higher than normal gellan gum. This has the benefit of combining the Development/Maturation and Partial Desiccation steps.

Method A.

1. Well developed cotyledonary somatic embryos are removed from the maturation medium and placed in a plastic 25 cell multi-well plates. The central well and the outer wells are ½ filled with sterile distilled water and approximately 10 somatic embryos are placed in each of the 8 remaining wells.
2. The multi-well plates containing the somatic embryos are wrapped with Parafilm and cultured for 3 weeks in the dark at 22 to 25 ^{O}C.
3. After the 3 weeks of partial desiccation the somatic embryos are transferred to Conversion medium.

Method B.

The use of higher than normal concentrations of gellan gum in the maturation media is to induce mild water stress in the somatic embryo (Klimaszewska and Smith, 1997).

1. EMS tissue directly from Initiation and Maintenance medium is transferred to Development and Maturation medium containing 4% Phytagel for 8 weeks in the dark at 22 to 25 ^{O}C.
2. After the 8 weeks the developed somatic embryos are transferred to Conversion medium.

Work is ongoing to document the benefits of Partial Desiccation in comparison with the costs of this step in order to determine if it has a place in a large-scale production protocol. Full desiccation treatments involving the use of Polyethylene Glycol have not been tested.

3.6 Conversion of Somatic Embryos to Emblings

After 3 weeks of Partial Desiccation the somatic embryos are removed from the multi-well plates and placed on petri dishes containing the Conversion medium.

1. In order to minimize the time required for this step, somatic embryos are selected without the aid of a stereo microscope for those that will go on to the Conversion medium.
2. Selection of "quality" somatic embryos is essential in the conversion of somatic embryos to emblings. A "quality" Sitka spruce somatic embryos is defined as a bipolar structure with a minimum of three distinct cotyledons. Somatic embryos on the Conversion medium (modified BMG media based on Krogstrup, 1988) is used and approximately 15 "quality" somatic embryos are cultured per Petri dish.

3. Good quality somatic embryos are cultured in the dark at 22 to 25 ^{O}C for the first 5 to 7 days and then transferred to the light (42 micromoles/second/m^2) under a 16 hour photoperiod.
4. After 4 to 8 weeks the shoot and root begins to elongate and the somatic embryos begin to develop into emblings.
5. As the shoot and the root elongate the emblings may need to be transferred to a larger container, especially if it will be necessary to hold the emblings before they can be transferred to soil.

3.7 Transfer to Soil

Transfer to ex-vitro conditions or "acclimitisation" is necessary not only to encourage the embling become autotrophic but also to help it adjust from the high levels of relative humidity and low light levels in the culture vessel to lower relative humidity and higher light levels ex-vitro. This is typically carried out in a greenhouse or plastic tunnel equipped with either a fogging or misting system. The timing of these operation may also be critical with mid-spring and mid-autumn perhaps the best times because of both moderate temperatures and acceptable light levels in the acclimitisation environment. Having said this Sitka spruce is a robust species and the acclimitisation process for this species presents no major problems even under relatively primitive misting systems, provided some care is taken.

1. Emblings with a shoots of 2 cm and a root of 1 cm are removed from the culture container, the agar washed from the roots. Emblings with smaller shoots and roots can be acclimitised, but they require greater care in the process. Care should be taken to prevent the emblings from drying out.
2. Emblings are planted in 100 cc cells of a HIKO or similar container containing 1:1 or 2:1 peat:perlite and placed under either a misting or fogging system initially set to maintain a high relative humidity (85 %). Slow release fertilizer can be incorporated into the potting media or liquid fertilizer can be applied during watering. Shading may need to be applied to the greenhouse or tunnel to prevent excessive air temperatures.
3. Over the next 4 to 8 weeks the relative humidity around the emblings is gradually reduced to reach ambient conditions and the emblings are then grown on under normal greenhouse conditions.
4. Embling will be grown for 1 growing season (6 months) in containers before they would either be lined out in a nursery bed or potted into larger pot for further growth.

3.8 Cryostorage of Embryonal Suspensor Mass

Cryogenic storage methods for the ESM of conifers, including Sitka spruce, will be described in another chapter of this volume.

4. CONCLUSIONS

Somatic embryogenesis offers a technique with the potential to multiply genetically improved material from tree improvement programmes for commercial-scale production faster than would normally be possible using conventional methods. However, claims have been made about the efficiency of the technique and the numbers of plants it is capable of producing are generally based on research-scale studies and should be viewed with caution.

All attempts to try and improve the efficiency of the somatic embryogenesis process in Sitka spruce to date including alterations in the Initiation/Maintenance, Development/Maturation and Conversion media including changes in both nutrients and the growth regulator regimes have not lead to any significant improvement in the process. The challenge is therefore to try and make the process more reliable (working efficiently with all families and individuals) as well as making it more efficient (to produce more "convertible" embryos). The high labour steps in the process are not in the growing up of large amounts of ESM tissue, but rather in the last several in vitro steps where individual emblings are handled. While automation may overcome this problem, it is likely to be an expensive solution.

Recognizing and selecting "high quality" somatic embryos is subjective as well as a very time consuming and thus a costly step. Therefore either a simple, rapid methods for screening out high quality somatic embryos or, preferably, treatments to increase the overall frequency of high quality embryos are needed.

Regarding the numbers of plants possible with this technology, while it is true that large numbers of somatic embryos can be produced in a small amount of space on a year-round basis, this ignores the fact that a very large number of these potential somatic embryos are lost during the process and never form somatic embryos capable of producing a useable propagule. More work needs to be done on comparing and contrasting the differences and similarities between zygotic and somatic embryos and the environment in which they form and develop. An increased knowledge of zygotic embryo development should be directly applicable to improving the production of somatic embryos.

While claims have been made that embling production costs will be less than that of rooted cuttings and even approach seedling production costs, there is little evidience to support these claims. Indeed very little information on the costs to produce emblings is available. Like all micropropagation methods, somatic embryogenesis is labour intensive, although not as labour intensive as organogenesis. As mentioed above automation is seen as the solution. Even if this is true, this will mean that embling production costs will probably always be more expensive than seedlings, but could be equal to or perhaps slightly less than rooted cutting production costs. Rooted cutting technology provides the greatest competition to somatic embryogenesis as an alternative vegetative propagation system.

In our tree improvement programme somatic embryogenesis is being used to multiply the limited amounts of seed of the best families from our breeding programme for use as stock plants which will provide cuttings for rooting which will deliver the improved material to the field (bulk propagation). Estimates of the production costs of Sitka spruce emblings are too high for direct planting in the field at this time. If however the production cost is spread over the several hundered rooted cuttings each stock plant will produce during its lifetime, then the higher embling production costs become much more acceptable.

Finally, the potential ability to initiate embryogenic cell lines from proven superior individuals is an objective that should not be forgotten.

REFERENCES

Von Arnold, S. and S. Woodward. 1988. Organogenesis and embryogenesis in mature zygotic embryos of *Picea sitchensis*. Tree Physiol. 4:291-300.

Drake, P.M.W., A. John, J.B. Power and M.R. Davey. 1997. Cytokinin pulse-mediated shoot organogenesis from cotyledons of Sitka spruce [*Picea sitchensis* (Bong. Carr.] and high frequency in vitro rooting of shoots. Plant Cell Tissue and Organ Culture 50:147-51.

El-Kassaby, Y.A., R.I. Alfaro. J. King, C.C. Ying, A. Yanchuk and I. Leaf. 2001. Somatic embryogenesis as a delivery system for specialty products with reference to resistant Sitka spruce to the white pine weevil. In: Proc. Of the 26th Southern Forest Tree Improvement Conference, June , 2001, Athens, Georgia (pp 154-68).

Gupta, P.K. and G.S. Pullman. 1993. Method for reproducing conifers by somatic embryogenesis using stepwise hormone adjustment. US Patent 5,236,841, 17 August 1993.

Klimaszewska, K. and D.R. Smith. 1997. Maturation of somatic embryos of *Pinus sylvestris* is promoted by high concentration of gellan gum. Physiol. Plant. 100: 949-57.

Krogstrup, P., E.N. Eriksen, J.D. Moller and H. Roulund. 1988. Somatic embryogenesis in Sitka spruce (*Picea sitchensis* (Bong.) Carr.). Plant Cell Reports 7:594-7.

Mac an tSaoir, S., J. O'Brien and C. Selby. 1992. The effect of explant type on the establishment of Sitka spruce (*Picea sitchensis*(Bong.) Carr.) in culture. Annals of Botany 69:161-5.

Roberts, D.R., B.C.S. Sutton and B.S. Flinn. 1990. Synchronous and high frequency germination of interior spruce embryos following partial drying at high relative humidity. Can. J. Bot. 68:1086-90.

PROTOCOL OF SOMATIC EMBRYOGENESIS OF *PINUS NIGRA* ARN.

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1. INTRODUCTION

Somatic embryogenesis in conifers is an effective tool for study of the conifer embryogenic pathway, and in general it contributes to understanding of fundamental questions of conifer biology (Kong et al.1999). In several conifer species the method appeared to be an effective method for plantlet production in large scale, available for plantation in forest (Grossnickle and Major 1994). Somatic embryogenesis provides a way to clonally propagate commercially valuable conifers of several species and offers an *in vitro* experimental sytem to study embryogenesis (Mirsa 1994), cell differentiation and totipotency. The main advantage of embryogenic cultures for clonal propagation of conifers is on the feasibility to automate this propagation, on the easiness to handle the final product, *i.e.* somatic embryos (Tautorus at al. 1991, Gupta et al. 1993) somatic seedlings (Gupta and Pullman 1990, 1991) and/or artificial seedlings (Gray and Purohit 1991, Attree and Fowke 1993).

In the genus Pinus somatic embryogenesis has been initiated in many species (Gupta and Durzan 1986, Finer et al. 1989, Salajova and Salaj 1992, Attree and Fowke 1993, Jones et al. 1993, Jain et al. 1995). The initiation process has been influenced by several factors, among them the developmental stage of zygotic embryo, the composition of culture medium and genotype have been important. Somatic embryogenesis initiation, as with other morphogenic responses, is in general genotype-

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dependent. Its is well known that embryogenesis induction in Pinus is influenced by the origen of the seed populations (Becwar et al. 1990). The influence of the seed population in achieving somatic embryogenesis induction can be due not only to yearly difference, but also the physiological stages. Since conifers show very irregular flowering habits, ther is not reason to assume that all the seeds collected in one period are the same physiological stage. Therefore storage nutrients and growth regulators content in the seed may vary considerably. Their role in embryogenic tissue induction has been already established (Tautorus et al. 1991). The importance of seed storage temperature seems to be also critical for succesful somatic embryogenesis induction.

Zygotic embryos at the precotyledonary stage of development have been the best explants for many *Pinus* species, e.g. *Pinus strobus* (Finer et al. 1989; Klimaszewska and Smith 1997), *Pinus pinaster* (Bercethe and Paques 1995; Lelu et al. 1999; Miguel et al. 2000), *Pinus sylvestris* (Keinonen-Mettala et al. 1996; Lelu et al. 1999; Hägmann et al. 1999), *Pinus caribaea* (Laine and David 1990), *Pinus taeda* (Becwar et al. 1990), *Pinus monticola* (Percy et al. 2000). The culture medium has also influenced the initiation. Plant growth regulators, auxins and cytokinins, have been used successfully in most of species. Plant growth regulator content in the medium has profoundly affected not only the initiation but later also the somatic embryo maturation (Klimaszewska et al. 2001). In *Pinus sylvestris* and *Pinus pinaster* somatic embryogenesis initiation occurred also in the absence of exogenous plant growth regulators (Lelu et al. 1999).

The embryogenic tissues usually are maintained on culture media for shorter or longer period. Somatic embryos, occurring in embryogenic tissues have been described as bipolar structures with embryonal part, consisted of meristematic cells and suspensor consisted of long vacuolised cells. These structures have been observed in most of cultures but differences in their morphology have been noticed among cell lines (Lainé and David 1990; Bercetche and Paques 1995; Keinonen-Mettala 1996).

After appropriate treatment and conditions, early somatic embryos underwent maturation. Well-structured somatic embryos have relatively high capacity for maturation and are capable of development on wide range of media including liquid media (Ramarosandratana et al. 1999). Maturation treatments usually include abscisic acid and osmotica. The ABA concentrations ranged from low levels e.g. 5.2 μM for *Pinus patula* (Jones and van Staden 1995) to as high as 120 μM for *Pinus strobus* (Klimaszewska and Smith 1997).

Extremely high concentrations (*e. g.* 300 μM for *Pinus monticola*) were less effective (Percy et al 2000). Carbohydrates have been found to be significant factor for

maturation process. In *Pinus pinaster* (Ramarosandratana et al. 2001) and *Pinus strobus* (Garin et al. 2000) sucrose improved somatic embryo maturation. In both cases sucrose was combined with high gellan gum content or manitol. The maturation process is terminated by formation of cotyledonary somatic embryos. These structures are capable of germination, usually on hormone free media, and plantlet regeneration. The regenerated emblings are transferred to soil.

2. INDUCTION OF EMBRYOGENIC TISSUE

Conifers, especially *Pinus* spp., have been recalcitrant to somatic embryogenesis induction. For the most part, induction in *Pinus* spp has been accomplished with explants of zygotic embryos at the pre-cotyledonar stages (Becwar et al.1988, Salajova et al. 1994). Thus the full potential of somatic embryogenesis as a means of propagating conifers is presently limited by the nature of the explants used (embryogenic and juvenile stages). In *Pinus nigra* Arn. embryogenic tissue has been initiated from immature zygotic embryos enclosed in megagametophytes (Salajova and Salaj 1992; Salajova et al. 1994; Salajova et al. 1999). Attempts to obtain embryogenic tissue from mature zygotic embryos or cotyledons dissected from seedlings used as explants are normally unsuccessful despite of testing various combination and concentrations of plant growth regulators and high number of explants. Preliminary firts results obtained with mature embryos are included (Radojevic et al. 1999).

2.1. Induction from immature zygotic embryos

The green cones are collected from open pollinated trees growing at natural stand. In the first series of experiments (years 1989, 1990, 1991) the collection period of cones containing seeds with zygotic embryos ranged from June to August. In these experiments we have investigated the explants response for initiation. The initiation frequencies drop by maturation of zygotic embryos (Salajova and Salaj 1992; Salajova et al. 1994). The most responsive explants (to initiation of embryogenic tissue) are collected in second half of June and in following experiments we collected explants exclusively in this period time (Salajova et al. 1999; Salajova and Salaj, in preparation).

The immature seeds are excised from green cones and surface sterilized with 10% H_2O_2 for ten min. Following, the seeds are rinsed with sterile distilled water 3-4 times. The megagametophytes are isolated from immature seeds and place on culture medium. The induction medium DCR consisted macro-and microelements and vitamins according to Gupta and Durzan (1985). Plant growth regulators 2,4-D and BA, L-glutamine, enzymatic caseine hydrolysate, and sucrose are also added. The

medium is solidified with 0.3% gelrite (Table 1). The pH is adjusted to 5.7-5.8 with 1M KOH before autoclaving at 121ºC 20 min. The medium is poured into petri plates (12.5 ml to plate 6 cm in diameter). In each plate 8 to10 explants are cultivated. The induction has been realized in culture room at 25ºC in dark.

Before culturing, several megagametophytes are opened (40-50 per experiment) and the zygotic embryo developmental stage is checked under dissected microscope.

The initiation of somatic embryogenesis starts by protruding of embryogenic tissue from micropylar end of megagametophyte around 12-14 days after explant culture (Figure 1A). Most of the initiation occurs during the first six weeks of cultivation of megagametophytes. After this period the initiation is occasional. The initiation frequency differs year by year, reaching values 1.53-24.1%. The initiation frequencies are profoundly affected by the developmental stage of zygotic embryo in the time of seed collection. The embryogenic tissues are transferred to maintenance medium when they reach at least 0.5 mm in diameter.

2.2. Induction from mature zygotic embryos

Mature seeds of salgareño pine, *Pinus nigra* Arn. ssp. salzmannii (Dunal) Franco form "hispanica", pop ESO74 obtained form selected open-pollinated trees in natural stands (La Dehesa de los Palancares, Cuenca, Spain) and kindly provided by ICONA (Instituto para la Conservación de la Naturaleza, Spain) in 1994, are stored at least 2 months at 4-5ºC before use. After removal of the seed coat, seeds are surface sterilized for 15 min with hydrogen peroxide (7.5% vol/vol) and rinse four times with sterile distilled water. After 48 h imbibition in sterile double distilled water at 4-5ºC, embryos (5 mm) with six cotyledons are isolated in sterile conditions and five embryos are placed in each petri dish with the defined medium. Before autoclaving, the pH of the media is adjusted to 5.8 and Rokoagar AB-50889 agar (Roko, S.A., Spain) is added at 7 g l^{-1}. Growth regulators are added before pH adjustment. Cultures are maintained for 15 to 80 days in a growth chamber at 25 $\pm$ 2ºC in the dark.

Both L1 and L2 media are effective in embryogenic induction, both media have the same phytohormone composition, but L1 has MS mineral salts, whereas L2 medium is based on DCR mineral salts (Table 2). After 2 weeks of culture on both media, the excised cotyledons proliferate into proembryogenic tissue. Callus is located at the basal and medial portions of the explants, and in subsequent cultures, leads to proliferation of white translucent mucilaginous masses. Furthermore, under these conditions, excised hypocotyls and radicles also show embryogenic tissue induction. However, the callus-derived lines from these two explant tissues are non-embryogenic and die after

two subcultures. The nature of induced embryogenic tissues is different depending on the mineral salts used, a prolific growth occurs on DCR mineral salts, which also supports greater development of suspensor masses. As the culture time increases, non- embryogenic callus also forms around the initial embryogenic tissues. After 6 weeks of culture the amount of embryogenic tissue is sufficient to transfer to the fresh media for embryo development. However, before transfer, somatic proembryo development is occasionally observed in two inductive media after 2 weeks of culture.

Embryogenic processes are facilitated and accelerated by using DCR mineral solution (Gupta and Durzan 1985). QLP (Quorin and Le Poivre 1977) medium stimulates further somatic embryos and plantlet development.

Transfer of isolated somatic embryos from the basal medium to L_3 medium (Quorin and Le Poivre modified mineral solution without hormones and supplemented with 0.3% activated charcoal), facilitate random embryo maturation and some development into plantlets.

For plantlet development, the effect of a 16 h photoperiod under 80 μmol m^{-2} s^{-1} provided by cool-white light fluorescence tubes (Sylvania, Germany, F40 w/154-RS) was assessed for a period of 30 days.

The success in the establishment of embryogenic tissue lines described, could be due to the origen of the explants used. Whereas protocols for immature embryos are defined for *P. nigra* form austriaca“, the cited responses obtained for mature embryos are achieved by the use of *P. nigra* form “hispanica“ (pop.ES074), maintained at 4^0C for a minimum period of 2 months as has been described for *Picea glauca* (Becwar et al.1988). In fact, Salajova et al (1992) described higly variable results for *Pinus nigra*, depending both on the year of seed collection and on the zygotic embryo stage, which affects not only embryogenic tissue induction, but also induction of non embryogenic callus. It is well known that several factors influence tissue behaviour in culture. These include influence of the genotype, effect of the stage of development, and induction potential of the culture media. These factors are also important when matures embryos are used. Even when majority of the tissues are embryogenic during first 2 weeks of culture, 50% tissues induced are non-embryogenic. Normally, these two tissue types are notsimultaneously found in *Pinus* species as they are in *Picea* (Becwar et al.1995). Once again this could be due to physiological state of the seed used for initiation. Results confirmed the effectiveness of auxin and cytokinin combinations in somatic embryogenesis, mainly when cultures are kept for long periods in their presence. But it must always consider the need to distinguish between embryogenic and nonembryogenic responses.

When L-proline, L-glutamine and casein hydrolysate are added to the DCR culture medium containing auxin and cytokinin, embryogenic induction is substantially superior and faster (Salajova et al. 1992). This combination is also effective for somatic embryo induction in *Pinus caribeae* (Lainé and Davir 1990), reinforcing the need to consider always the genotype-dependent nutritional requirements.

In this context, the present results provide the first succesful report of somatic embryogenesis induction and embryo development from mature zygotic embryos of *Pinus nigra,* but control of somatic embryogenesis from mature embryos is insufficient, and further efforts must be done to fill this gap.

3. MAINTENANCE OF EMBRYOGENIC TISSUES

The embryogenic tissue is maintained on the same medium as for induction (as they have been initiated). Although the initiation frequencies in average are not high in comparison with other conifer species, the survival of embryogenic tissues is 81.96 to 94.11 % according to our results from the years 2000 and 2001 (Salajova and Salaj, in preparation). The cultures are sub-cultured every 16-21 days. Longer growth of tissues than 21 days without transfer to fresh medium resulted decline in growth, degeneration and necrosis.

According to our experiences, culture of 6-8 pieces of tissues per petri plate (8 cm in diameter) gives vigorous growth.

The embryogenic tissues are white, mucilaginous cell masses, containing early somatic embryos, revealed by microscopic investigation. These structures have been present in all embryogenic tissues during maintenance but we have observed differences in their morphology among cell lines. In general somatic embryos were bipolar structures consisted of embryonic “head” with meristematic cells and long vacuolised suspensor cells. According to morphological features of somatic embryos, the cell lines can be divided into three group, characterized by presence of well formed, less organized and poorly organized somatic embryos (Salajova et al. 2001).

The embryogenic tissues can be maintained also in liquid medium as suspension cultures. In this case 1 g of embryogenic tissue is dispersed in liquid DCR medium of the same composition as for maintenance, in 25 ml Erlenmeyer flasks. After one week of culture 5 ml of dense suspension is pipetted onto 20 ml fresh maintenance medium. The change of fresh medium occurred in 14-days intervals. The structural features of somatic embryos in liquid cultures are similar as on solid media.

The flasks suspension cultures were placed on shaker at 80-110 rpm in culture room at 25ºC in dark.

4. MATURATION

For somatic embryo maturation different treatments have been tested. DCR medium (Gupta and Durzan 1985) containing abscisic acid (ABA) develops somatic embryos, which are mostly precotyledonary. However, the later developmental stages of somatic embryos as well as plantlet regeneration are infrequent (Salajova et al. 1994). Polyethylene glycol (PEG-4000) combined with sucrose as carbohydrate degenerate developing somatic embryos (Salajova et al. 1998). The best maturation treatment for the development of cotyledonary somatic embryos, capable of germination and plantlet regeneration, is DCR medium containing ABA (94.6 μM) and maltose (6-9%). Somatic embryo development and maturation are cell line dependent.

Maturation protocol: Seven or eight days after transfer, 10 gm embryogenic tissue is resuspended in 25 ml liquid DCR medium containing only macro- and microelements. Following, the dense suspension is diluted to volume 75-80 ml. Three ml of this suspension is pipetted on the filter papers placed on the surface of maturation medium (Table 1). The cells are allowed to settle for 15-20 min and the remnant of liquid is absorbed with sterile cotton wool. The petri plates (6 cm in diameter) are sealed by parafilm and place them in the culture room at 25ºC in the dark. Observe samples under the dissected microscope to detect developmental stages of somatic embryos at 3-5 day interval. After four weeks of cultivation the filter papers with developing somatic embryos are transferred to fresh maturation medium. The tissues are kept on maturation medium for 8 weeks and after that are transferred to a medium without ABA and low maltose (3%). Precotyledonary somatic embryos develop on all the tested media but quantitative differences are seen among treatments and cell lines. The development of cotyledonary somatic embryos is also dependent on cell line and maturation medium. Cell line E-15 produces the highest number of cotyledonary somatic embryos (Figure 1B) on medium containing 6 or 9 % maltose.

5. GERMINATION OF SOMATIC EMBRYOS

Somatic embryos with well-developed cotyledons (Figure 1B) are selected for germination. Prior to germination the somatic embryos are partially desiccated. For desiccation somatic embryos are carefully isolated with forceps and put into empty petri plates. The unsealed petri plates are placed in a desiccator containing water at the bottom. Desiccation continues for 2 weeks in dark at 25ºC. After partially drying the somatic embryos are transferred to germination medium (Table 1) and culture them in

the dark. Finally the elongated plantlets with protruding roots (Figure 1C) are cultivated in 100 ml Magenta baby food jars on the same medium as for germination under light with intensity 110 $\mu mol.m^{-2}.s^{-1}$ for 14 hours per day. Later the regenerated emblings, possessing at least 5 mm long roots are transplanted to soil in small plastic containers. After 5-6 month, emblings develop root system in soil (Figure 1D).

6. CONCLUSIONS AND FUTURE PROSPECTS

Somatic embryogenesis from immature embryos is already induced and the protocol presented here assures the production of plantlets, but initiation frequencies are affected by the developmental stage of zygotic embryos and time of seed collection. Control of somatic embryogenesis from mature embryos is still far from success in most of the woody trees. Further efforts are needed to address this problem. Research on the automatisation of somatic embryogenesis process from immature embryos must be carried out. On the other hand efforts on the comprehension of the maturation process must be continued. The maturation process begins during embryogenesis. Many morphological modifications related to maturation are accompanied by an increase in endogenous methylcytosine levels. So methylation of the genomic DNA could be one of the main molecular mechanisms involved, new insights concerning embryogenesis from mature tissues could be obtained by controlling the methylation though reinvigoration techniques or by exogenously added demethylation drugs.

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7. REFERENCES

Attree, S.M. & L.C. Fowke, 1993. Somatic embryogenesis and synthetic seeds of conifers. Plant Cell Tiss. Org. Cult. 35: 1-35

Beccwar, M.R., S.R. Wann, M.A. Johnson, S.A. Verhagen, R.P. Feirer & R. Nagmani, 1988. Development and characterisation of *in vitro* embryogenic systems in conifers. In: M.R. Ahuja (Ed). Somatic cell genetics of woody plants. Pp 1-18. Kluwer Academic Publishers. Dordrecht, Netherlands.

Becwar, M.R., R. Nagmani & S.R. Wann, 1990. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can. J. For. Res. 20: 810-817

Bercetche, J. & M. Paques, 1995. Somatic embryogenesis in maritime pine (*Pinus pinaster*) (p. 221-242). In: S.M. Jain, Gupta, P.K., Newton, R.J. (Eds) Somatic Embryogenesis in Woody Plants, Vol. 3, Gymnosperms. Kluwer Academic Publishers, Dordrecht, The Netherlands 1995, 388.

Finer, J.J., H.B. Kriebel & M.R. Becwar, 1989. Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus*). Plant Cell Rep. 8: 203-206

Garin, E., M. Bernier-Cardou, N. Isabel, K. Klimaszewska & A. Plourde, 2000. Effect of sugars, amino acids, and culture technique on maturation of somatic embryos of *Pinus strobus* on medium with two gellan gum concentrations. Plant Cell Tissue Org. Cult. 62: 27-37

Gray D.J. & A. Purohit, 1991. Somatic Embryogenesis and development of synthetic seed technology. Critical Review Plant Sciences 10: 33-61

Grossnickle, S.C. & J.E. Major, 1994. Interior spruce seedlings compared with emblings produced from somatic embryogenesis. III. Physiological response and morphological development on a reforestation site. Can. J. For. Res. 24: 1397-1407

Gupta, P.K., D.J. Durzan, 1985. Shoot multiplication from mature trees of Douglas fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep. 4: 177-179

Gupta, P.K., D.J. Durzan, 1986. Somatic polyembryogenesis from callus of mature sugar pine embryos. Biotechnoloy 4: 643-645

Gupta, P.K., G.S. Pullman, R. Timmis, M. Kreitinger, W. Carlson & J. Grob & E. Welty, 1993. Forestry in 21th century: the biotechnology of somatic embriogenesis. Biotechnology 11: 454-459

Gupta, P.K. & G.S. Pullman, 1990. Method for reproducing coniferous plants by somatic embryogenesis. US Patent No 5: 036, 007.

Gupta, P.K. & G.S. Pullman, 1991. Method for reproducing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. US Patent No 5: 036, 007.

Häggman, H., A. Jokela, J. Krajnakova, A. Kauppi, K. Niemi, T. Aronen, 1999. Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction. J. Exp. Bot. 50: 1769-1778

Jain, S.M., P.K. Gupta, R.J. & R.J. Newton, 1995. Somatic Embryogenesis in Woody Plants, Vol. 3 Gymnosperms, Kluwer Academic Publishers, Dordrecht, The Netherlands. 1995, 388 pp.

Jones, N.B. & J. van Staden, 1995. Plantlet production from somatic embryos of *Pinus patula*. J. Plant Physiol. 145: 519-525

Keinonen-Mettala, K., P. Jalonen, P. Eurola, S. von Arnold & K. von Weissenberg, 1996. Somatic embryogenesis of *Pinus sylvestris*. Scand. J. For. Res. 11: 242-250

Klimaszewska, K. & D.R. Smith, 1997. Maturation of somatic embryos of *Pinus strobus* is promoted by high concentration of gellan gum. Physiol. Plant. 100: 949-957

Klimaszewska, K., Y.S. Park, C. Overton, J. Maceacheron & J.M. Bonga, 2001. Optimized somatic embryogenesis in *Pinus strobus* L. In Vitro Cell. Dev. Biol.- Plant 37: 392-399

Kong, L., S.M. Attree, D.E. Evans, P. Binarova, E.C. Yeung & L.C. Fowke, 1999. Somatic embryogenesis in white spruce: Studies of embryo development and cell biology (p. 1-28) In:

S.M. Jain, P.K. Gupta & R. Newton (Eds), Somatic Embryogenesis in Woody Plants, Vol. 4. Kluwer Academic Publishers, Dordrecht, The Netherlands, 547 pp.

Laine, E. & D. David, 1990. Somatic embryogenesis in immature embryos and protoplasts of *Pinus caribaea*. Plant Sci. 69: 215-224

Lelu, A.M., C. Bastien, A. Drugeault, M.L. Gouez, & K. Klimasyewska, 1999. Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. Physiol. Plant. 105: 719-728

Miguel, C., S. Concalves, S. Teraso, L. Marum & M.. Oliveira, 2000. Induction of somatic embryogenesis from mature zygotic embryos of maritime pine (Pinus pinaster). In: COST 843 "Quality enhancement of plant production through tissue culture", pp63. WG2 -Advanced propagation techniques. Tampere, Finland.

Mirsa, S., 1994. Conifer zygotic embryogenesis, somatic embryogenesis and seed germination: Biochemical and molecular advances. Seed Science Research 4: 357-384.

Percy, R.E., K. Klimaszewska & D.R. Cyr, 2000. Evaluation of somatic embryogenesis for clonal propagation of western white pine. Can. J. For. Res. 30: 1867-1876.

Quorin M. & P. Le Poivre, 1977. Etude des milieux adaptes aux cultures *in vitro* de *Prunus*. Acta Horticulturae 78 : 437-442.

Radojevic Lj., C. Alvarez, M.F. Fraga & R. Rodríguez, 1999. Somatic embryogenesis in European black pine (*Pinus nigra* Arn). Biol. Plant. 35: 206-209.

Ramarosandratana, A., L. Harvengt, E. Garin, M. Paques & R. Calvayrac, 1999. Factors influencing the development of mature somatic embryos of maritime pine (*Pinus pinaster* Ait.) In: Espinel S., Ritter E. (Ed). Proceedings of Application of Biotechnology to Forest Genetics, pp 271-274. Biofor 1999, Vitoria-Gasteiz, Spain.

Ramarosandratana, A., L. Harvengt, A. Bouvet, R. Calvayrac & M. Paques, 2001. Effect of carbohydrate source, polyethylene glycol and gellan gum concentration on embryonal - suspensor mass (ESM) proliferation and maturation of maritime pine somatic embryos. In Vitro Cell. Dev. Biol.-Plant 37: 29-34.

Salajova, T. & J. Salaj, 1992. Somatic embryogenesis in European black pine (*Pinus nigra* Arn.). Biol. Plant. 34: 213-218.

Salajova, T., J. Salaj, J. Jasik & A. Kormutak, 1994. Somatic embryogenesis in *Pinus nigra* Arn. In: Jain, S.M., Gupta, P.K., Newton, R.J. (Ed.). Somatic Embryogenesis in Woody Plants, vol. 3, pp 207-220. Gymnosperms. Kluwer Academic Publishers, Dordrecht.

Salajova, T., J. Salaj, & A. Kormutak, 1998. Plantlet regeneration in *Pinus nigra* Arn. through somatic embryogenesis. In: COST 822 - 5th Meeting of WG 3, pp 33-35.Identification and control of phase changes in rejuvenation., Budapest.

Salajova, T., J. Salaj & A. Kormutak, 1999. Initiation of embryogenic tissues and plantlet regeneration from somatic embryos of *Pinus nigra* Arn. Plant Sci. 145: 33-40.

Salajova, T., J. Salaj, R. Matusova & A. Kormutak, 2001. Embryogenic tissue initiation and somatic embryo development in *Pinus nigra* Arn.. In: COST 843, 2nd meeting of WG2, pp. 27-28 Quality enhancement of plant production through tissue culture. Advanced propagation techniques. Thessaloniki.

Tautorus, T.E., L.C. Fowke & D.I. Dunstan, 1991. somatic embryogenesis in conifers. Can. J. Bot. 69: 1873-1899.

Table 1 Medium composition for different steps of *Pinus nigra* Arn. somatic embryogenesis initiated from immature zygotic embryos.

	Induction and maintenance medium (mg l^{-1})	Maturation medium (mg l^{-1})	Germination medium (mg l^{-1})
NH_4NO_3	400	400	400
KNO_3	340	340	340
Ca $(NO_3)_2$. 4 H_2O	556	556	556
KH_2PO_4	170	170	170
$MgSO_4$. $7H_2O$	370	370	370
$CaCl_2.2H_2O$	85	85	85
H_3BO_3	6.2	6.2	6.2
$MnSO_4$. H $_2^o$	22.3	22.3	22.3
$ZnSO_4$. $7H_2O$	8.6	8.6	8.6
$CuSO_4.5H_2O$	0.25	0.25	0.25
KJ	0.83	0.83	0.83
$FeSO_4.7H_2O$	27.8	27.8	27.8
Na_2EDTA	37.3	37.3	37.3
$CoCl_2.6H_2O$	0.025	0.025	0.025
$NiCl_2$	0.025	0.025	0.025
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
Thiamine HCl	1.0	1.0	-
Pyridoxine HCl	0.5	0.5	-
Nicotinic acid	0.5	0.5	-
Glycine	2.0	2.0	-
Myoinositol	200	200	-
CH	500	500	-
L-glutamine	50	50	-
Sucrose	20 000	-	
Maltose	-	3,6,9 %	2%
BA (µM)	2.22	-	-
2,4-D (µM)	9.00	-	-
ABA (µM)	-	94.6	-
Activated charcoal	-	-	1%
Gelrite	3000	4000	5000

Table 2. Culture media used for induction, proliferation, manifestation and mantenance of somatic embryogenesis in mature zygotic embryos of *Pinus nigra* Arn.

Salt solution component	**Murashige and Skoog (1962) (MS)**	**Gupta and Durzan (1985) (DCR)**
Sucrose (g L^{-1})	30.0	30.0
Agar (g L^{-1})	7.0	7.0
Vitamins (mg L^{-1})		
B1	2.0	2.0
B6	0.5	0.5
Nicotinic acid	5.0	5.0
Biotin	2.0	2.0
Glycine	2.0	2.0
Myo-inositol	200.0	200.0
Amino acids (mg L^{-1})		
L-glutamine	250.0	250.0
L-proline	250.0	250.0
Casein hydrolysate (mg L^{-1})	500.0	500.0
Growth regulators (µM)	L_1 = NAA 10.7 + BA 8.8 L_{1a} = without hormones L_{1b} = NAA 10.7 + BA 8.8 L_{1c} = NAA 2.6 + BA 8.8 L_{1d} = NAA 2.6 + BA 6.6	L_2 = NAA 10.7 + BA 8.8 L_{2a} = without hormones L_{2b} = NAA 10.7 + BA 8.8 L_{2c} = NAA 2.6 + BA 8.8 L_{2d} = NAA 2.6 + BA 6.6 L_{2e} = NAA 10.7 + BA 2.2
L_{1a-d} and L_{2a-d} media with 10 g L^{-1} sucrose (S). L_{2e} medium with 50 g L^{-1} sucrose		

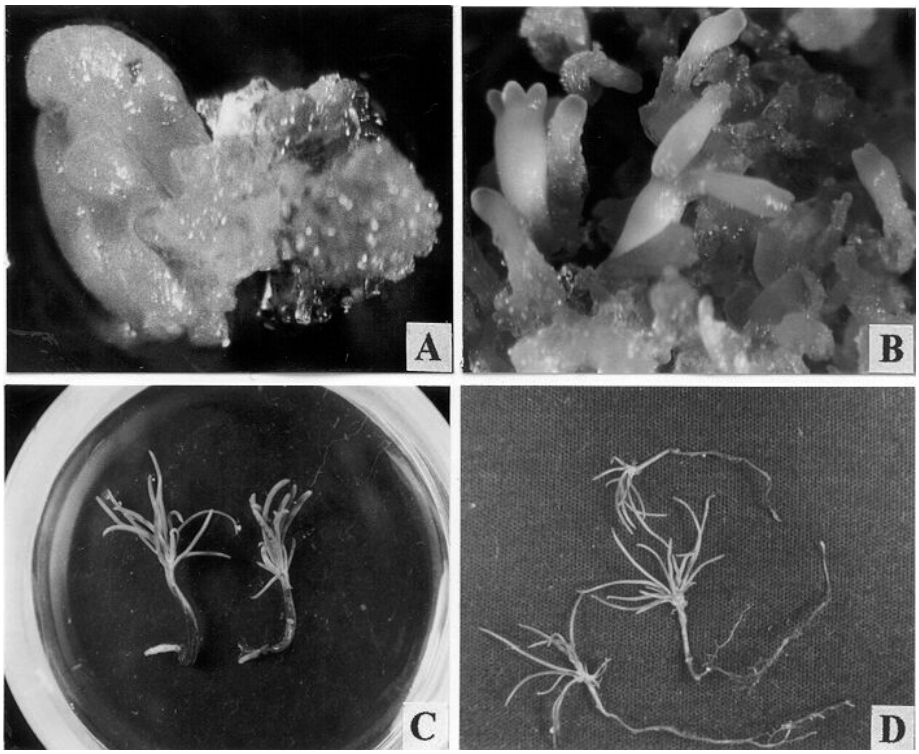

Figure 1. Different developmental stages of somatic embryogenesis in *Pinus nigra* Arn. 1A-embryogenic tissue protruding from megagametophyte. 1B-Cotyledonary somatic embryos around the 9th week of culture (cell line E15). 1C – Germinating emblings with protruding roots. 1D-Plantlets after 5-6 months of growth in soil.

LOBLOLLY PINE (*PINUS TAEDA*)

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1. INTRODUCTION

Somatic embryogenesis is a promising alternative technique for clonal propagation of superior and genetically engineered forest tree families in coniferous species (Attree and Fowke, 1993; Gupta and Durzan, 1991; Jain et al. 1995). It is also widely considered to be an efficient system for genetic transformation and transgenic plant recovery (Gupta et al. 1988; Wenck et al. 1999). Somatic embryogenesis in conifers was first described relatively by three independent groups: (1) Hakman and von Arnold (1985) achieved plant regeneration through somatic embryogenesis from cultured immature embryos of *Picea abies*; (2) Chalupa (1985) described somatic embryogenesis and plantlet regeneration from immature and mature embryos of *Picea abies*; and (3) Nagmani and Bonga (1985) reported the induction of haploid European larch (*Larix decidua* Mill.) somatic embryos from megagametophytic tissue. Mature conifer somatic embryos produced via plant tissue culture techniques appear to have the same quality as mature zygotic embryos, so somatic embryogenesis is a potential choice and resource for the production of artificial seeds (Attree and Fowke, 1993; Jain et al. 1995). In conifers, plantlet regeneration via somatic embryogensis includes: (1) induction of embryogenic callus; (2) maintance of embryogenic cultures; (3) maturation of somatic embryos; and (4) germination of somatic embryos and plant formation (Tautorus et al. 1991; Jain et al. 1995).

Progress on somatic embryogenesis of coniferous woody plants has been reviewed (Attree and Fowke, 1993). However, plant regeneration via somatic embryogenesis from mature zygotic embryos of conifers has been only reported in *Picea abies* (Jalonen and von Arnold, 1991), *Picea glauca* (Tremblay, 1990), *Picea mariana* (Attree et al.1990), *Picea pungens* (Afele et al.1992), *Picea rubens* (Harry and Thrope 1991), and *Picea sitchensis* (Krogstrup 1990). In *Pinus*, plant regeneration via somatic embryogenesis from mature zygotic embryos of sugar pine (*Pinus lambertiana*) was reported

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(Gupta and Durzan, 1986). This chapter describes the protocol to regenerate plantlets via somatic embryogenesis from embryogenic cultures derived from mature zygotic embryos of loblolly pine.

Loblolly pine (*Pinus taeda* L.) is an economically important coniferous species that is widely planted in temperate and subtropical regions (Tang et al. 2001). In vitro regeneration of loblolly pine has proven to be very difficult. There are only a few reports on plant regeneration via somatic embryogenesis or organogenesis in loblolly pine. Gupta and Durzan (1987) first reported somatic embryogenesis and plant regeneration from immature zygotic embryos with megagametophytes cultured on modified Murashige and Skoog (1962) medium. Becwar et al (1990) reported somatic embryogenesis from different genotypes of immature zygotic embryos of loblolly pine. Somatic embryogenesis is a promising method for large-scale propagation of loblolly pine as it offers the potential for developing automated systems to produce large numbers of synthetic seeds (Attree and Fowke, 1993; Becwar and Pullman, 1995; Gupta et al. 1993; Handley et al. 1995; Jain et al. 1995). However, limitations due to low initiation frequency, the restriction to a certain time window for initiation, and the genetic specificity of explants are problems associated with somatic embryogenesis when immature zygotic embryos were used as explants. But some of above problems can be resolved if mature zygotic embryos are used as explants. Although immature zygotic embryos have proven to be the most responsive explants (Gupta and Durzan 1987), fully developed mature zygotic embryos from dry seeds can be also used (Gupta and Durzan 1986). The procedure described in this chapter removes the narrow time constraints imposed by using immature embryos.

2. MATERIALS

1.Mercuric chloride, ethanol, sterile distilled water, 125 ml Erlenmeyer flasks
2.Flow hood, petri dishes, forceps, scalpel, and pipettes
3.Seeds and mature female cones
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-Insoyil, casein hydrolysate, and L-glutamine, polyethylene glycol (PEG), and activated charcoal
7.Shaking incubators and tissue culture chambers
8.Media (see Tables 1 and 2)

TABLE 1: LOBLOLLY PINE BASIC CULTURE MEDIUM

Chemicals	Quantity in mg/l	Chemicals	Quantity in mg/l
$Ca(NO_3)_2.4H_2O$	1900	$Na_2MoO_4.H_2O$	0.25
KNO_3	720	$CoCl_2.6H2O$	0.025
$CaCl_2.2H_2O$	440	$CuSO_4.7H_2O$	0.025
NH_4NO_3	400	$FeSO_4.7H_2O$	27.8
$MgSO_4.7H_2O$	370	NaEDTA	37.3
KCl	250	Myo-inositol	10
KH_2PO_4	170	Nicotinic acid	0.1
$ZnSO_4.7H_2O$	25.8	Pyridoxin HCl	0.1
$MnSO_4.H_2O$	25.35	Thiamine HCl	0.1
HBO_3	6.2	Glycine	0.4
KI	0.83	pH	5.8

The composition of supplementary organic compounds of LOB basal medium was the same as the LP medium (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.

TABLE 2: FORMULATIONS OF LOBLOLLY PINE MEDIA

Chemicals	LOB-1	LOB-2	LOB-3	LOB-4	LOB-5
	Stage I Initiation	Stage II Maintenance	Stage III Development	Stage IV Maturation	Stage V Germination
Myo-Inositol	500[(1)]	500	300	300	-
L-Glutamine	500	500	300	300	-
Casein hydrolysate	500	500	300	300	-
Sucrose	30,000	20,000	20,000	15,000	15,000
PEG 6000	-	-	-	75,000	-
2,4-D	10	2	1	-	-
IBA	5	1	0.5	-	0.5
BA	4	0.8	0.4	0.5	1
Kinetin	4	0.8	0.4	-	-
ABA	-	-	-	40	-
GA_3	-	-	-	-	0.1
Activated charcoal	-	-	-	5,000	500
Agar[(2)]	7,000	7,000	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

Basic medium composition is listed in Table 1. Required modifications for different culture stage 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°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.

3. METHOD

The regeneration procedure includes four steps: embryogenic culture initiation from explants, maintenance and proliferation of embryogenic cultures, embryo development and maturation, and embryo germination and acclimatization and field transfer

Embryogenic Culture Initiation

Use mature zygotic embryos for embryogenic culture initiation. Collect mature female cones in early October, dry the cones and harvest mature seeds. Store cones and seeds in plastic bags at 4°C for 2 months before use.

1. Remove the seeds from the mature cones.
2. Wash the seeds with tap water and agitate for 20 min.
3. Treat the seeds with 70% v/v ethanol for 30 sec.
4. Rinse 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 in a petri dish.
8. Remove the seed coat with sterile scalpel and forceps and aseptically isolate mature zygotic embryos from the megagametophyte under a dissecting microscope.
9. Put horizontally the isolated mature zygotic embryos on the surface of 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 the dark at 23°C.

Maintenance and Proliferation of Embryogenic Cultures

Callus is formed on cotyledons, hypocotyls, and radicles of mature zygotic embryos after 9 weeks on callus induction medium (LOB-1). Conifer embryogenic tissue has a distinctly different phenotype than organogenic callus. In this protocol, four types can be distinguished: (1) white to

translucent, glossy, mucilaginous; (2) light yellowish, loose, glossy globular; (3) light green globular; and (4) light brown globular. White to translucent, glossy, mucilaginous callus develop from the cotyledons of the mature zygotic embryos (Fig.1a); whereas light yellowish, loose, glossy globular callus; light green globular callus; and light brown globular callus form from the hypocotyl as well as from the radicle of the explants. White to translucent, glossy, mucilaginous callus is embryogenic and is proliferated on solidified LOB-2 medium with 2mg/l 2,4-D, 0.8 mg/l BA, 1 mg/l IBA, 0.8 mg/l Kn, 500 mg/l myo-inositol, 500 mg/l casein hydrolysate (CH), 500 mg/l L-glutamine, and 2 % sucrose (Fig.1b). After an additional 9 weeks on the LOB-2 medium, white to translucent, glossy, mucilaginous calli containing embryogenic suspensor masses (ESMs) is observed. It is subsequently transferred to a medium consisting of LOB basic medium as described above, but supplemented with 1 mg/l 2,4-D, 0.4 mg/l BA, 0.5 mg/l IBA, and 0.4 mg/l Kn (LOB-3). The ESMs cultures are maintained by subculture every three weeks on fresh LOB-3 medium and incubated in the dark at 23^{o}C.

Making of Cell Suspension Cultures

1. Transfer 1 gram of embryogenic cultures to a liquid proliferation medium supplemented with 2 mg/l 2,4-D, 0.8 mg/l BA, 1 mg/l IBA, 0.8 mg/l Kn, 500 mg/l myo-inositol, 500 mg/l CH, and 500 mg/l L-glutamine, and 2% sucrose.
2. Put the flask on a rotary shaker with shaking at 120 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. During the early stage, 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 3 min) and resuspended 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.
3. The suspension cultures are subcultured weekly. After two weeks, embryogenic cells and ESMs are established. ESMs are defined as polarized structures organised into an embryogenic region subtended by elongated suspensor cells (Attree and Fowke 1993). Proliferating embryogenic tissues consist primarily of single embryogenic cells (Fig.1c), ESMs (Fig.1d and 1e) and immature somatic embryos. ESMs continuously initiate embryos by cleavage polyembryogenesis (Fig.1f). 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).

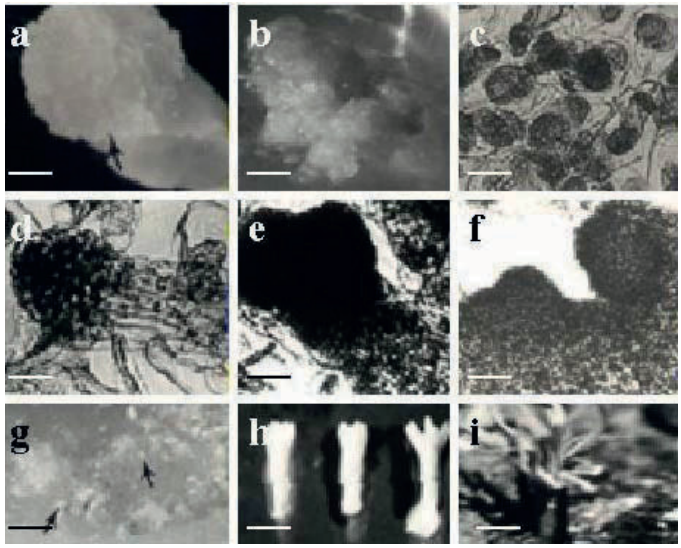

Figure 1 *Plant regeneration via somatic embryogenesis in loblolly pine.*
(a) Embryogenic callus obtained from cotyledons of a mature zygotic embryo on LOB-1 (bar = 0.5 cm); (b) Proliferation of embryogenic callus on LOB-2 (bar = 0.5 cm); (c) Embryogenic suspension cells on liquid LOB-2 (bar = 0.15 mm); (d) Embryogenic suspensor masses (bar = 0.25 mm); (e) Globular somatic embryo (bar = 0.25 mm), (f) Cleavage somatic polyembryos (bar = 0.5 mm); (g) Precotyledonary somatic embryos on LOB-3 (bar = 0.5 cm); (h) Cotyledonary somatic embryos on LOB-4 (bar = 0.5 cm); (i) Regenerated plantlet established in soil (bar = 0.8 cm)

Staining to Confirm Embryogenic Nature

The presence of ESMs is confirmed by double staining as described by Gupta and Durzan (1987).

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. 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. 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.

Embryo Development, Maturation, and Germination

To obtain the maturation of loblolly 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 (LOB-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. 2a)) on a proliferation medium with decreased auxin and cytokinin concentrations (LOB-3).
3. Transfer ESMs suspension cultures with stage 3 embryos (embryos with small cotyledons (Fig. 1g, Fig. 2b)) to medium devoid of auxin (LOB-4).
4. Transfer ESMs suspension cultures with stage 4 embryos (embryos with fully developed cotyledons (Fig. 1h, Fig. 2c and d)) on LOB-5.
5. Mature somatic embryos with cotyledons are transferred to solidified LOB medium containing 0.5 mg/l IBA, 0.1 mg/l gibberellic acid (GA_3), 1 mg/l BA, and 0.5% activated charcoal for 4-12 weeks.
6. Somatic embryo desiccation is performed according to the method of Takahata et al. (1993). 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 LOB-5 medium containing 2.2 μM BA for further development (Fig. 2c and d).

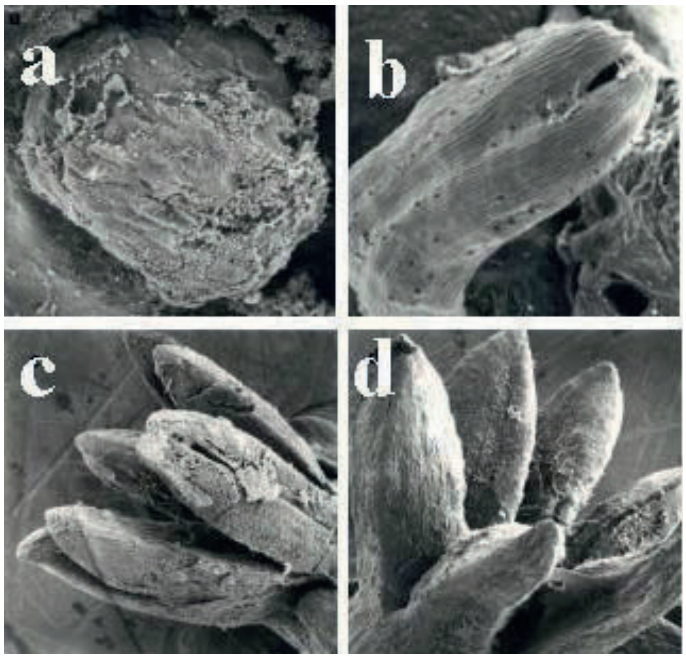

***Figure** 2 Scanning electron microscopy of somatic embryogenesis in loblolly pine*
(a) Globular somatic embryo (Bar = 0.1mm), (b) Cotyledonary somatic embryo (Bar = 0.2mm), (c) Desiccation-sensitive somatic embryo with an irregular cell surface (Bar = 0.3mm), (d) Desiccation-tolerant somatic embryo with regularly arranged epidermal cells (Bar = 0.3mm).

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.
2. For acclimatization, plantlets are covered with glass beakers for one week. Then the acclimatized plantlets are exposed to greenhouse conditions by recovering the cover for 16 days.
3. Then the plants are transplanted to soil in the field (Fig. 1i). 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 (Fig.1h).

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 (Fig.1i) is obtained when the acclimatization time was 16 days.

4. APPLICATIONS

Embryogenic cultures of loblolly pine can be used to isolate protoplasts for physiological study and to produce large-scale somatic embryos in bioreactors for clonal forestry. Somatic embryos can be encapsulated to form artificial seeds that provide an efficient embryo delivery system. Combining available genetic transformation techniques with somatic embryogenesis systems can result in transgenic pine with economically useful traits such as insect and disease resistance, which are difficult to obtain by conventional breeding programs. Although somatic embryogenesis is an efficient system of regeneration, it has the disadvantage of causing genetic deviations called somaclonal variation. This problem could appear when embryogenic masses are maintained on proliferation media during an extended period. In order to prevent such phenomena, it is best to cryopreserve the embryonic masses as soon as they are produced. Cryopreservation has proven successful with a variety of conifers (Attree and Fowke 1993, Jain et al. 1995).

Protoplasts

1. Embryogenic cell suspension cultures are incubated for 5 hours in an enzyme solution containing cellulose R-10 “Onozuka”, Pectolyase Y-23 and Biovine Serum Albumin.
2. Protoplasts are purified on a 70% (w/v) Ficoll-containing solution by centrifuging at 5000 rpm for 8 min. One ml of cell suspension yields approximatively 2×10^5 protoplasts.
3. Protoplasts are cultivated at a density of 2×10^3, 5×10^3, and 10×10^3 per ml in 10 ml of culture medium. Cells derived from protoplasts divide after two days of culture.
4. Two months after protoplast plating, the protoplast-derived cell cultures are transferred to a solid proliferation medium and subcultured at two-week intervals on the same medium.
5. After six weeks, the appearance, growth rate and nutritional requirement of the protoplast-derived materials are similar to the embryogenic cultures used to initiate the cell suspension.

Cryopreservation

1. Cultured cells and embryos originate from single cells and are cryopreserved by vitrification. The vitrification solution (PVS1) contains (w/v) 22% glycerol, 15% ethylene glycol, 15% propylene glycol and 7% DMSO in 0.5M sorbitol Murashige-Skoog medium (MS).
2. After cryoprotection with 0.5M-sorbitol MS medium containing 12% ethylene glycol, cells or embryos are exposed stepwise to 85% PVS1 at 0°C.
3. They are loaded within a transparent straw, and then plunged directly into liquid nitrogen. After rapid warming, PVS1 is removed and diluted stepwise. The highest survival of vitrified cells and embryos is 65% and 50%, respectively. Surviving embryos develop into plantlets

Encapsulation

1. Mature somatic embryos are encapsulated using different concentrations of sodium alginate (3–6%) and calcium chloride (50–100 mM) in order to determine the effect of bead rigidity on the conversion frequency of somatic embryos.

2. LOB medium supplemented with 2% sucrose is used for germination. Variability is observed between sodium alginate and calcium chloride concentrations with respect to the percentage of germination.
3. Plant regeneration frequency decreases with an increase in sodium alginate concentration above 4.5% and 3% sodium alginate, and 75 mM calcium chloride gives the best conversion and optimal bead rigidity and shape.
4. The germination frequency is reduced from 85% to 60% for cotyledonary somatic embryos, compared to the control. This is lower than the germination frequency of intact zygotic seeds from the same seedlot.

5. IDENTIFY STEPS REQUIRED FURTHER PROTOCOL MODIFICATIONS

Plant regeneration via somatic embryogenesis in loblolly 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).

REFERENCES

Afele, J.C.; Senaratna, T.; McKersie, B.D.; Saxena, P.K. Somatic embryogenesis and plant regeneration from zygotic embryo culture in blue spruce (*Picea pungens* Engelman.). Plant Cell Rep. 11:299-303; 1992.

Attree, S.M.; Budimir, S.; Fowke, L.C. Somatic embryogenesis and plantlet regeneration from cultured shoots and cotyledons of seedlings germinated from stored seeds of black and white spruce (*Picea mariana* and *Picea glauca*). Can. J. Bot. 68:30-34;1990.

Attree, S.M.; Fowke, L.C. Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tiss. Org. Cult. 35: 1-35; 1993.

Becwar, M.R.; Nagmani, R.; Wann, S.R. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can. J. For. Res. 20:810-817; 1990.

Becwar, M.R.; Pullman, G.S. Somatic embryogenesis in loblolly pine (*Pinus taeda* L). In: Jain, S.M.; Gupta, P.K.; Newton, R.J. (eds), Somatic embryogenesis in woody plants, vol.3, Kluwer Academic Publishers, the Netherlands, pp 287-301; 1995.

Chalupa, V. Somatic embryogenesis and plantlet regeneration from immature and mature embryos of *Picea abies* (L.) Karst. Comm. Inst. For. 14:57-63; 1985.

Gupta, P.K.; Durzan, D.J. Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Technology 4:643-645; 1986.

Gupta, P.K.; Durzan, D.J. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio/Technology 5:147-151; 1987.

Gupta, P.K.; Dandekar, A.M.; Durzan, D.J. Somatic proembryo formation and transient expression of a luciferase gene in Douglas fir and loblolly pine protoplasts. Plant Sci. 58: 85-92; 1988.

Gupta, P.K.; Durzan, D.J. Loblolly pine (*Pinus taeda* L.). In: Bajaj, Y.P.S. (ed), Biotechnology in agriculture and forestry, vol 16, Trees III, Springer-Verlag, Berlin, pp 383-407; 1991.

Gupta, P.K.; Pullman, G.; Timmis, R.; Kreitinger, M.; Carlson, W.C.; Grob. J.; Welty, E. Forestry in the 21st Century: The biotechnology of somatic embryogenesis. Bio/Technology 11:454-459; 1993.

Hakman, I.; von Arnold, S. Plant regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). J. Plant Physiol. 121:49-158; 1985.

Hakman, I.; Fowke, L.C. Somatic embryogenesis in *Picea glauca* (white spruce) and *Picea mariana* (black spruce). Can. J. Bot. 65: 656-659; 1987.

Handley, L.W. Method for regeneration of coniferous plants by somatic embryogenesis. U.S. Patent No. 5,731,203. Issued March 24, 1998.

Handley, L.W.; Becwar, M.R.; Chesick, E.E. Research and development of a commercial tissue culture system in loblolly pine. Tappi J. 78(5): 169-175; 1995.

Harry, I.S.; Thorpe, T.A. Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. Bot. Gaz. 152:446-452; 1991.

Jain, S.M,.Gupta, P.K., Newton, R.J. Somatic embryogenesis in woody plants, vol 3, Gymnosperms, Kluwer Academic Publishers, 1995.

Jalonen, P.; von Arnold, S. Characterization of embryogenic cell lines of *Picea abies* in relation to their competence for maturation. Plant Cell Rep. 10:384-387; 1991.

Krogstrup, T. Effect of culture densities on cell proliferation and regeneration from embryogenic cell suspension of *Picea sitchensis*. Plant Sci. 72:115-123; 1990.

Murashige, T.; Skoog, F. A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant. 15: 473-497; 1962.

Nagmani, R.; Bonga, J.M. Embryogenesis in subcultured callus of *Larix decidua*. Can. J. For. Res. 15:1088-1091; 1985.

Takahata, Y.; Brown, D.C.W.; Keller, W.A.; Kaizuma, N. Dry artifical seed and desiccation tolerance induction in microspore-derived embryos of broccoli. Plant Cell Tiss. Org. Cult. 35: 121-129; 1993.

Tang W. Peroxidase activity of desiccation-tolerant loblolly pine somatic embryos. In Vitro Cell. Dev.-Plant 36: 488-49; 2000.

Tang W., Guo Z.C., Ouyang F. Plant regeneration from embryogenic cultures initiated from mature loblolly pine zygotic embryos. In Vitro Cell. Dev.-Plant 37: 558-563; 2001.

Tautorus, T.E.; Fowke, L.C.; Funstan, D.I. Somatic embryogenesis in conifers. Can. J. Bot. 69:1873-1899; 1991.

Tremblay, F.M. Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. Can. J. Bot. 68:236-240; 1990.

von Arnold, S. Eriksson, T. Bud induction on isolated needles of Norway spruce (*Picea abies* L. Kast.) grown in vitro. Plant Sci. Lett. 15:363-372; 1979.

Wenck, A.R.; Quinn, M.; Whetten, R.W.; Pullman, G.; Sederoff, R. High-efficiency *Agrobacterium*-mediated transformation of Norway spruce (*Picea abies*) and loblolly pine (*Pinus taeda*) Plant Mol. Biol. 39: 407-416; 1999.

SOMATIC EMBRYOGENESIS IN MARITIME PINE (*PINUS PINASTER* AIT.)

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1. INTRODUCTION

Maritime pine (*Pinus pinaster* Aït.) covers more than 4 million hectares in Europe, with France, Portugal and Spain averaging 1.3 million ha each. In France, maritime pine is the major species used for reforestation. Due to its higher tolerance to salinity and drought comparatively to radiata, this species is in significant extension in Australia where it would reach 1 million ha by the next decade. Somatic embryogenesis in conjunction with cryopreservation could offer a competitive solution for speeding up the availability of plant material selected according to the requirements of wood chain industries. We will present hereafter the standard technique corresponding to the state of the art for routine culture in our lab at the beginning of the SEP project (started in 2000, http://www.vbsg.slu.se/sep/) aiming to enhance the performance of somatic embryogenesis in both *Pinus sylvestris* and *Pinus pinaster*. This deal with embryogenesis from immature zygotic embryos (immature seeds) but of course, for practical reasons, it has been extended to mature seeds. Moreover, the initiation of somatic embryos from mature trees selected after full field evaluation would be of paramount interest to maximize the genetic gain and therefore the economic value of plants produced from somatic embryos. This technique is emerging for a few broadleaves and conifers species (see Harvengt *et al.*, 2001) and is the subject of extensive research regarding important pines species.

While initiation and multiplication are not problematic in most cases, the limiting step is the maturation of immature embryo culture (ESM, embryonal-suspensor mass, Gupta 1995) into cotyledonary embryos able to evolve properly into well-growing plants. The technique detailed here can however produce several plants per Petri dish of ESM subjected to maturation treatment in skilled hands.

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 107–119.

2. MATERIALS

2.1. Basic material and equipment

- dark and lighted culture room at 23± 2°C
- autoclave
- magnetic stirrer
- laminar flow hood (horizontal is better)
- stainless steel scalpels and forceps
- gas burner or glass bead sterilizer
- ethanol, ultrapure water , bleach and detergent (or tween)
- sterile Petri dishes
- filter paper Whatman n°2
- Dissecting microscope
- Strong forceps or hatchet to open cones
- Immature pine cones
- Cling film (PVC food film)
- For cryopreservation: DMSO, cryoboxes, cryovials, sterile centrifuge tubes, ice, programmable freezer, cryotank, liquid nitrogen

2.2. Culture media

From culture initiation to plant growth, we used a single mineral basis for culture media, comprising the macronutrients of Gupta and Durzan (1985) and the micronutrients of Murashige and Skoog (1962) supplemented with sucrose as a carbon source. The medium is prepared from 5 stock solutions detailed in table 1.

Table 1. Medium mineral basis composition. Macro nutrients are prepared as 2 separate stock solutions (sol A and B). Micro nutrients are prepared as a 1000X concentrated stock solution (sol C, sodium molybdate need to be diluted apart and then added to the other salts). Iron chelate is prepared as a 20X solution. Solutions A to D are kept at +4°C. Vitamins (sol E) are prepared as a 2000 X stock solution conserved in the freezer (-20°C) in small aliquots corresponding to one liter of medium.

Culture media Basis – Components					
Stock solution	Component	Final concentration in mg l^{-1}	Stock solution	Component	Final concentration in mg l^{-1}
A	$NH_4 NO_3$	400	C	$CuSO_4.5H_2O$	0.025
A	$K NO_3$	340	C	$CoCl_2.6H_2O$	0.025
A	$KH_2 PO_4$	170	D	$FeSO_4.7H_2O$	27.8
A	$Mg SO_4, 7H_2O$	37	D	$Na_2EDTA.2H_2O$	37.2
B	$Ca(NO_3)_2, 4H_2O$	55.6	-	Inositol	200
B	$Ca Cl_2, 2H_2O$	85	E	Glycine	1
C	KI	0.83	E	Nicotinic Acid	0.25
C	H_3BO_3	6.2	E	Pyridoxin HCl	0.25
C	$MnSO_4.H_2O$	16.9	E	Thiamin HCl	0.5
C	$ZnSO_4.7H_2O$	10.6	-		
C	$Na_2MoO_4.2H_2O$	0.25	-	Casein Hydrolysate	500

The solidification of the medium is obtained by adding gellan gum (G-1910, Sigma Chemical Co., St. Louis, MO, USA) before adjusting the pH to 5.8. The medium is then autoclaved at 110°C, 110 kPa for 30 min (temperature and pressure inside the sterilizing room, *i.e.* autoclave parameters). After addition of heat-labile components, media are poured at 60°C into Petri dishes (Greiner, 94x15 mm, 25 ml medium per dish), sealed with cling film and then stored at room temperature for 1 week before use.

Table 2. Hormonal content of culture medium

	Initiation and Maintenance	Maturation	Conversion
BAP (µM)	2.2	0	0
2,4-D (µM)	13.5	0	0
ABA (µM)	0	80	0
Gelrite (g l^{-1})	30	90	30
Sucrose (g l^{-1})	30	60	20

ABA (MW = 264.3) is prepared as a stock solution at 5 g l^{-1} (18.92 mM) in a few milliliters of KOH 1M completed up to 200 ml with ultrapure water (18 MΩ.cm reverse osmosed water). Before final volume adjustment, the pH of the solution is adjusted to 5.8. The solution is immediately filter-sterilized and stored in the dark in the fridge (+4°C). ABA is added to the culture medium after autoclaving.

BAP (MW = 225.3) is dissolved at 1 g l^{-1} (4.44 mM) in absolute ethanol and the volume is adjusted to 100 ml with ultrapure water. 2,4-D (MW = 221) is prepared as a 1 g l^{-1} (4.52 mM) solution in absolute ethanol and stored in the dark. BAP and 2,4-D are stored in the dark until used and autoclaved with the culture medium.

Glutamine is solubilized in water (solubility: 1 g per 30 ml at room temperature), filter-sterilized and added to autoclaved medium just before pouring. The high degradability of glutamine in solution request frozen storage or immediate use.

The liquid maturation medium used for making a suspension of the culture for spreading on filter paper is the same as solid medium but without gelrite.

EMBRYOGENIC CULTURE INITIATION

2.2. Initiation

Figure 1. Immature cones and seed of maritime pine at the time of sampling for initiation of somatic embryo lines.

Figure 2. View of the stage at which immature zygotic embryos are inoculated on initiation culture medium.

Initiation of Embryogenic Cultures

Use immature embryos at the globular to pre-cotyledonary stage for initiation of embryogenic cultures. Collect female cones in a period between the first week of July until the first appearance of cotyledon primordia (middle of July to middle of August according to your local climate and genetic background). The optimal embryo stage for initiation is when the apical dome well developed but before the full appearance of the cotyledons.

1. Remove the seeds *(Figure 1)* from the green cones.
2. agitate the seeds for 15 min in 5% w/v sodium hypochlorite (diluted household bleach) with a few drops of Tween or household detergent.
3. Wash the seeds 3 – 4 times with sterile (autoclaved) distilled water in the laminar-flow hood.
4. Sterilize the seeds with 70% (v/v) Ethanol for 15 min.
5. Wash the seeds 3 – 4 times with sterile water in the laminar-flow hood.
6. Transfer the sterile seeds in a Petri dish.
7. Remove the seed coat with scalpel and forceps and excise the embryos from the female gametophyte (see Figure 2). Work under a dissecting microscope.
8. discard the female gametophyte and put the excised embryos horizontally on to initiation medium. No more than 10 embryos are put in a single Petri dish.
9. Wrap plates with a double layer of parafilm and incubate the cultures in the dark culture room.
10. Check daily for the presence of eventual bacterial or fungal development for one week and then at a decreasing frequency.
11. The embryos are transferred to fresh medium every 3 weeks. After 3 months, embryos without any development of embryonic culture are discarded while growing ESM are regularly picked and transferred to separate dishes.

After their exposure to a hormonal treatment ("induction" on culture medium containing auxin and cytokinin as described by Bercetche and Pâques, 1995), immature zygotic embryos reacts by the production ("initiation" per se) of proliferating embryonic cultures), the latter production being called "initiation". The embryonic cultures are to be maintained on the "initiation medium" at least until filling a full Petri dish (9 cm diameter, around 3 g FW of ESM).

Recently initiated lines need special attention to remove necrosing material and to avoid the spread of infection from inefficiently disinfected zygotic embryos and accidental contamination by operator. The average initiation rate reaches 19 to 50% (Bercetche and Pâques, 1995 and unpublished) while for some particular crosses it can reach 80% (our unpublished data, at least 500 seeds per cross per

year). But the success rate can be highly variable from year to year, even from the same parent tree with the same protocol operated by the same people.

2.3. Culture multiplication (maintenance or proliferation)

The maintenance of embryonic cultures is done on the same medium as initiation with bi-weekly subculture. As much as possible of the colored (non-whitish) parts of ESM are discarded. Due to high growth rate, the quantity of plant material per container needs to be limited to a maximum of 2.5 g FW (for a 9 cm wide Petri dish containing 25 ml of culture medium). The vigorous proliferation (fresh weight at least doubled in one week) of the plant material can be maintained for some weeks on old or very diluted medium but the regularity of subculturing is necessary to the maintenance of a high plant regeneration potential, *i.e.* stresses are to be avoided as much as possible. Liquid culture in shaked culture flasks can be easily launched through progressive increase of the volume ratio of culture medium to ESM. But the optimal culture conditions varied widely between lines. Regarding the easy subculturing and high proliferation rate achieved in solid culture while liquid culture is more susceptible to infection, suspension culture can only be justified when producing a huge quantity of plant material from a restricted numbers of lines.

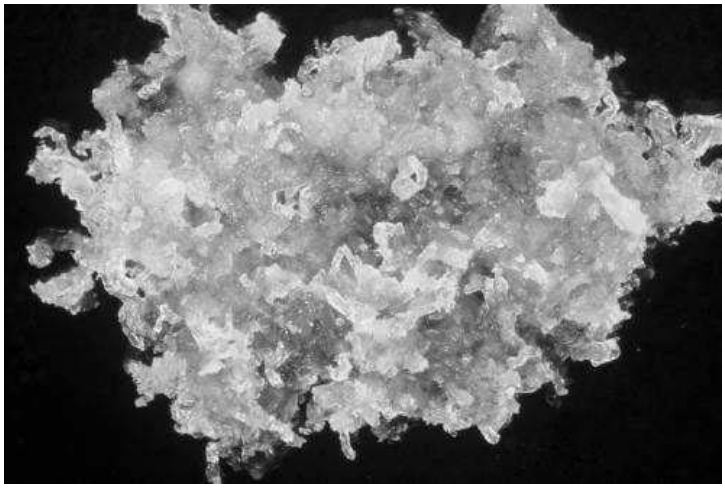

Figure 3. Proliferation and maintenance on solid culture medium.

2.4. Embryo maturation

Similarly to other pine species, the maturation can mainly be obtained by osmotic stress through the use of PEG (Attree and Fowke, 1991) or by reduced water availability through the use of high content of gelling agent (Klimaszewska and Smith, 1997). The later approach has been shown to give regenerated plants of a better quality in *Pinus pinaster* (Ramarosandratana *et al.*, 2001).

1. A suspension of ESM (3-5 g per 20 ml) is prepared with the outer part of colonies collected from several dishes of the same line, resuspended in liquid maturation medium and shaken vigorously.
2. Thereafter, 1 ml of this suspension is spread on a round piece of filter paper (Whatman n°2, 5.5 cm in diameter) placed on the gelled medium.
3. Dishes are sealed with cling film and kept in total darkness (22±1°C).
4. The filter with growing embryos is subcultured on new medium every 3 weeks.
5. After three months under maturation condition, embryos are harvested and converted into plants ("germination").

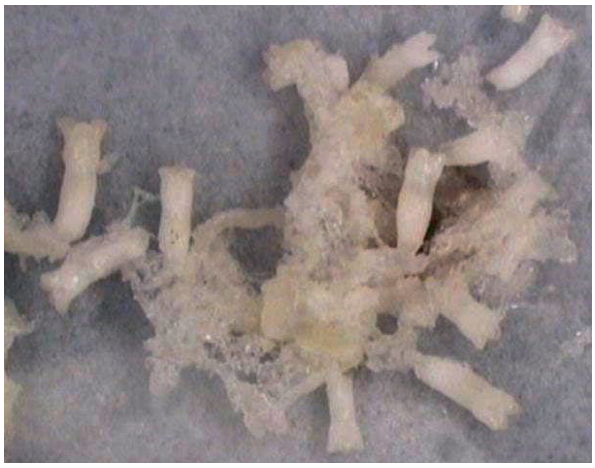

Figure 4. Close up of maturation of a high-yield ESM line.

2.6. Embryo germination

1. Harvested mature embryos are placed horizontally onto germination medium.
2. They are incubated in the dark at 22±1°C for a week before being directly transferred under cool white fluorescent light (80 µE.m-2.s-1, 16-h photoperiod). The first visible changes are the swelling of the whole embryo's body and the appearance of a red ring at the level of the root collar and the yellowing and then greening of cotyledons.

3. After one week, the embryos are transferred to fresh medium every month.
4. When too large for the Petri dish (cotyledons and/or root touching the wall), the plantlets are transferred to larger containers like Magenta boxes (air to medium volume ratio of 2:1).

Figure 5. Plantlets after one month of growth after the conversion (graduation on the scale = 2.5 cm).

2.7. Acclimatization and field transfer

1. After a maximum of 2.5 months or when the plantlets are more than 2.5 cm long, they are transferred to a 1:1 mix of peat and composted pine bark in the greenhouse under a translucent plastic sheet keeping air humidity to saturation.
2. After one month, the plastic sheet is progressively removed and the plantlets treated as standard seedlings. Fortunately, plantlets lacking primary root development are usually able to form secondary root following the transfer to ex vitro conditions.
3. The plants are established in the field at the normal planting season when their size is above 15 cm length.

Figure 6. 18 months-old plants in the field in Nangis, in June 1999 (stick = 1 m high , plant beside a white stick = control seedling).

3. APPLICATIONS (LINK WITH OTHER BIOTECHNOLOGIES)

3.1. Cryopreservation

The main difficulty of cryopreservation is not to have and to follow a good protocol but it is the organization of the work and of the data. Our method was adapted from the protocol detailed in Bercetche *et al*., 1990.

a. Freezing

1. 5 days after the last subculturing on standard (0.09 M sucrose) maintenance medium, the ESM are transferred to solid proliferation medium with sucrose concentration increased to 0.3 M
2. After 1 day, the ESM are transferred to 0.5 M sucrose solid proliferation medium
3. After 1 day on 0.5 M sucrose solid medium, the ESM are suspended in ice-cold liquid maintenance medium containing 0.5 M sucrose in order to obtain a 10% w/v suspension which is incubated in sterile centrifuge tubes on ice
4. filter-sterilized DMSO is added stepwise along one hour until reaching a concentration of 5% v/v. The centrifuge tube containing the suspension is briefly agitated by hand after each DMSO addition.
5. the cultures are incubated on ice for a further 2 hours
6. the cultures are manipulated without disturbing the spontaneous sedimentation and supernatant is removed in order to reach a ESM to liquid ratio of 30%
7. the resulting suspension is dispensed aseptically into 2 ml cryovials which are closed hermetically and put inside a cryobox
8. the cryobox is put inside a programmable freezer set at 0°C
9. the temperature of the incubating chamber of the programmable freezer is dropped slowly until –10°C in 15 min. After a 15 min pause at –10°C, the cooling restart to reach –35°C in a further 50 min.
10. The cryobox is taken from the freezer at –35°C and plunged directly in liquid nitrogen.
11. The input of data describing the freshly cryostored samples is confirmed and terminated before the operator shift to another task. The data have been prepared before and during the treatment of the samples. They are precisely describing the nature of the samples (with subculture numbers, mediums used, genetic parameters…) with the name of the operator, the freezing date and overall where precisely in the cryotank the samples are stored.

b. Thawing:

1. The cryovials of interest are localized in the cryotank using the associated database in order to minimize the duration of the operation.
2. The cryovials are removed from the cryotank and quickly plunged in a warm water bath pre-warmed to 40°C.
3. After the complete thawing of the content, the cryovials are brought to the laminar flow hood and thoroughly washed with 70% ethanol.
4. They are then aseptically manipulated. After the complete drying of their outside, cryovials are carefully opened and the content of each is poured on a piece of sterile filter paper placed on the top of a pile of sterile filter or absorbing paper.
5. When all the liquid phase has been removed, the top filter supporting the thawed plant material is transferred to a Petri dish containing 0.5 M sucrose proliferation medium. The material is incubated in the dark culture room for one day.
6. Afterwards, the filter paper is transferred to a Petri dish containing 0.3 M sucrose proliferation medium.
7. After one day, the filter paper is transferred to a Petri dish with normal proliferation medium and subcultured bi-weekly as usual. The plant material is removed of the supporting filter paper as soon as possible.

As soon as one cubic centimeter (about 1 g) of healthy ESM is obtained, half of this quantity can be cryopreserved while the rest is maintained in culture. Step by step, each time a cubic centimeter is available, the cryopreserved stock is increased until having a suitable stock of "young" culture (typically ten to twenty cryovials per new line).

The early check of regrowth capacity from cryopreserved samples is necessary in order to be allowed to discontinue as early as possible the maintenance of the lines whose the initial cryopreservation is completed without compromising the possibility of their further culture. The lines of which the initial cryopreservation is completed can be either removed from the set of active cultures or maintained at the minimal scale needed for further characterization.

3.2. Genetic transformation

Genetic transformation of maritime pine ESM is currently developed by AFOCEL in partnership with INRA (French National Agricultural Research Institute). Protocols for both biolistic and *Agrobacterium tumefasciens transformation* are presented *in* Trontin *et al.*, 2002

3.3. Molecular markers for genetic fidelity

As in other plant production operations, it is important to check the absence of any mislabeling or clones admixture. Microsatellites, also called SSR (simple-sequence repeats), are the only kind of molecular markers allowing a sufficiently precise identification of each clone. At present for maritime pine, only a few nuclear microsatellites (highly species-specific and expansive to discover in the genome but they have a very high discriminative power of apparented individuals due to very high polymorphism) are available (Gonzalez-Martinez *et al*., 2002) in addition to the widely used chloroplastic microsatellites (Genus specific, paternally inherited with moderate variability so limited in discrimination power) designed initially from DNA sequence of Pinus thunbergii (Vendramin *et al*., 1996) but it is enough for most situations. An international consortium comprising the most important *Pinus pinaster* breeders is under work to obtain suitable pairs of primers for around 200 new SSR loci. These tools will be of great value for a handful of applications like genetic diversity monitoring and management and quality control in experimental and commercial seeds and plants production.

REFERENCES

Attree SM and Fowke LC (1991) Micropropagation through somatic embryogenesis in conifers. *In:* Bajaj YPS (*eds*) High-Tech and Micropropagation I, Springer-Verlag, Berlin,17:53-70.

Bercetche J and Pâques M (1995) Somatic embryogenesis in maritime pine (Pinus pinaster). *In:* Jain SM, Gupta PK and Newton RJ (*ed*) Somatic embryogenesis in woody plants. Volume 3 - Gymnosperms., 221-242.

Bercetche J, Galerne M and Dereuddre J (1990) Enhancement regeneration from embryogenic callus of *Picea abies* L. karst after freezing in liquid nitrogen. Bull. Soc. Bot. Fr. - Actual. bot. 137:3; 4):136-137.

Gonzalez-Martinez S, Gerber S, Cervera M, Martinez-Zapater J, Gil L and Alia R (2002) Seed gene flow and fine-scale structure in a Mediterranean pine (*Pinus pinaster* Ait.) using nuclear microsatellite markers. Theor. Appl. Genet. 104:1290-1297.

Gupta PK (1995) Somatic embryogenesis in sugar pine (*Pinus lambertiana* Dougl.). *In:* Jain S, Gupta P and Newton R (*ed*) Somatic embryogenesis in woody plants, Kluwer Academic Publishers, 3:197-205.

Gupta pK and Durzan DJ (1985) Shoot multiplication from mature trees of Douglas fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Reports 4:177-179.

Harvengt L, Trontin J, Reymond I, Canlet F and Pâques M (2001) Molecular evidence of true-to-type propagation of a 3-year-old Norway spruce through somatic embryogenesis. Planta 213:828-832.

Klimaszewska K and Smith DR (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. Physiol Plant 100:949-957.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Ramarosandratana A, Harvengt L, Bouvet A, Calvayrac R and Pâques M (2001) Effects of carbohydrate source, polyethylene glycol and gellan gum concentration on embryonal-suspensor

mass (ESM) proliferation and maturation of maritime pine somatic embryos. *In vitro* Cellular and Developmental Biology - Plant 37:29-34.

Trontin JF, Harvengt L, Garin E, Lopez-Vernaza M, Arancio L, Hoebeke J, Canlet F and M. P (2002) Towards genetic engineering of maritime pine (*Pinus pinaster* Ait.). Annals of Forest Sciences 59:5/6:687-697.

Vendramin GG, Lelli L, Rossi P and Morgante M (1996) A set of primers for the amplification of 20 chloroplast microsatellites in Pinaceae. Mol Ecol 5:4:595-598.

SOMATIC EMBRYOGENESIS IN *PINUS PATULA*

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1. INTRODUCTION

Softwood species make up about 50 % of the plantation area in South Africa, of which *Pinus patula* is the most important, occupying approximately half of this (Department of Water Affairs and Forestry, 1998). Introduced from Mexico, this species is deployed in commercial plantations over a range of sites in the summer rainfall regions of the country, typically characterized by a minimum of 800 mm MAP, located above an altitude of 1200 m and MAT less than 18°C (Morris and Pallett, 2000). Under these conditions, yields of 22 m^3 ha^{-1} y^{-1} can be expected from the best quality sites (Evans, 1999), while mean annual increments (MAI) of about 17 m^3 ha^{-1} y^{-1} are derived from lower productivity sites (Pallett, 2000). The timber products derived from softwood species include saw timber, pulp and paper among others (Department of Water Affairs and Forestry, 2001).

In order to ensure that material of current highest genetic gain is deployed as rapidly as possible, breeding and propagation strategies need to be aligned. *Pinus patula* is conventionally propagated from open pollinated (OP) seed derived from seed orchards. Further gains can be expected through the deployment of selected OP or controlled pollinated (CP) families. Although deployment of softwoods is conducted mainly through seedling planting stock, vegetative propagation has played an important role in maximising genetic gains from forest breeding programmes and promises to continue adding value to the forest industry.

A valuable strategy used to align breeding and propagation methods, is the practice of family forestry, defined by Griffin (2001) as the deployment of open pollinated seed from top performing clones, in the clonal seed orchard (CSO), by means of cuttings. Although, this strategy is being

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implemented, the importance of *P. patula* to the South African forest industry, would justify the investment in clonal forestry (Bayley and Blakeway, 2002). The major constraint associated with this approach, particularly for *P. patula*, is the phenomenon of physiological maturation (or aging), which prevents sustained clonal propagation of cuttings due to their decreased rooting and poorer in-field growth compared to their seedling counterparts (Bayley and Blakeway, 2002). The implication of this is that when the tissue is most plastic and amenable to vegetative propagation, the genotype is untested and a prediction of final performance is unreliable (John, 2002). Furthermore, the hedges of *P. patula* are not conducive to cycling (establishing hedges from cuttings), which results in cuttings that are morphologically different to those derived from seedling hedges (Bayley and Blakeway, 2002). The lower productivity of older (2.5-year-old) *P. patula* hedges, coupled with an inability to maintain the hedges through cascading techniques implies that the mass propagation of individual clones is not feasible and is the reason that clonal forestry has not been possible (Jones, 2002).

Another more recent restriction, to the deployment of *P. patula*, has been the outbreak of *Fusarium circinatum* (pitch canker fungus), observed in South African forestry nurseries. This is currently one of the most serious threats to the softwood forestry industry, and has highlighted the susceptibility of *P. patula* seedlings and cuttings to this pathogen. The presence of this disease poses problems for the commercial establishment of *P. patula*, in that poor stocking of improved material influences final yield and negates the breeding effort. Some of the long-term strategies suggested to curb the spread of the disease in order to limit its destructive impact are: to hybridize *P. patula* with other more tolerant species, to identify and propagate tolerant families of pure *P. patula*, or to implement a combination of both these strategies. It is in providing a solution to the clonal deployment of selected *P. patula* genotypes that the value of somatic embryogenesis could potentially be realized. Further value-added traits that could be captured through this process include improved yield and wood quality (Sutton *et al*., 1993).

Somatic embryogenesis offers a means of bulking up specifically selected genotypes. Embryonal suspensor mass (ESM) tissue has several advantages over other *in vitro* propagation methods in that it has potentially high multiplication rates, potential for scale-up and delivery via bioreactor and synthetic seed technologies, amenability to cryogenic storage, and the suitability of the target tissue for gene transfer (Merkle and Trigiano, 1992; Percy *et al.*, 2000). Cryostorage is also a means of circumventing the effects of somaclonal variation (De Verno *et al*., 1999) and loss of cultures to contamination. Furthermore, this technique is a prerequisite for the production of plants from selected lines following

completion of long-term clonal trials, as it allows the storage of embryogenic tissue in a juvenile state (Park *et al.*, 1993; Percy *et al.*, 2000).

Somatic embryogenesis in *P. patula* was first achieved in 1992 (Jones *et al.*, 1993). The embryogenic induction frequencies range from 2.6 to 8.5 % and at least 90 % of the families tested in culture, respond (Fig. 1), although with varying levels of success (large variation in the number of clones initiated per family). This represents first time exposure of cultures to *in vitro* clonal selection. Embryogenic tissue is multiplied and prepared for cryostorage, where the recovery rate is excellent, if the tissue placed into storage is semi-translucent and rapidly proliferating. However, the recovery rate rapidly declines if the ESM are compact, slow to proliferate and semi-opaque. This is a further factor contributing towards *in vitro* clonal selection. The most significant obstacle in the production of *P. patula* embryogenic plantlets is the maturation process, in that not all genotypes respond to the maturation treatment (Fig. 1). In some genotypes, abnormal cotyledonary embryos can develop, while in others the tissue may loose its embryogenic capacity and embryo development does not progress beyond stage 2 (opaque, pre-cotyledonary embryos). Initially polyethylene glycol (PEG 8000) was included in the maturation medium as an osmoticum. This treatment produced high numbers of well formed embryos, but the inhibitory effects of the PEG led to poor development of germinated plantlets (Jones and van Staden, 2001). To date the combination of maltose and ABA have yielded well-developed embryos, but the yields are limited (Jones and van Staden, 2001). The efficiency of the embryogenic process is further determined by the conversion rate, which, for *P. patula* is still significantly low (Fig. 1), but linked to the quality of embryo produced during the maturation phase.

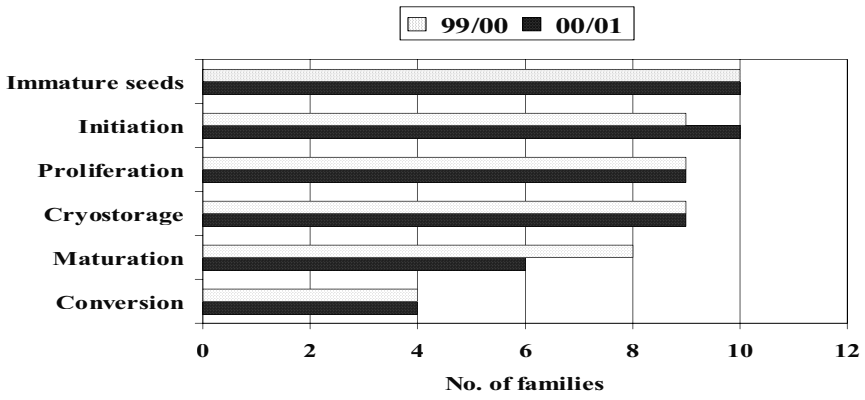

Figure 1*:* Constraints in the somatic embryogenesis process in *P. patula* cultures initiated over two seasons, indicating the *in vitro* selection in family responses.

Application of somatic embryogenic techniques to *P. patula* offers the opportunity to explore the potential for clonal forestry with this species. Furthermore, with the application of biotechnology, scope exists to consider the genetic modification of selected genotypes, particularly for disease tolerance. The aim of this chapter is to report on the current best operating protocols used in establishing, maintaining, manipulating and maturing embryogenic tissue of *P. patula* derived from selected families in order to produce quality somatic plantlets.

2. EMBRYOGENIC CULTURE INITIATION

2.1 Explant

One-year-old green cones are harvested from selected trees between December and January (Southern Hemisphere). The cones are collected in the morning on a weekly basis and placed into brown paper packets. The packets are stored in a cooler bag with freezer blocks to maintain the temperature at about 4 ºC. The cones are transported to the laboratory and stored in the refrigerator until they are placed into culture (within 4d of harvesting).

The cones are washed under running water to remove surface dirt. They are then submerged in 75% ethanol, containing a few drops of Tween 20® for 5 min, with frequent agitating. The ethanol is decanted and a 1.3 % NaOCl solution with a few drops of Tween 20® is added. The cones are left in solution for 20min, agitating frequently. The liquid is decanted and the cones are washed 3 times in sterile distilled water.

2.2 Culture Medium

Initiation medium: Megagametophytes are placed onto MSG3 medium (Becwar *et al.*, 1990). This medium comprises MSG basal salts (Table 1), 3% sucrose, 0.1 $g.l^{-1}$ myo-inositol, 1.5$g.l^{-1}$ L-glutamine and 0.3% Gelrite®. The L-glutamine is filter sterilised (using a Sartorius filter sterilizing unit) and added to the medium when it has cooled to 60°C, just prior to pouring into 65 mm plastic petri dishes.

Proliferation/maintenance medium: The proliferating tissue is subcultured every two weeks onto fresh maintenance medium. The medium is prepared using the MSG basal salts (Table 1), 3% maltose, 0.1$g.l^{-1}$ myo-inositol, 1.5$g.l^{-1}$ L-glutamine and 0.3% Gelrite®. The maltose is autoclaved separately and added to the medium after autoclaving. The L-glutamine is filter sterilised and added to the medium when it has cooled to

60 °C, prior to dispensing.

Liquid medium: This medium is used to bulk up tissue in suspension and also for singulation of the somatic embryos prior to placing them onto 240- maturation medium. The medium is prepared using the MSG basal salts (Table 1), supplemented with 3 % sucrose, 0.1g.l^{-1} myo-inositol and 1.5g.l^{-1} L-glutamine. The L-glutamine is filter sterilized and added to the medium when it has cooled.

Table 1. Formulation of MSG and 240 basal media

Components	MSG [1] (mg.l^{-1})	240 [2] (mg.l^{-1})
$MgSO_4.7H_2O$	370	246.5
NH_4NO_3	-	200
KNO_3	100	909.9
KH_2PO_4	170	136.1
$Ca(NO_3).4H_2O$	-	236.2
$Mg(NO_3)_2.6H_2O$	-	256.5
$MgCl_2.6H_20$	-	101.7
KCl	745	-
$CaCl_2.2H_2O$	440	-
KI	0.83	4.15
H_3BO_3	6.2	15.5
$MnSO_4.H_2O$	16.9	10.5
$ZnSO_4.7H_2O$	8.6	14.4
$Na_2MoO_4.2H_2O$	0.25	0.125
$CuSO_4.5H_2O$	0.025	0.125
$CoCl_2.6H_2O$	0.025	0.125
$FeSO_4.7H_2O$	27.8	13.9
Na_2EDTA	37.3	18.65
Nicotinic acid	0.5	0.5
Pyridoxine	0.1	0.5
Thiamine. HCl	0.1	1.0
Glycine	-	2.0
Additives:		
Myo-inositol	100	100
Casein acid hydrolysate	-	500
L-glutamine	1500	450
Maltose	30000	60000
Gelrite®	3000	3000
Abscisic acid	-	10
2,4-D	20	-
Benzyladenine	10	-

[1] Becwar *et al.* (1990)
[2] Pullman and Webb (1994)

Maturation medium: Singulated embryos are plated onto 240-maturation

medium (Pullman and Webb, 1994). The medium is prepared using 240 basal salts (Table 1), 6% maltose, 0.5g.l^{-1} casein acid hydrolysate, 0.45 g.l^{-1} L-glutamine and 10mg.l^{-1} abscisic acid. The dissolved L-glutamine and the abscisic acid stock solution are filter sterilized and added to the cooled, autoclaved medium, prior to pouring.

Germination medium (with activated charcoal): Embryos that have been partially dried under high relative humidity are placed onto 240- germination medium (Pullman and Webb, 1994). The medium is prepared using 240-basal salts (Table 1), 3% maltose, 0.5g.l^{-1} casein acid hydrolysate, 5g.l^{-1} activated charcoal and 0.45g.l^{-1} L-glutamine. The L-glutamine is filter sterilized and added to the cooled, autoclaved medium.

Germination medium (for GA7 flasks): Embryos that have germinated on 240-germination medium, containing activated charcoal (Pullman and Webb, 1994), are transferred into Magenta GA7 flasks for further development in the light. The medium is prepared using 240-basal salts (Table 1), 3% maltose, 0.5g.l^{-1} casein acid hydrolysate and 0.45g.l^{-1} L-glutamine. The L-glutamine is filter sterilized as described previously, before adding to the medium.

2.3 Inoculation

The scales of the decontaminated cones are pealed back under aseptic conditions and the immature seeds extracted. The seeds are placed into a sterile glass petri dish and moistened with a little sterile, distilled water. Using a dissecting microscope, the seed coat is removed and the female gametophyte containing the immature embryo excised as the explant. The megagametophyte is placed onto the MSG3 medium and the petri dish is sealed with commercial cling wrap. Five explants are usually placed into each petri dish. The cultures are stored at 25°C ± 2°C in the dark. Attempts at inducing ESM from mature seeds have to date not been successful.

2.4 Culture Initiation

After 4-6 weeks, small amounts of semi-translucent tissue begin to proliferate from the micropylar end of the megagametophyte (Fig. 2A). When significant amounts of tissue have begun to proliferate, the tissue should be placed onto a fresh plate.

2.5 Culture Maintenance

Once proliferating, embryogenic cultures are sub-cultured every 14d (Fig. 2B). Small pea-sized nuggets of tissue are pinched off from the culture

using sterile forceps. The tissue is placed onto fresh MSG3 maintenance medium. The petri dishes are sealed with commercial cling wrap and the cultures are transferred to a dark growth room at 25°C ± 2°C.

2.6 Preparing Cell Suspensions

To prepare 100ml Erlenmeyer flasks, 35 ml aliquots of liquid MSG3 medium are measured aseptically and transferred into the sterile Erlenmeyer flasks. Approximately 200 mg of tissue is plucked from the maintenance cultures. The tissue is selected from the outer perimeter of the culture, where it is more mucilaginous and transferred to the flask. The neck of the flask is stoppered with a sterile cotton wool bung and covered with a foil cap. The flask is wrapped in foil to exclude light and is then placed onto a rotary shaker at 120 rpm at 25°C ± 2°C.

2.7 Staining to Confirm Embryogenic Nature

Tissue is stained with 0.5% acetocarmine stain to confirm the embryogenic nature. This is prepared by bringing 45ml glacial acetic acid and 55ml distilled water to boil under a fume hood. Carmine powder (0.5g) is added to the boiling liquid and boiled for 10-15 min, stirring until the colour becomes bright red. The solution is allowed to cool before being filtered into a dark bottle and stored at 4 °C. This stain is used to check the developmental stage of the zygotic embryos housed in the megagametophyte, and to determine whether the embryogenic tissue has produced “polar” or “solar” embryos (described below).

2.8 Embryo Development

Stage 1 embryos (characterized by a translucent embryonal head) are generally visible protruding from the surface of embryogenic cultures. When these cultures are placed into liquid MSG3 medium, the embryos separate out and form polar embryos (a well-defined embryonal head subtended by a suspensor tail). After two weeks, the tissue is usually ready to be placed onto 240-maturation medium. In some cases, the tissue develops into solar embryos (the embryonal head is not well defined and the suspensor tissue forms a “halo” around the head). These embryos do not develop further on the 240-maturation medium. The tissue can be left in suspension culture for a little longer to determine whether polar embryos develop. When embryos are placed onto 240-maturation medium, they develop further, to stage 2-4 embryos. Stage 2 embryos are characterised by a smooth, opaque embryonal head. Stage 3 embryos are also opaque and have cotyledonary initials starting to develop. Stage 4 embryos characteristically, are white, have well-defined cotyledons and the hypocotyls have elongated (von Arnold and Hakman, 1988).

2.9 **Embryo Maturation**

When the tissue in suspension is at the polar stage (generally after 10-14d), it is ready to be placed onto maturation medium. A Sartorius filter sterilising unit is used to transfer the liquid cultures back onto solid maturation medium. Whatman No. 2 filter paper discs (42.5 mm) are used as supports for the tissue. A one ml aliquot of the suspension culture is pipetted onto the filter paper. When all of the liquid has been drawn off from the tissue (using a vacuum pump), the disc is lifted off and placed onto 240-maturation medium. The plates are sealed with commercial cling wrap and kept in a dark culture room at 25°C ± 2°C. The cultures are subcultured every 3 weeks onto fresh medium. After 4-8 weeks, stage 2-3 embryos are visible, and from 8-12 weeks in culture, stage 4 embryos develop. When the cotyledons are open wide and the hypocotyl is well developed, the embryos are plucked from the medium and dried under high relative humidity. This is achieved by placing the embryos into 4-well multi dishes. The wells are lined with sterile Whatman No. 2 filter paper discs (cut to size) and the center of the dish is filled with 3 ml sterile distilled water. The dishes are sealed with a double layer of commercial cling wrap and the cultures are placed into a dark growth room at 25°C ± 2°C for 3 weeks. Care must be taken to prevent water from getting into the wells and in contact with the embryos.

2.10 Embryo Germination

The filter paper discs (supporting the embryos) in the 4-well multi-dishes are removed from the dish under sterile conditions and placed onto the 240-germination medium (0.5% activated charcoal) for a few minutes. Once the filter paper has hydrated, the embryos are gently removed from the filter paper and plated out on the germination medium. They are arranged in rows, with no more than 25 explants per petri dish (Fig. 2D). The petri dishes are wrapped in commercial cling wrap and left in the dark at 25 °C ± 2 °C for 2 weeks.

After 2 weeks, embryos that have undergone hypocotyl elongation and are showing signs of radicle initiation (i.e. germinated) are removed from the 240-germination medium (0.5% activated charcoal) and inserted into 240-germination medium in GA7 flasks. The GA7 flasks are sealed with a double layer of commercial cling wrap and placed in the light at 25°C ± 2°C until the roots have emerged and the cotyledons have greened (Fig. 2E).

2.11. Acclimatisation and Field Transfer

The commercial cling wrap is removed and the GA7 flasks are placed in the greenhouse for 7-10d to begin the acclimatisation process. A mixture of composted pine bark: Irish peat moss: Vermiculite (7:7:3) moistened with tap water is autoclaved, prior to filling disinfected, 200 cavity polystyrene seedling trays. A small hole is made in the medium with a blunt object. The somatic plantlets are removed from the GA7 flasks, taking care not to damage the roots, and transferred into the seedling trays. The trays are drenched with a 1% Kelpak solution, covered with clear plastic covers to maintain high humidity and placed under a double layer of 20% shade cloth in the greenhouse. After 24 h, the plants are drenched again with 0.2% Previcur-N and 3% Benlate. After 3-4d, the plastic covers are removed from the trays. The somatic plantlets are thoroughly watered every 2d depending on the weather and fertilized with Hortichem Orange fertilizer ($0.4g.l^{-1}$). Thereafter, the plantlets are watered twice a week depending on the weather (during very hot periods this is increased to 3 times a week) and fertilized when necessary.

When the plantlets have been acclimatized for 3 months, they are transferred to the nursery (Fig. 2F). At 6 months, the somatic plantlets are transferred into 10 litre black bags filled with well-composted pine bark, growing medium. The plantlets are allowed to acclimatize for a further month before cuttings can be taken from the hedges. The cuttings are set in sterilized seedling trays filled with bark: Vermiculite: Perlite (4:1:1). The cuttings are placed onto heated mist beds so that the temperature of the growing medium lies between 25 – 28°C for 2 months in summer and 3 months in winter. The cuttings derived from the somatic hedges are moved out into natural sunlight. They are watered as necessary (taking care not to leave the cuttings too moist as this encourages disease) and fertilized with Hortichem Orange and Hortichem Blue once a week. Kel-P-Max (a foliar feed) can also be used periodically. The cuttings produced in this manner are planted out into clonal field trials on selected sites 60 d after leaving the misting camp.

3. APPLICATIONS

3.1. Cryopreservation

3.11. Freezing

Healthy embryogenic tissue (7-10d after subculture) is selected. Following the protocol developed for *P. patula* (Ford *et al*., 2000a), 3ml MSG3 liquid medium for cryopreservation (containing 0.3 M sorbitol)

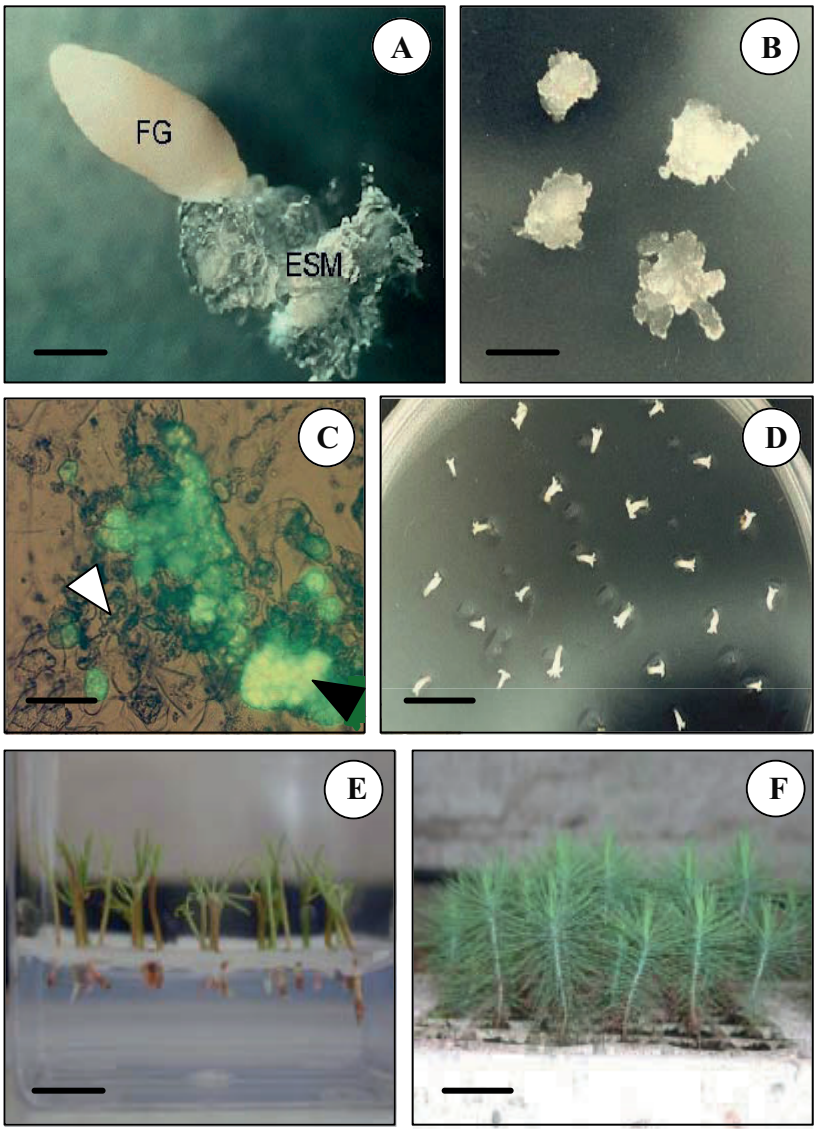

Figure 2. The somatic embryogenesis process: (A) Initiation from immature seeds, FG, female gametophyte; ESM, embryonal suspensor mass (Bar=1.4 mm); (B) Embryogenic tissue from four different families showing the different tissue morphologies (Bar=8 mm); (C) Tissue stained with fluorescene diacetate (FDA) after 8 weeks in cryostorage. Intact embryonal head is indicated with the black arrowhead, while the highly vacuolated suspensor tissue that has not fluoresced (white arrowhead) is no longer viable (Bar=80 µm); (D) Somatic embryos with well-developed hypocotyls and cotyledons after partial drying (Bar=9 mm); (E) Embryos germinating in Magenta GA7 flasks (Bar=10 mm); and (F) Somatic plantlets that have been acclimatised and are ready for bagging into hedges (Bar=5 cm).

is pipetted into a sterile 25ml Erlenmeyer flask with a cotton wool bung and foil cap. Using a sterile balance and a sterile plastic petri dish, 1 g of embryogenic tissue is weighed from the selected plate (ensure that the most friable tissue is selected and avoid the core tissue). The tissue is suspended in the cryopreservation medium. The flask is wrapped in foil and placed on a rotary shaker at 120 rpm for 24h.

A 10% DMSO solution is prepared using the MSG3 liquid medium for cryopreservation by adding 1ml DMSO to every 9 ml MSG3 cryo-medium. The medium is chilled on ice and the pre-treated tissue also placed on ice. The tissue and medium is allowed to equilibrate for approximately 10min. Three ml of the 10% DMSO solution is pipetted into the flask (this results in a final concentration of 5% DMSO). The tissue is left for a further 10min to equilibrate. One ml aliquots of the tissue are pipetted into the sterile cryovials. These are placed into a Mr Frosty (Nalgene® Cryo 1°C Freezing Container) containing iso-propyl alcohol. The container is placed into a –70°C freezer for 2h. After this slow-cooling period, the cryovials are removed from the freezer and clipped onto canes. The canes are placed into their allotted canisters and plunged into the liquid nitrogen dewars.

3.12. Thawing

A water bath filled with distilled water is heated to 42°C. Selected cryovials are removed from the canes, and placed into the cryovial boat (found inside the Mr Frosty container). The boat is placed into the water bath and the samples are allowed to thaw for 5-6min (until the solid ice core has melted, but the suspension should not be allowed to heat up).

The cryovials are transferred onto the laminar flow bench, and sprayed with 80 % ethanol. Using a sterile filter-sterilizing unit, a fresh filter paper disc is placed onto the filter sterilizing support, followed by a sterile 4x4 cm, 32µm polyester grid. Vacuum pressure is applied and the contents of the cryovial are poured onto the grid. When all of the supernatant has been drawn off, remove the grid and place it onto MSG3 maintenance medium. Remove the filter paper disc and replace with a fresh one before placing the next genotype onto the filter. Subculture the recovered tissue by lifting the polyester grid and placing it onto fresh MSG3 maintenance medium after 24 h. Subculture the tissue onto fresh MSG3 maintenance medium 7 d later, and then every 14d. When sufficient tissue has recovered, the tissue can be removed from the grid and place directly onto the MSG3 maintenance medium, subculturing every 14d.

3.13. Staining

Preparation of FDA stain: Tissue can be stained with 0.05% fluoreceine diacetate (FDA) and viewed under an ultraviolet light (UV) to assess whether cells are still viable. Viable tissue fluoresces apple green under UV light (Fig. 2C). Prepare fluoreceine diacetate stain solution by adding
5mg FDA in 1ml acetone. The solution should be stored in a sealed bottle in the freezer to prevent the acetone from evaporating.

3.2 Genetic Transformation

3.21 Treatment of Target Tissue

Aliquots (1.5ml) of suspended embryogenic tissue (ESM) are filtered onto Whatman No. 3 filter paper supports and are placed onto MSG3 medium to achieve 6 d growth, or longer for slow-growing genotypes. Material used for gene transfer studies should not be more than one-year-old from the date of initiation and cryopreservation protocols should be implemented to maintain tissue juvenility. There is a need to select for genotypes that not only have good embryogenic potential but also the inherent ability to undergo genetic transformation and subsequent regeneration. The successful transformation of selected lines can depend on their proliferative nature and ease of culture.

3.22 DNA-Coating of Microparticles

Tungsten microparticles are sterilised by incubating 100mg, 1.5μm-sized particles in 2ml 70 % ethanol overnight. The particles are briefly spun down at 2374.6g. The ethanol is then removed and replaced with 2ml sterile dH_2O. Washing of the microparticles (resuspension followed by centrifugation) with sterile dH_2O should be repeated twice more. The sterile particles are stored in autoclaved 50% glycerol. Microparticle precipitation should be prepared to a concentration of 4μg DNA mg^{-1} tungsten particles according to the method described by Perl *et al.* (1992). The vector construct used was a *bar*-GUS cassette under the control of the *ubiquitin* promoter.

3.23. Bombardment Procedure

Day 0: The filter paper discs, supporting the ESM, are placed onto MSG3 medium supplemented with 0.25M sorbitol (Walter *et al.*, 1999) and left with unsealed lids, overnight, on a laminar flow bench. Microscopic analysis, after particle transfer, showed cell-burst in cultures without osmotic treatment, while a marked positive effect on the integrity of culture cells was observed after inclusion of an osmoticum in the medium. Osmotic enhancement was suggested to result from plasmolysis of the cells that may have reduced cell damage by preventing or making

protoplasm extrusion less likely from bombarded cells (Vain *et al.,* 1993; Li *et al.,* 1994) and may have improved particle penetration itself (Sanford *et al.*, 1993).

Day 1: The laminar flow bench, as well as the interior and exterior of the GENEBOOSTER^{TM}, is wiped with 70% alcohol at least 15min prior to bombardment. Macroparticles, stored in 100% ethanol overnight, are placed onto an autoclaved petri dish using sterile forceps and left to air dry. Twenty shots are prepared by adding 25µg isolated plasmid DNA to 125µl sterile tungsten particle suspension and mixed well, prior to the addition of 125µl ice-cold 1M $CaCl_2$. The mixture is vigorously shaken to prevent DNA and tungsten aggregation, and to allow precipitation onto the tungsten particles. Incubation on ice for 10-12min allows precipitation and sedimentation to occur, after which time 200µl of the clear supernatant is discarded. The DNA-coated microparticles are resuspended in the remaining liquid. Bombardment conditions using the gene gun (GENEBOOSTER^{TM}-ELAK Ltd., Budapest, Hungary), in a sterile environment, remain constant with the shelf supporting the target tissue set at 70 mm from the stopping shelf. Ten µl DNA-coated tungsten particles are loaded onto a macroprojectile and then inserted into the barrel prior to bombarding the tissue at -0.4 bar vacuum and at 40 bar for shooting pressure of the Nitrogen gas. The target tissue, bombarded once only, is removed, replacing the lid immediately to prevent contamination, and incubated in the dark at 25°C. Remove the macroprojectile from the stopping plate and discard.

3.24. *GUS* Assay

Samples of bombarded tissue are tested for transient expression on the same or the following day. Using sterile forceps, random samples of ESM (size of drawing pin head) are removed and placed in separate wells of a microtitre plate. Thirty µl 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-glcA) buffer (0.3% X-glcA [Sigma, Johannesburg, South Africa],
5mM K-ferrocyanide, 5 mM K-ferricyanide, 0.005% Triton X-100, 100 mM Na-phosphate buffer [0.5M NaH_2PO_4, 0.5M $Na_2HPO_4.2H_2O$], [pH 7], dissolved in methanol) is added to cover tissue samples entirely. Samples are incubated with the X-glcA buffer in a thermostat for 6h to overnight at 37°C. β-glucuronidase, encoded by the GUS gene, causes the oxidative dimerisation of X-glc, (a chromogenic substrate) and results in a deep blue coloured stable and insoluble precipitate when the GUS gene is transiently expressed. Bombarded ESM samples exhibit a range of expression strength of β-glucuronidase enzyme, although higher magnification may be utilised to reveal that the embryonal heads express the β-glucuronidase enzyme (transient GUS activity) and turn a turquoise-blue colour. It has been established that osmoticum-supplemented media increases transient

and stable transformation efficiency (Perl *et al.*, 1992; Vain *et al.*, 1993; Walter *et al.*, 1994; Clapham *et al.*, 1995; Walter *et al.*, 1998).

3.25. Diphasic Selection

Day 4: This step is dependent on tissue recovery, which is indicated by the disappearance of the tungsten particles. The bombarded ESM disc is transferred onto selection medium, which in this instance is MSG3 solid medium supplemented with BASTA® (AgrEvo South Africa (Pty) Ltd.), containing water soluble glufosinate ammonium at 200g.l^{-1}). The first subculture is transferred to 1 mg.l^{-1} followed by 3 mg.l^{-1} bioactive ingredient at the next subculture. Samples of bombarded tissue for stable integration analysis using PCR, should be taken once there has been an indication of tissue survival after selection, and DNA extracted immediately or frozen with liquid nitrogen prior to storage at –70 °C.

3.26. Regeneration

Growth on the outer perimeter of potentially transformed tissue is placed into liquid MSG3 medium at 1g regenerating tissue / 10ml liquid MSG3 medium. This is then filtered onto 10 plates for bulking up and later transferred to 240-maturation medium (Table 1) to stimulate embryo development. Harvested mature somatic embryos are partially dried (for 10d) before being placed onto charcoal-supplemented germination medium (240-hormone-free). Some genotypes selected for biolistic experimentation exhibited good regeneration ability and underwent all stages of maturation. At times, genotypes exhibited decreased maturation potential and change in tissue phenotype (loss of mucilaginous appearance) irrespective of particle transfer. This may have been attributed to a genotypic response, or to tissue age resulting in loss of productivity.

3.27. Molecular Characterization

Genomic *P. patula* DNA that will be tested for stable integration of the desired transgene is extracted by grinding 0.1g ESM with liquid nitrogen to a fine powder using a pestle and mortar. The cellular powder is transferred to sterile 1.5ml microfuge tubes in which 500µl urea extraction buffer (7M urea crystals, 5M NaCl, 1M Tris/Cl [pH 8.0], 0.5M EDTA, 20% sarkosyl [v/v]) is added and vortexed for 10 sec. The phenol:chloroform (1:1) is added to the cell extract and shaken on an orbital shaker at 120 rpm for 1 h at room temperature. After centrifugation (15min at 14 841.5g), the supernatant is transferred to fresh microfuge tubes. The nucleic acids are precipitated with a tenth volume 4.4M ammonium acetate and an equal volume ice-cold iso-propanol. This is

mixed well by inversion and placed at – 20°C for 15min. Nucleic acids are collected by 15min centrifugation at 14 841.5g and subsequently purified using 70% ethanol and air-dried for 3–5min on a laminar flow bench. Isolated genomic DNA can be stored in 20µl ultra pure water (BDH, Poole, England) at –20°C. Genomic DNA extractions from 0.1g putatively transformed ESM usually yield a concentration range of 5µg –20µg DNA samples.

PCR protocols are both species and vector specific. The GUS primer sets 5'-GGTGGGAAAGCGCGTTACAAG-3'/5'-GTTTACGCGTTGCTTCCGCCA-3', are used to amplify the GUS reporter gene and yield a fragment of 1.2 kb using a modified regime described by Hare (1998), after electrophoretic analysis on a 0.8% TAE (0.04M Tris-acetate, 0.002M EDTA, pH 8.5) agarose gel after PCR. The GUS amplification cocktail (for all samples plus one) consists of a 50µl reaction with 100ng genomic template DNA, 1.25 units of *Taq* DNA polymerase (Roche Biochemicals), 0.5µM of each primer, 10mM of each dNTP: dATP, dTTP, dCTP and dGTP, and5µl PCR buffer (Roche Biochemicals). To enhance the efficiency of the PCR, 10% Dimethyl sulphoxide (DMSO) (v/v) is also included in the reaction mix (Winship *et al.,* 1989). The PCR contents are mixed well and all samples are overlaid with an equal volume of paraffin oil prior to undergoing 36 amplification cycles (Hybaid thermal cycler), (Table 2). An estimated 40% transformation efficiency was concluded from the samples tested.

Amplification of the *bar* gene can be problematic and this is attributed to the high GC content (68.3%) present in the *bar* gene. The *bar* primer sets 5'-CATCGAGACAGCACGGTCAACTTC-3'/5' ATATCCGAGCGCCTCGTGCATGCG-3' (Wan and Lemaux, 1994) (Roche Products, Randburg, South Africa), are used to yield a 0.34 kb *bar* fragment (Fig. 3) using the PCR conditions with Expand^{TM} High Fidelity *Taq* polymerase (Roche) described by Vickers *et al.* (1996).

Table 2. A PCR-program for GUS amplification products in *Pinus patula* (adapted from Hare, 1998)

Steps	Temperature (°C)	Duration (sec)	Process
Time delay	94	60	Denaturation
35 cycles	94	30	Denaturation
	60	30	Annealing
	72	45	Extension
Time delay	72	5 min	Final extension
Storage	4		

A higher transformation efficiency of the *bar* amplicon than the GUS gene (47%) was observed. This indicates that co-integration of both reporter GUS gene and the herbicide resistant *bar* gene did not always

occur; perhaps the smaller gene is easier to incorporate and is expressed at a higher rate during selection.

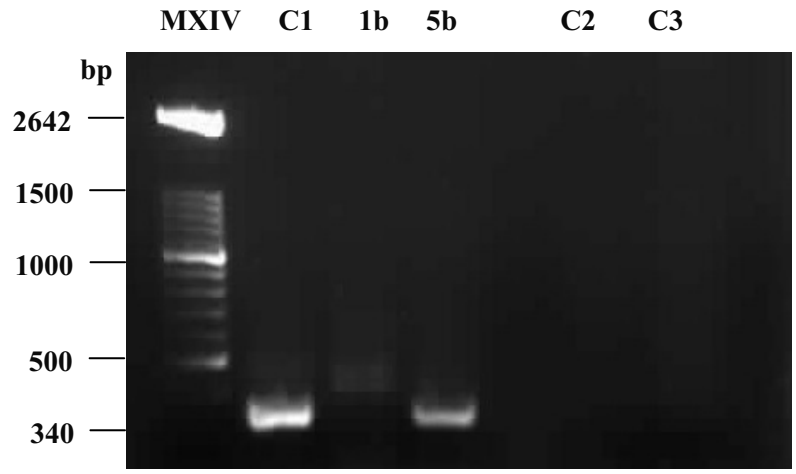

Figure 3. PCR-mediated amplification of *bar* products from genomic DNA of transformed *P. patula* ESM. Amplification products obtained from a PCR-regime for *bar* gene products described by Vickers *et al.* (1996). DNA contents of the lanes are: Molecular Weight Marker XIV (MXIV) Roche Biochemicals; positive control of pAHC25 (C1); genomic samples of bombarded *P. patula* coded as 1b and 5b; negative control (C2), genomic sample of unbombarded *P. patula*; and negative control (C3), no genomic DNA included in reaction.

Stable integration of the GUS and *bar* transgenes elucidates that *P. patula* ESM is amenable to genetic modification using the biolistic process. A regime for the genetic transformation of *P. patula* embryogenic tissue can be successfully established.

4. FURTHER PROTOCOL MODIFICATIONS

Since the first report of somatic embryogenesis in *P. patula* (Jones *et al*., 1993), further protocol modifications have been reported (Jones and van Staden, 1995; Jones and van Staden, 2001) which have helped to optimise the process. In addition to this, progress has been made in developing essential applications such as the cryopreservation protocols (Ford, 1999; Ford *et al*., 2000a; Ford *et al*., 2000b), which are now routinely implemented. More recently, genetic modification of the *P. patula* genome was achieved (Nigro *et al*., 2003), highlighting a further milestone in the accomplishments that have been achieved with this species. Research has also been attempted in embryo encapsulation techniques of this species (Sparg *et al*., 2002), but despite the ability to coat somatic embryos, storage and germination was poor. Nevertheless,

the major obstacles in the implementation of somatic embryogenesis of *P. patula* remain the low induction frequencies and the poor embryo maturation. It is particularly in these areas that greater focus is required in the future.

While development of microsatellite probes as breeding tools for screening genotypes for desirable growth characteristics would reduce the time needed for testing of the clones and genetic manipulation of the selected genotypes, particularly for disease tolerance would greatly benefit the forestry industry, it is only with improvements in the production and quality of somatic plantlets that these goals can be achieved.

5. CONCLUSIONS

With the implementation of the current protocols, sufficient somatic plantlets have been produced for the establishment of clonal hedges. Cuttings derived from these hedges have been used to initiate clonal trials. The aim of these trials has been primarily to determine the performance of cuttings derived from somatic hedges versus cuttings produced from seedling hedges. Seedling stock from the same families has also been included in the trials. Furthermore, the trials will allow the evaluation of the performance of clones that have been subjected to cryostorage. In this manner, the potential of somatic embryogenesis can be assessed to determine its suitability in applying a clonal forestry approach to *P. patula.*

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REFERENCES

Bayley, A., Blakeway, F. Deployment strategies to maximise value recovery from tree improvement: The experience of two South African companies. S. Afr. For. J. 2002; 195:11-22.

Becwar, M.R., Nagmani, R., Wann, S.R. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can. J. For. Res. 1990; 20:810-817.

Department of Water Affairs and Forestry. Report on commercial timber resources and primary roundwood processing in South Africa 1997/98. Department of Water Affairs and Forestry, 1998.

Department of Water Affairs and Forestry. Abstract of South African forestry facts for the year 1998/99. Forest Owners Association, 2001.

De Verno, L.L, Park, Y.S., Bonga, J.M., Barret, J.D. Somaclonal variation in cryopreserved embryogenic clones of white spruce (*Picea glauca* (Moench) Voss.). Plant Cell Rep. 1999; 18:948-953.

Clapham, D., Manders, G., Yibrah, H.S., von Arnold, S. Enhancement of short- and medium-term expression of transgenes in embryogenic suspensions of *Picea abies* (L.) Karst. J. Exp. Bot. 1995; 46: 655-662.

Evans, J. Sustainability of forest plantations. A review of evidence concerning the narrow-sense sustainability of planted forests. The Department for International Development 1999; pp Ford, C.S. Cryopreservation of *Pinus patula* Scheide et Deppe embryogenic tissue. M. Sc. Thesis, University of Natal Pietermaritzburg, South Africa, 1999.

Ford, C.S., Jones, N.B, van Staden, J. Cryopreservation and plant regeneration from somatic embryos of *Pinus patula*. Plant Cell Rep. 2000a; 19:610-615.

Ford, C.S., Jones, N.B, van Staden, J. Optimization of a working cryopreservation protocol for *Pinus patula* embryogenic tissue. In vitro Cell. Dev. Biol. 2000b; 36:366-369.

Griffin, A.R. Deployment decisions – capturing the benefits of tree improvement with clones and seedlings. In *Developing the Eucalypt of the Future*, Proceedings of IUFRO Symposium, Valdivia, Chile, 10 – 15 September, 2001, INFOR.

Hare, P. A regulatory role for proline metabolism in *Arabidopsis thaliana* (L.) *Heynth.* Ph.D. Thesis, University of Natal, Pietermaritzburg, South Africa, 1998.

John, A. The technology of clonal forestry of conifers. Nordic Group for the Management of Genetic Resources: Scotland, September 2002.

Jones, N.B., van Staden, J., Bayley, A.D. Somatic embryogenesis of *Pinus patula*. J. Plant Physiol. 1993; 142:366-372.

Jones, N.B., van Staden, J. Plantlet production from somatic embryos of *Pinus patula*. J. Plant Physiol. 1995; 145:519-525

Jones, N.B., van Staden, J. Improved somatic embryo production from embryogenic tissue of *Pinus patula*. In Vitro Cell. Dev. Biol. – Plant 2001; 37:543-549.

Jones, N.B. Somatic embryogenesis as a tool to capture genetic gain from tree breeding strategies: Risks and benefits. S. Afr. For. J. 2002; 195:93-101.

Li, Y-H., Tremblay, F.M., Séguin A. Transient transformation of pollen and embryogenic tissues of white spruce (*Picea glauca* (Moench.) Voss) resulting from microprojectile bombardment. Plant Cell Rep. 1994; 13:661-665.

Merkle, S.A., Trigiano, R.N. *In vitro* propagation of hardwoods. Applications of vegetative propagation in forestry. Proceedings of the 1992 SRIEG Biennial Symposium on Forest Genetics: 1992, July 8-10; Huntsville, AL. New Orleans, LA USDA Forest Service General Technical Report SO-108: Southern Forest Experiment Station.

Morris A., Pallett, R. Pines. In South African Forestry Handbook 2000, Vol. 1. South African Institute of Forestry, 2000 pp. 80-84.

Nigro, S.A., Makunga, N.P., Jones, N.B, van Staden, J. Towards transgenic *Pinus patula* with *uid*A and *bar* genes resulting from microprojectile bombardment of embryonal suspensor masses (ESM). Poster presented at 27th Annual Congress of South African Association of Botanists (SAAB), Pretoria, South Africa, 2003, 7-11 January.

Pallett, R. Growth and fibre yield of *Pinus patula* and *Pinus elliottii* pulpwood plantations at high altitude in Mpumalanga. S. Afr. J. For. 2000; 187:11-17.

Park, Y.S., Pond, S.E., Bonga, J.M. Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for plant breeding. Theor. Appl. Genet. 1993; 86:427-436.

Percy, R.E., Klimaszewska, K., Cyr, D.R. Evaluation of somatic embryogenesis for clonal propagation of western white pine. Can. J. For. Res. 2000; 30:1867-1876.

Perl, A., Kless, H., Blumenthal, A., Galili, G., Galun, E. Improvement of plant regeneration and GUS expression in scutellar wheat calli by optimization of culture conditions and DNA-microprojectile delivery procedures. Mol. Gen. Genet. 1992; 235:279-284.

Pullman, G.S., Webb, D.T. An embryo staging system for comparison of zygotic and somatic embryo development. Biol. Sci. Sym., 1994.

Sanford J.C., Smith, F.D., Russel, J.A. Optimizing the biolistic process for different biological applications. Method Enzymol. 1993; 217:483-509.

Sparg, S.G., Jones, N.B., van Staden, J. Artificial seed from *Pinus patula* somatic embryos. S. Afr. J. Bot. 2002; 68:234-238.

Sutton, B.C.S., Grossnickle, S.C., Roberts, D.R., Russell, J.H., Kiss, G.K. Somatic embryogenesis and tree improvement in interior spruce. J. For. 1993; 91:34-38.

Vain, P., Kee, N., Murillo, J., Rathus, C., Nemes, C., Finer, J.J. Development of the particle inflow gun. Plant Cell Tiss. Org. Cult. 1993; 33:237-246.

Vickers, J.E., Graham, G.C., Henry, R.J. A protocol for the efficient screening of putatively transformed plants for *bar*, the selectable marker gene, using the polymerase chain reaction. Plant Mol. Biol. Rep. 1996; 4:363-368.

von Arnold, S., Hakman, I. Regulation of somatic embryo development in *Picea abies* by abscisic acid (ABA). J. Plant Physiol. 1988; 132:164-169.

Walter, C., Smith, D.R., Connet, M.B., Grace, L., White, D.W.R. A biolistic approach for the transfer and expression of a *gus*A reporter gene in embryogenic cultures of *Pinus radiata.* Plant Cell Rep. 1994; 14:69-74.

Walter, C., Grace, L.J., Wagner, A., White, D.W.R., Walden, A.R., Donalson, S.S., Hinton, H., Gardner, R.C., Smith, D.R. Stable transformation and regeneration of transgenic plants of *Pinus radiata* D.Don. Plant Cell Rep. 1998; 17:460-468.

Walter, C., Smith, D.R. Genetic transformation of *Pinus radiata*. In *Biotechnology in Agriculture and Forestry* Vol 44. Transgenic Trees, Y.P.S. Bajaj ed. Springer-Verlag, Berlin, 1999, 193-211.

Wan, Y., Lemaux, P.G. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 1994; 104:37-48.

Winship, P.R. An improved method for directly sequencing PCR amplified material using dimethyl sulphoxide. Nucl. Acids Res. 1989; 17:1266.

SOMATIC EMBRYOGENESIS IN NORWAY SPRUCE

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1. INTRODUCTION

Norway spruce is most important conifer for wood production in central and northern Europe. Somatic embryogenesis of Norway spruce (*Picea abies* L. Karst.) was first reported in 1985 (Chalupa 1985, Hakman and von Arnold 1985). This method is a powerful tool for rapid *in vitro* micropropagation of desired genotypes. Almost two decades of research resulted in a relatively high degree of knowledge and graduated protocols for somatic embryogenesis. Norway spruce thus belongs to a few coniferous species, which are used as "model system" for studying of somatic embryogenesis.

2. CULTIVATION OF EMBRYOGENIC CULTURES

2.1. Preparation of the culture media

Somatic embryogenesis is regulated by changes of plant growth regulators exogenously supplied in the culture medium. Other changes include slight difference in the composition of nutrient media during specific phases of somatic embryogenesis. Several cultivation media are used for embryogenic cultures of Norway spruce in different laboratories (e.g. modified MS, LP, DCR, MSG, N6, NIII, for overview see Tautorus et al. 1991). The composition of slightly modified nutrient media, according to Gupta and Durzan (1986), including plant growth regulators and other compounds, is represented schematically in Table 1. Laboratory praxis, stock solutions in detail and protocol for preparation of nutrient media are summarized in Tables 2 and 3.

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 141–155.

2. 2. Initiation of embryogenic culture

Table 1. Overview of main changes in nutrient media during different phases of somatic embryogenesis of Norway spruce:

	induction	*proliferation*	*maturation*	*desiccation*	*germination*
medium	full GD			filter paper	½ GD
agar	0.8%			none	0.8%
pH	5.8			-	5.8
auxin	5 μM 2,4-D		none		
cytokinins	2 μM BA, 2 μM kinetin		none		
ABA	none		20 μM	none	
charcoal	none				0.5%
PEG	none		5 %	none	

Table 2: Media composition, stock solutions (A – macro salts, B – micro salts, C- Fe, D –organic compounds, E – auxin and cytokinins, F – abscisic acid

A: Macro salts:

	stock solution (20 x concentrated) (g/l)	**concentration in the medium**	
		(mg/l)	mM
KNO_3	46.8	2340	23.14
NH_4NO_3	5.5	275	3.44
$CaCl_2 . 2 H_2O$	4.4	220	1.50
$MgSO_4 . 7 H_2O$	3.7	185	0.75
KH_2PO_4	1.7	85	0.62

B: Micro salts:

	stock solution (100 x concentrated) (mg/l)	**concentration in the medium**	
		(mg/l)	μM
$MnSO_4 . H_2O$	1115	11.15	66
$ZnSO_4 . 5 H_2O$	430	4.30	17
H_3BO_3	310	3.10	50
KI	41.5	0.415	2.5
$CuSO_4 . 5 H_2O$	1.25	0.0125	0.05
$CoCl_2 . 6 H_2O$	1.25	0.0125	0.05
$Na_2MoO_4 . 2 H_2O$	12.5	0.125	0.52

Stock solutions of macro and micro salts are prepared from sterilized water in a semisterile way. After component dilution, solutions are kept refrigerated.

C: Fe:

	stock solution (100 x concentrated) (mg/l)	**concentration in the medium**	
		(mg/l)	µM
Na_2EDTA . 2 H_2O	1890	18.9	50
$FeSO_4$. 7 H_2O	1390	13.9	50

Both compounds are diluted in water and boiled (10 min). The volume is adjusted, the stock solution is left overnight and stored in refrigerator.

D: Organic compounds:

	stock solution (40 x concentrated) (g/l)	**concentration in the medium**	
		(mg/l)	µM
myo-inositol	40	1000	5550
caseinhydrolysate	20	500	-
L-glutamine	18	450	3080
glycine	0.04	1	13.32
thiamine HCl	0.02	0.50	1.48
nicotinic acid	0.01	0.25	2.03
pyridoxine HCl	0.01	0.25	1.22

Components are diluted in water, pH adjusted close to 5.8 and solution is sterilized through an 0.22 µm filter. Sterile stock solution is stored in freezer.

E : auxin and cytokinins:

	stock solution (40 x concentrated) (mg/100 ml)	**concentration in the medium**	
		(mg/l)	µM
2,4-D	4.4	0.110	5
BA	1.72	0.043	2
kinetin	1.72	0.043	2

All compounds are diluted separately (2,4-D in drop of ethanol, cytokinins in drop of 1 N KOH, after dilution add water, mix solutions together and adjust volume with water). Adjust pH to 6.0 (with HCl). Sterilize with an 0.22 µm filter and store in freezer.

F: abscisic acid:

	stock solution (40 x concentrated) (mg/100 ml)	**concentration in the medium**	
		(mg/l)	μM
ABA	26.4	0.66	20

Dilute ABA in a few drops of 1 N KOH; after dilution adjust volume with water. Adjust pH to 6.0 aproximately (with HCl). Sterilize with an 0.22 μm filter and store in freezer. Thawing of ABA stock solution should be done carefully as ABA is sensitive to high temperature and UV light.

Table 3: *Preparation of the medium:*

	components of 400 ml medium for:		
	induction and proliferation	embryo development and maturation	germination
water	352 ml	354 ml	352 ml
A (macro salts)	20 ml	20 ml	10 ml
B (micro salts)	4 ml	4 ml	2 ml
C (Fe)	4 ml	4 ml	2 ml
sucrose	12 g	12 g	2 g
PEG 4000	-	15 g	-
active charcoal	-	-	2 g
agar*	3.2 g	3.2 g	3.2 g
pH	5.8	5.8	5.8
autoclaving of the media, after cooling add filter sterilized solutions:			
D (organics)	10 ml	10 ml	5 ml
E (2,4-D + cytokinins)	10 ml	-	-
F (ABA)	-	8 ml	-

* for solidified media only

2.2.1. Primary explant

Embryogenic cultures are mainly initiated from immature or mature zygotic embryos. The initiation rate of immature zygotic embryos changes only slightly during embryo ontogeny and can reach up to 100% (Hakman and von Arnold 1985). The initiation rate of mature zygotic embryos is somewhat lower. On the other hand, this way of induction is not seasonally dependent, sufficient initiation rate is reached if zygotic embryos from older seeds are

used as a primary explant. The initiation rate varies among different genotypes of primary explant (Jain et al. 1988).

Embryogenic culture is also initiated from somatic embryos and juvenile tissues of young plants (cotyledons, emblings). Their initiation rate is still markedly lower compared to zygotic embryos. Moreover, these ways still do not solve the major problem: induction of embryogenic culture from a mature tree, which is of great practical importance. However a few successful attempts were made (induction of embryogenic culture from needles, Ruaud et al. 1992, Harvengt et al. 2001), efficiency of these techniques is extremely low and the method is not still routinely used.

2.2.2. Initiation media

Both auxin and cytokinin(s) are necessary for the induction of embryogenic culture of Norway spruce. 2,4-D (2,4-dichlorophenoxyacetic acid) or NAA (naphtaleneacetic acid), and BA (N^6-benzyladenine) or/and kinetin are the preferred auxins and cytokinins for induction of embryogenic cultures.

Specific composition of media (composition of macro and micronutrients) is not a crucial factor for initiation of cultures. Embryogenic cultures are initiated from primary explants on media either solidified with agar or gelrite.

Immature Norway spruce cone could serve as a source of primary explant, zygotic embryo (Figure 1A), but it is difficult to determine an optimal harvest time. Initiation rate of early immature zygotic embryos is the highest, but it changes with the ontogeny of an embryo, and is usually sufficient during all stages of embryo development and maturation. Younger cones are more compact, which prevents mold infections. On the other hand, dissection of more developed immature seeds is more simple. Dissection of seeds and extraction of primary explants should follow immediately after a harvest of cones to decrease possible danger of mold and bacterial infection resulting from the storage of immature cones or seeds.

Wash the harvested Norway spruce cones in 70 % (v/v) ethanol (5 min), then rinse repeatedly with sterile distilled water. In a semisterile way, remove developing seeds. Then, in a flow-box, sterilize the seeds with 0.1% $HgCl_2$ (5 min, mix occasionally), and thoroughly wash the seeds at least three times with sterile distilled water. Dissect the seed (unarmed eye or under binocular microscope). The testa and endosperm are removed and the zygotic embryo is placed on induction medium.

If mature zygotic embryos are used as primary explant, surface of seeds should be sterilized as described above. In some cases, dissection of seeds and

extraction of zygotic embryo is difficult. Imbibition of sterilized seeds improves this.

Two types of cultivation are recommended: either in disposable petri dishes (60 or 90 mm, 10 – 15 embryos per dish) or in vials (1 embryo per vial, slanting medium). Slightly modified GD medium (Gupta and Durzan, 1986) solidified with 0.8 % agar (Sigma) is used (Table 1).

Induction of embryogenic tissue proceeds in dark plant growth room or thermostat (24 °C, darkness). Embryogenic tissue starts to differentiate on explant surface of within 3-4 weeks (Figure 1B). Developing embryogenic tissue is white, translucent and mucilaginous, and sharply contrasts either with brown turning tissue of primary explant or with developing nonembryogenic tissue (compact hard callus composed of small rounded cells, yellow, white or greenish with velvet surface). At this stage, a long subcultivation interval (4-6 w) is recommended. Do not separate developing embryogenic structures from primary explant prematurely; wait until embryogenic culture grows to 8 – 10 mm in diameter. Primary explant is able to generate embryogenic tissue for a relatively long time, usually for several months.

2.3. Proliferation of embryogenic culture

For a few weeks after induction of embryogenic culture, their appearance and growth become uniform. On the other hand, various cell lines differ markedly in growth parameters and structure of culture. Repeatedly passage on proliferation medium (Table 1), cultures could sustain their character for many years.

There are several ways to cultivate embryogenic cultures. The use of agar-solidified medium is most frequent. Cultures could be either proliferated in a liquid medium in flasks placed on a roller or on a gentle shaker. Growth rate of liquid grown cultures is usually higher compared to solidified media. Although some cell lines grow well even after long cultivations in liquid, others suffer from morphological aberrations (mainly shortening and disaggregating of suspensor cells) followed in decrease of growth rate and cell viability. Generally, cultivation in liquid medium could be used only for a shorter time and should be limited only to the proliferation phase. In practice, liquid media are used for cultivation of embryogenic cultures prior to cryopreservation.

Cultivation of cultures on support floating on the surface of the medium (*e.g.* membrane rafts, Osmotec) represents the third way of cultivation (Figure 1E). This method gives excellent results for the majority of cell lines, and is recommended mainly for the last proliferation passage(s) prior to maturation. The method saves labor costs and is ideal to the culture (whole rafts are

transferred to a fresh medium without touching of embryogenic tissue). Disadvantages of the method lie in the high cost of membrane rafts and frequent problems with optimal degree of wet ability of membrane.

For routine maintenance of cultures transfer small (up to 10 mm size) pieces of embryogenic tissue to the agar-solidified proliferation medium (Table 1). Manipulation of embryogenic cultures should be done carefully and in consideration to the tissue (nor cut the tissue with scalpel neither transfer to a fresh media in upside down orientation). Cultivation in Magenta boxes (Sigma) is more convenient than plastic petri dishes due to greater volume of air inside. Cultivation should proceed in total darkness at 24 °C, cultures are relatively sensitive to temperature variation. Subculture intervals differ among cell lines and depend on growth rate of culture (usually 1-2 w).

2.3.1. Visualization of embryogenic cultures

For simple microscopic observation, embryogenic cultures during proliferation stage could be stained with trypan blue (0.04 %, diluted in water) which is added directly to a small piece of tissue (approx. 2 mm size) without previous fixation. After a few seconds, a drop of water is added and the excessive dye is wiped with cellulose. This method could be also applied to a early somatic embryo maturation stages (up to 2 w on maturation medium).

2.4. Development and maturation of somatic embryos

Development of somatic embryos in embryogenic tissue is triggered by changes in plant growth regulators in the culture medium. Auxin and cytokinins are removed and abscisic acid (ABA), in relatively high concentration, is added to the maturation medium. Non-penetrating polyethylene glycol (PEG 4000 or 6000) is added to the maturation medium in order to increase osmotic strength.

Embryogenic cultures could either be transferred from the proliferation medium to the maturation medium, or for a short period (1-10 d) pre-culture on the proliferation medium without plant growth regulators (Bozhkov et al. 2002). Pre-culture leads to decrease of endogenous IAA. Thereafter, majority of embryogenic cultures synchronize well in the maturation medium containing ABA and PEG.

Transfer small pieces (up to 10 mm size) of embryogenic tissue, grown on agar-solidified media in Magenta box (or petri dish), to a membrane raft floating on nutrient medium. From this point, whole rafts are transferred to a fresh medium without manipulation of embryogenic tissue. Allow one or two more passages of cultures on proliferation medium (depends on growth of

tissue). Then rafts should be transferred onto the proliferation GD medium without plant growth regulators for another week, and thereafter onto a maturation medium containing 20 μM ABA and 5 % PEG 4000. Developing somatic embryos are visible after 1 - 2 weeks. After 2 - 3 w, cotyledons start to develop in apical pole of somatic embryos. The time necessary to get mature somatic embryos varies among cell lines (4-7 weeks) (Figure 1C).

Alternatively, the development and maturation of somatic embryos could also be completed on a solidified medium. Pieces of tissue must be transferred weekly onto the fresh medium. The rapidity of somatic embryo development on solidified medium is somewhat lower when compared with membrane rafts. Although liquid cultures are routinely used for somatic embryo production of several conifer species (e.g. *Pseudotsuga*), cultivation of Norway spruce embryogenic cultures submersed in liquid maturation medium (roller, shaker, bioreactors) do not bring satisfactory results.

2.4.1. Staining of embryogenic cultures and embryos

Shortly after the start of embryo development on maturation medium, developing somatic embryos become too big to be studied under a microscope without further preparation. For that it is necessary to prepare paraffin sections (thickness 12 μm) (Johnson 1940). The sections are stained in a 2-step procedure using alcian blue and nuclear fast red (Poláčková and Beneš, 1975). Alcian blue (0.1%) stains cell walls, whereas nuclear fast red (1%) visualizes chromatin structures in the nucleus.

2.4.2. Analysis of embryogenic cultures

Embryogenic cultures are analysed by the system of computer image analysis, which allows counting and measurement of different structures. In order to be analysed, the embryogenic culture is recorded either directly by digital TV camera (magnification 1 – 10 x), or by microscope (recommended magnification 15 – 150 x) and digital camera.
Microscope paraffin sections are prepared as described above (2.4.1.), or pieces of tissue are vitally stained as in 2.3.1. Paraffin sections are suitable for analysis of developing tissues of somatic embryo, whereas the vital staining is more convenient for observation of ESM (somatic embryos and suspensor cells).

In lower magnification, embryogenic tissue is recorded directly by a digital TV camera. The culture is placed in open petri dish against contrast backround, lightened by several cold light sources to avoid shadows, and recorded. Program of computer image analysis (Lucia, ver. 4.61, Laboratory Imaging, Czech Republic) enables to record series of images of a more spatial object, focusation continually changes from the top to the base of the object.

The final image with high depth of sharpness is then combined of the individual pictures. For the determination of the embryogenic capacity of the culture, a known amount of embryogenic culture is gently mixed with water in petri dish and recorded by the camera. The image is further processed by a computer image-analysing software. A number of macros were created with the aim to distinguish particular structures (e.g. embryos), count them and measure additional parameters.

2.5. Desiccation of somatic embryos

The germination rate of mature somatic embryos is usually improved by desiccation before the transfer of somatic embryos to the germination medium. However, the growth and anatomical development of somatic embryos has been already completed, whereas storage compounds still continue to accumulate; and the levels of endogenous ABA and water content decrease during this stage. The somatic embryos are in the process of germination. The necessity of this step is variable among different cell lines. A number of desiccation protocols is used. Generally somatic embryos are exposed to high relative humidity (>98 %) at the same or slightly decreased temperature (+ 18 °C) for 2-5 w.

Uniform mature somatic embryos are harvested by forceps and aseptically placed on a dry filter paper in a petri dish. Several small petri dishes are placed into a big sterile one on wet filter paper (sterile distilled water) and sealed with parafilm tape (Figure 1D). Sealed dishes should be placed at 20 - 24 °C. The role of light is not clear up to now. Slightly better germination is achieved if somatic embryos were exposed to dim light during desiccation compared to desiccation in total darkness. Desiccating somatic embryos could be kept in dim light/dark regime 12h/12h, intensity of light should not exceed 18 $\mu mol.m^{-2}s^{-1}$. Optimal time needed for desiccation varies from 2-5 weeks and also depends on cell line and previous procedure of maturation. If the desiccation lasts longer than optimal period, malformations in apical pole of emblings are visible, if the desiccation is too short, protruding primary root tends to stop growth and occur recallusing. Filter paper in lower petri dish should remain wet during the desiccation procedure and add additional sterile water if necessary.

2.6. Germination of somatic embryos

Desiccated somatic embryos are fully prepared to germinate. Germination medium consists of solidified half-strength GD medium without plant growth regulators. Usage of different gelling agents (agar, gelrite, transfer agar, phytoagar) gives similar results. At this stage, PEG is replaced by 0.5 %

Figure 1: *A - primary explant: developing zygotic embryo, B - embryogenic culture composed of meristematic heads and long suspensor cells, C - mature somatic embryos, D - desiccation, E - cultivation on membrane raft, F - germinated somatic embryo with apical bud*

active charcoal. It is recommended to fill Magenta boxes (or petri dishes) with an autoclaved medium, which is cooled sufficiently (temperature bellow 50 °C) to prevent sedimentation of active charcoal in the boxes. Desiccated somatic embryos are placed on the surface of the medium. Cultivation temperature should be set to around 20 °C; temperature in dark phase could be set somewhat lower. Germination is not as dependent on optimal temperature as it is an proliferation of embryogenic culture and maturation of somatic embryos. Mild variations of temperature from optimal do not seriously affect germination. Germination of

embryos starts immediately, visible growth could be seen after few days. At first primary root rapidly extrudes. Growth of apical pole is much slower and cells of apical meristem start to divide and form apical bud; hypocotyl slowly elongates along longitudinal axis during first weeks. Role of light is crucial during the germination. The most important for that is the photoperiod, but the intensity of light has minor effect. The effect of light quality is negligible and it has not been sufficiently studied so far. Short day conditions lead to formation of apical bud, long day conditions promote growth of the shoot. In this first phase, lasting 6 – 10 w after start of germination, photoperiod composed of shorter light phase (8 - 10 h) and longer dark phase (14-16 h) are recommended. After apical bud formation light phase of photoperiod should be prolonged to 16h light/8h darkness. Intensity of light should gradually increase during this period (to 80 – 20 $\mu mol.m^{-2}s$) (Figure 1F).

After 3–4 weeks, germinating somatic embryos should be transferred to a fresh medium (the same as for germination except active charcoal is omitted). Primary roots of small plantlets penetrate the solidified medium and secondary roots emerge. Long day conditions trigger longitudinal growth of apical buds.

2.7. Transfer of emblings *ex vitro*

Small seedlings with developed or growing apical bud and root are transferred to nonsterile substrate comprised of peat and sand (1:1). Alternatively a fresh natural substrate originating from a spruce forest is used. Seedlings are kept in a glasshouse in shadow. For the first few weeks after the transfer *ex vitro*, it is necessary to avoid high cultivation temperatures (over 35 °C) and keep the relative humidity high (more than 80 %). The humidity later gradually decreases. After plantlets grow to several centimeters and root systems are well developed; acclimatization to low temperatures and drought could start. Well acclimatized plants could be transferred to the field.

There is a distinct gap in the rate of growth after the *ex vitro* transfer. Even then, for several months, the growth of seedlings is slower than their zygotic counterparts. No significant differences in growth were found in trees 3 – 7 years of age grown naturally or through somatic embryogenesis.

3. CRYOPRESERVATION OF EMBRYOGENIC TISSUE

Cryopreservation of embryogenic cultures is a unique way for long-term storage of germplasm. Cultures should be kept genetically unchanged for a long time until clones are evaluated in the field test. Cryopreservation is also a potent tool for routine preservation of large numbers of cell lines in the laboratory.

Successful cryopreservation of embryogenic cultures of Norway spruce was first published in 1987 (Gupta et al. 1987). Bercetche et al. (1990) reported that cryopreservation enhances embryogenic capacity. Different cell lines showed different cryotolerance, however, no conclusions could be reached on the relationships of cryo-tolerance and distinct morphological or biochemical characteristic of cell lines (Nørgaard et al. 1993).

3.1. Pretreatment of cultures

The cryopreservation protocol of Bozhkov is used and slightly adapted (Bozhkov, pers. comm., 2002). Embryogenic cultures grown on solidified medium are passed to roller flasks (3 g of fresh weight per 30 ml liquid medium) and cultivated for 1 w. Cultures are then treated with sorbitol (0,16 ml of 4 M sorbitol is added to the flask ten times during 30 min, resulting concentration of sorbitol was 0.2 M) and return to the roller till the second day, when this procedure is repeated (concentration of sorbitol is then 0.4 M). On the third day the cultures in flasks are placed on ice and treated with DMSO (0,175 ml DMSO ten times during 30 min, final concentration of DMSO is 5 %), left for another 15 min and then filter through the sieve. Ice-cooled cryotubes are filled with cell suspension and then closed.

3.2. Cooling program

Two different cooling systems are used. Cryotubes are either put in simple cryobox (Mr. Frosty, Nalgene) filled with isopropanol and cool in laboratory freezer, or cool in the programmed cryomachine (Glacier, Sy-Lab) (Figure 2). Temperature in the first system is monitored directly by thermometer inserted in one separate cryotube. After the end of cooling, the cryotubes are transferred to the liquid nitrogen.

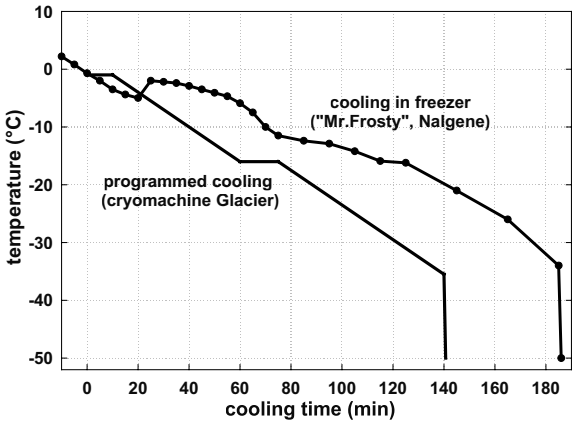

Figure 2: Temperature gradient of both two cooling systems.

3.3. Thawing

Cryotubes are transferred to sterile water (45 °C) for approx. 1 min. At this time the contents of tubes melt, the cryotubes are placed in sterile water (+ 4 °C) for a couple of minutes. After surface sterilization of vials in 70 % (v/v) ethanol, the cultures are layered on filter paper placed on proliferation medium in petri dish. Filter paper with culture is further transferred onto fresh medium after 1 h, and once more after 1 d. Cultures layered on filter paper are further transferred to fresh proliferation medium weekly. After tissue growth started, the filter paper is removed.

Cryopreservation using both cooling system yield in regrowth of embryogenic cultures. After thawing, the growth of embryogenic cultures is visible in 1-2 w. Cells of meristematic heads (the only part of culture which remained alive) start to divide. Osmotic sensitive suspensor cells, damaged by cryopreservation, recover. The growth rate of cultures is changed during the period of lag-phase. This period is variable for different cell lines. There is no doubt that cryopreservation represents a strong selection pressure which eventually could change the embryogenic capacity of the culture.

Chance for successful regrowth of cultures is slightly lower using an inexpensive cooling system (Mr. Frosty, Nalgene) compared to a sofisticated, programmed cryomachine due to unpredictable deviations from optimal cooling rates. On the other hand, even this simple system give satisfactory results.

4. CONCLUSION AND PROSPECTS

During almost twenty years, which passed since the first report on Norway spruce somatic embryogenesis, reliable cultivation protocols were elaborated. Norway spruce became one of the most studied species among other conifers in the aspect of somatic embryogenesis. These protocols comprise the induction of embryogenic tissue, development and maturation of somatic embryos, germination and transfer of plantlets *ex vitro*, cryopreservation of embryogenic tissue and construction of transgenic trees. On the other hand, at least two big issues still remain to be solved: 1) induction of embryogenic cultures from vegetative organs of mature tree, 2) elaborating of successful protocols in liquid media and bioreactors which are the must for automation and decrease of labour costs. Our knowledge on regulation of the somatic embryogenesis (signal pathways, the role and action of phytohormones, gene expression) is still low but gradually increases.

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5. REFERENCES

Bercetche J., Galerne M., Dereuddre J. Efficient regeneration of plantlets from embryogenic callus of *Picea abies* (L.) Karst after freezing in liquid nitrogen. Compt Rend Acad Sci Ser III 1990; 310:357-366

Bozhkov P.V., Filonova L.H., von Arnold S. A key developmental switch during Norway spruce somatic embryogenesis is induced by withdrawal of growth regulators and is associated with cell death and extracellular acidification. Biotechnol Bioeng 2002; 77:658-667

Chalupa V. Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. Commun Inst Forest Cech 1985; 14:57-63

Gupta P.K., Durzan D.J. Plantlet regeneration via somatic embryogenesis from subcultured callus of mature embryos of *Picea abies* (Norway spruce). In Vitro Cell Dev Biol - Plant 1986; 22:685-688

Gupta P.K., Durzan D.J., Finkle B.J. Somatic polyembryogenesis in embryogenic cell masses of *Picea abies* (Norway spruce) and *Pinus taeda* (loblolly pine) after thawing from liquid nitrogen. Can J Forest Res 1987; 17:1130-1134

Hakman I., von Arnold S. Plantlet regeneration through somatic embryogenesis in *Picea abies* (Norway spruce). J Plant Physiol 1985; 121:149-158

Harvengt L., Trontin J.F., Reymond I., Canlet F., Paques M. Molecular evidence of true-to-type propagation of a 3-year-old Norway spruce through somatic embryogenesis Planta 2001; 213:828-832

Jain S.M., Newton R.J., Soltes E.J. Enhancement of somatic embryogenesis in Norway spruce (*Picea abies* L.). Theor Appl Genet 1988; 76:501-506

Johnson D.A., *Plant Microtechnique.* Mc Graw-Hill Book Co. Inc., New York, 1940.

Nørgaard J., Durzan V., Johnsen Ö., Krogstrup P., Baldurson S., von Arnold S. Variations in cryotolerance of embryogenic *Picea abies* cell lines and the association to genetic, morphological and physical factors. Can J For Res 1993; 23:2560-2567

Poláčková D., Beneš K. The staining of chromosomes and nuclei in squashes of root tip with aluminium lake of nuclear fast red. Biol Plant 1975; 17:374-375

Ruaud J.N., Bercetche J., Paques M. 1st evidence of somatic embryogenesis from needles of 1-year-old *Picea abies* plants. Plant Cell Rep 1992; 11:563-566

Tautorus T.E., Fowke L.C., Dunstan D.I. Somatic embryogenesis in conifers. Can J Bot 1991; 69:1873-1899

CASHEW (*ANACARDIUM OCCIDENTALE* L.)

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1. INTRODUCTION

Cashew (*Anacardium occidentale* L.) is an evergreen tropical tree. The chief product of this tree, the cashewnuts, is globally popular as dessert. Cashewnut ranks third in the world market after almonds and walnut in the tree nut category, and its trade is valued at more than U.S. $ 2 billion annually (Montealegre *et al*., 1999). Raw cashewnuts have to be processed before consumption, and the processing industry provides employment to about half a million people in India alone, of which, majority are women from rural areas. Thus, cashew plays a role in the socio-economic development of India, as well as other cashewnut producing countries (Balasubramanian, 1997).

It is necessary to cultivate superior varieties of cashew through vegetative methods, in order to maintain the desired traits. Presently, the selected varieties are multiplied vegetatively by softwood grafting (Bhaskara Rao *et al*., 1993). However, propagules produced by this method fall short of the existing demand for quality planting material. Introduction of new methods of cultivation like high-density planting have further increased the gap between supply and demand. There is a need to develop a method, which can generate the requisite amount of plants necessary for large-scale commercial plantations.

Use of in vitro plant production techniques can effectively supplement the present method of vegetative propagation, due to their ability to produce great number of plants in short period (George, 1993). In cashew, mainly juvenile explants have been responsive to in vitro culture conditions (Philip 1984; Jha, 1988; Laxmi Sita, 1989; Sy *et al*., 1991; D'Silva and D'

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Souza 1992; Hegde *et al*., 1994; Das et al., 1996; Ananthakrishnan *et al*., 1999; Boggetti et al., 1999; Cardoza and D'Souza, 2000; Ananthakrishnan et al., 2002; Cardoza and D'Souza, 2002; Mneney and Mantell 2002; Thimmapaiah et al., 1999 and 2002).

Work was initiated in our laboratory to investigate the possibilities of developing a system of in vitro plantlet regeneration in cashew. The protocol for induction of somatic embryogenesis from two explants, nucellus, and immature zygotic embryo (Gogte and Nadgauda, 2000; Gogate and Nadgauda, 2003) is described here. The nucellus is a maternal tissue, hence plantlets derived from this tissue would be identical to the mother plant, and could be used for clonal propagation. Plantlets derived from immature zygotic embryo can be used for propagation of chosen hybrid varieties.

2. SOMATIC EMBRYOGENESIS FROM NUCELLAR TISSUE AND IMMATURE ZYGOTIC EMBRYO

2.1 Culture medium

The basal culture medium to be used for all experiments is MS (Murashige and Skoog, 1962) salts and vitamins at full strength. The basal medium is supplemented with 0.5% activated charcoal, and gelled with 0.5% purified agar. The medium used for somatic embryo germination is gelled with 0.2% gelrite (Sigma Co., U.S.A.).

For nucellar tissue, three media (Initiation, Maintenance, and Expression Medium) are used in sequence. Initiation Medium (for initiation of callus from nucellus) is supplemented with 3% sucrose, 2,4-D 5 μM, GA_3 15 μM and BA 5 μM. Maintenance Medium (used for maintenance of nucellar callus) is supplemented with 4% sucrose, 0.05% casein hydrolysate, 10% coconut water, 2,4-D 10 μM, and GA_3 15 μM. Expression Medium (for differentiation of somatic embryos from nucellar callus) contains 4% sucrose, 0.05% casein hydrolysate, 10% coconut water, 2,4-D 5 μM, and GA_3 30 μM.

For immature zygotic embryo only one medium is required for formation of somatic embryos, and this Embryo Formation medium contains 3% sucrose, 2,4-D 5 μM, GA_3 3 μM and BA 5 μM.

2.1.1. Embryo Development Media

Somatic embryos of nucellar origin are transferred to medium with sucrose 3% and AC 0.5% for further development. Somatic embryos of zygotic embryo origin are transferred to medium with ABA 20 μM + maltose 3% for three- four weeks for maturation. Somatic embryos arising from nucellus as well as zygotic embryo are subsequently transferred to medium with sucrose 3% and AC 0.5% for germination.

All growth regulators are added to media before autoclaving, except ABA, which is added after autoclaving as a filter sterilized solution. After dissolving all the ingredients except for solidifying agent, pH of the medium is adjusted between 5.8-6.0 using 1.0 N HCl/ NaOH. The final volume is adjusted and gelling agent is added before sterilization. Media are autoclaved at 1.1 kg/cm^2 pressure at 121°C for 20 minutes, and dispensed in petri-plates in laminar airflow after sterilization. All cultures (nucellar and immature zygotic embryo) are incubated in dark at 25±2°C.

2.2 Explant preparation

Immature nuts (Fig. 1a) of cashew are collected at 3-4 weeks post-fertilization stage (for variety Vengurla 1) (the ovule should fill approximately one-third of the ovary space; the correct stage is different for different varieties, and would have to be standardized separately). Nuts are processed as early as possible after collection, to minimize wastage due to deterioration of tissue. Nuts are washed thoroughly under running tap water, followed by laboratory detergent wash (1% Labolene®, Qualigens, India) for 20 minutes. They are surface sterilized with 0.1% $HgCl_2$ for 20 minutes and finally, rinsed 3-4 times with sterile distilled water in laminar airflow cabinet. Immature nuts are then cut open in laminar airflow cabinet (Fig. 1b), by holding the biconvex nut with sterilized forcep, and taking a longitudinal incision with sterilized blade/ scalpel, along the convex surface, keeping the notched side down. The ovules (Fig. 1b) are carefully taken out, and bisected longitudinally into halves. The ovular halves contain the nucellar tissue. Ovular halves are then inoculated on nutrient medium, with nucellus exposed to view. The

immature zygotic embryos (Fig. 2a) present in the ovules are removed and inoculated separately.

2.3 Culture of explants and embryogenic response

1. The ovular halves (with nucellus), and immature zygotic embryos are placed in disposable petri dishes (55 mm x 15 mm or 85 mm x 15 mm).
2. Ovule halves are first inoculated on callus Initiation Medium. The nucellus proliferates rapidly in three weeks immediately following inoculation, to form a yellowish callus (Fig. 1c). This callus fills up the ovular half cavity completely.
3. After three weeks, the ovular halves (along with the proliferated nucellar callus) are transferred to callus Maintenance Medium. Soon after transfer, the yellowish callus starts browning, and later, blackens completely. The rapid growth of the callus also slows considerably. The ovular explants are transferred to fresh medium after every four-five weeks. The completely blackened primary callus from some explants forms a whitish granular mass (Fig. 1d). This mass may be formed 9-16 weeks after transfer to Maintenance Medium.
4. The whitish granular mass is then transferred to embryo Expression Medium. Differentiation of somatic embryos occurs from the granular mass after 8-12 weeks.
5. The somatic embryos formed from nucellar callus are transferred at heart or torpedo stage (Fig. 1e) to MS + sucrose 3% + activated charcoal 0.5% for further development (Fig. 1f, g).
6. The immature zygotic embryos are inoculated on MS + 2,4-D 5 μM + GA_3 3 μM + BA 5 μM + sucrose 3% + AC 0.5% + 0.6% agar.
7. Somatic embryos arise directly in three to five weeks from the radicle tip of the precociously germinating immature zygotic embryos. Initially, a knoblike structure is observed at the radicle tip (Fig. 2b) of the precociously germinating IZEs, which develops into a single or a cluster of globular somatic embryos (Fig 2c – single globular somatic embryo). Somatic embryos develop through the normal heart (Fig. 2d) torpedo and cotyledonary (Fig. 2e) stages while still attached to the parent zygotic embryo. Somatic embryos frequently get detached from the parent IZE at the torpedo or cotyledonary stage. The detached embryos continued their further development even after separation from the parent explant. The somatic embryos develop normally on the same medium till cotyledonary stage.

8. The somatic embryos of zygotic embryo origin are transferred to MS + ABA 20 μM + maltose 3% + gelrite 0.2% for maturation, and incubated for 3-4 weeks.
9. Somatic embryos are transferred to MS + sucrose 3% + activated charcoal 0.5% + gelrite 0.2 % for germination (Fig. 2g).
10. It must be mentioned that the embryogenic response obtained from both explants occurs at low frequency, hence, a large number of explants need to be inoculated. Also, suitable media for maturation of somatic embryos of nucellar, and zygotic embryo origin need to be worked out, for proper development of somatic embryos, and their effective conversion to plantlets.

Accumulation of storage substances is a key step in zygotic embryogenesis, when compounds are stored for later use by the germinating zygotic embryo, until it attains autotrophy. Lack of storage substances like triglycerides, adversely affect the later stages of development, and subsequent conversion of somatic embryos into plantlets (Feirer et al., 1989). In cashew, this step is prolonged and occupies major part of the developmental phase, as cotyledons (2-2.5 cm in length in mature seed) form the major bulk of the seed.

It may be noted that in experiments done with the mature zygotic embryo in our laboratory, it was observed that the mature zygotic embryo developed into a normal healthy plantlet only when cotyledons were left intact. The isolated mature zygotic embryo axis (without cotyledons) always failed to develop into a plantlet. This indicates that fully developed cotyledons are essential for development of embryo axis to form plantlet (conversion).

In the present system, poor development of cotyledons may be the major factor adversely affecting the subsequent maturation and conversion of somatic embryos. Considering the importance and indispensability of cotyledons for germination and conversion (in mature zygotic embryos), proper growth and complete development of the cotyledons of the somatic embryos would certainly help in better maturation and conversion rates. Difficulties in the conversion of somatic embryos obtained from nucellus and immature zygotic embryos of cashew have also been reported in earlier studies (Jha, 1988; Hegde *et al*., 1994; Ananthakrishnan *et al*., 1999; Cardoza and D'Souza, 2000; Cardoza and D'Souza, 2002).

3.RESEARCH PROSPECTS

The protocol for formation of somatic embryos from nucellus and immature zygotic embryos has been described. However, further studies and experimentation is essential to devise a maturation medium for these somatic embryos, which will help in increasing the conversion frequency of somatic embryos. The embryogenic systems then can be used as methods of multiplication of the selected superior variety.

Improvement in the conversion rates of somatic embryos of zygotic embryo origin could assist in using the system effectively for propagation of rootstocks and hybrid varieties. Used in combination with an appropriately designed breeding program, the embryogenic system could contribute to propagation and improvement of cashew. As somatic embryos of zygotic embryo origin are formed directly, they can be used for genetic transformation procedures, and subsequent multiplication of transformants. Similarly, somatic embryos of nucellar origin can be effectively used as propagules for clonal multiplication of the chosen elite mother plant.

Once the complete protocol has been established, both the systems would undoubtedly be useful for propagation and improvement of varieties of cashew.

4. ABBREVIATIONS USED

ABA: abscisic acid; BA: N^6benzyl adenine; CH: casein hydrolysate; CW: coconut water; 2,4-D: 2,4-dichlorophenoxyacetic acid; Fig.: figure; GA_3: gibberellic acid

5. REFERENCES

Ananthakrishnan G, Ravikumar R, Prem Anand R, Vengadesan G and Ganapathi A (1999) Induction of somatic embryogenesis from nucellus-derived callus of *Anacardium occidentale* L. *Sci. Hort*. 79: 91-99.

Ananthakrishnan G, Ravikumar R, Girija S, Ganapathi A (2002) *In vitro* adventitious shoot formation from cotyledon explants of cashew (*Anacardium occidentale* L.). *Sci. Hort*. 93: 343-355.

Balasubramanian PP (1997) Indian Cashew Development: an introspection and strategies for 9th Five Year Plan. *The Cashew* XI (1): 5-15.

Bhaskara Rao EVV, Swamy KRM, Yadukumar N, Dixit S (1993) *Cashew Production Technology PP 36,* NRCC Puttur. (Pub: MK Nair, CPCRI, Kerala, India)

Boggetti B, Jasik J and Mantell S (1999) *In vitro* multiplication of cashew (*Anacardium occidentale* L.) using shoot node explants of glasshouse raised plants. *Plant Cell Rep*. 18(6): 456-461.

Cardoza V and D'Souza L (2000) Direct somatic embryogenesis from immature zygtoic embryo of cashew. *Phytomorphology* 50(2): 201-204.

Cardoza V and D'Souza L (2002) Induction, development, and germination of somatic embryos from nucellar tissue of cashew *Anacardium occidentale* L. *Sci. Hort.* 93: 367-372.

Das S, Jha TB and Jha, S (1996) *In vitro* propagation of cashewnut. *Plant Cell Rep*. 15: 615-619.

Feirer TP, Conkey JH, Verhagen SA (1989) Triglycerides in embryogenic conifer calli: a comparison with zygotic embryos. *Plant Cell Rep*. 8: 207-209.

George EF (1993) *Plant Propagation By Tissue Culture I – The Technology*, Exegetics Ltd., Edington, London.

Gogte S and Nadgauda R (2000) Induction of somatic embryogenesis in cashew (*Anacardium occidentale* L.) *In Vitro Cell Dev. Biol.-Plant* 36 (1): 41-46.

Gogate SS and Nadgauda RS (2003) Induction of direct somatic embryogenesis in immature zygotic embryo of cashew (*Anacardium occidentale* L.). *Sci. Hort*. 97 (1): 75-82.

Hegde M, Kulashekaran M, Shanmughavelu K G and Jayashankar S (1994) *In vitro* embryogenesis in cashew (*Anacardium occidentale* L.). *Ind. Cashew J*. 21(4): 17-25.

Jha T B (1988) *In vitro* morphogenesis in cashewnut, *Anacardium occidentale* L. *Ind. J. Exp. Biol*. 26: 505-507.

LakshmiSita G (1989) Differentiation of embryos and leafy shoots from callus cultures of cashew (*Anacardium occidentale* L). *Abstr*. XIII Plant Tissue Culture Conference, October 18-20, Shillong, Abstract 71.

Mneney EE and Mantell SH (2002) Clonal propagation of cashew by tissue culture. *J. Hort. Sci. Biotechnol.* 77 (6): 649-657.

Montealegre J C, Childers N F, Sargent S A, Barros L M and Alves R E (1999) Cashew (*Anacardium occidentale* L.) Nut and Apple: A review of current production and handling recommendations. *Fruit Varieties J*. 53(1): 2-9.

Philip VJ (1984) *In vitro* organogenesis and plantlet formation in cashew (*Anacardium occidentale* L). *Ann. Bot*. 54: 149-152.

Sy MO, Martinelli L and Scienza A (1991) *In vitro* organogenesis and regeneration in cashew (*Anacardium occidentale L.). Acta Hort*. 289: 267-268.

Thimmapaiah, Shirly RA, Sadhana PH (2002) *In vitro* propagation of cashew from young trees. *In Vitro Cell. Dev. Biol*. 38 (2): 152-156.

Thimmappaiah and Samuel S R (1999) *In vitro* regeneration of cashew (*Anacardium occidentale* L.) *Ind. J. Exp. Biol*. 37(4): 384-390.

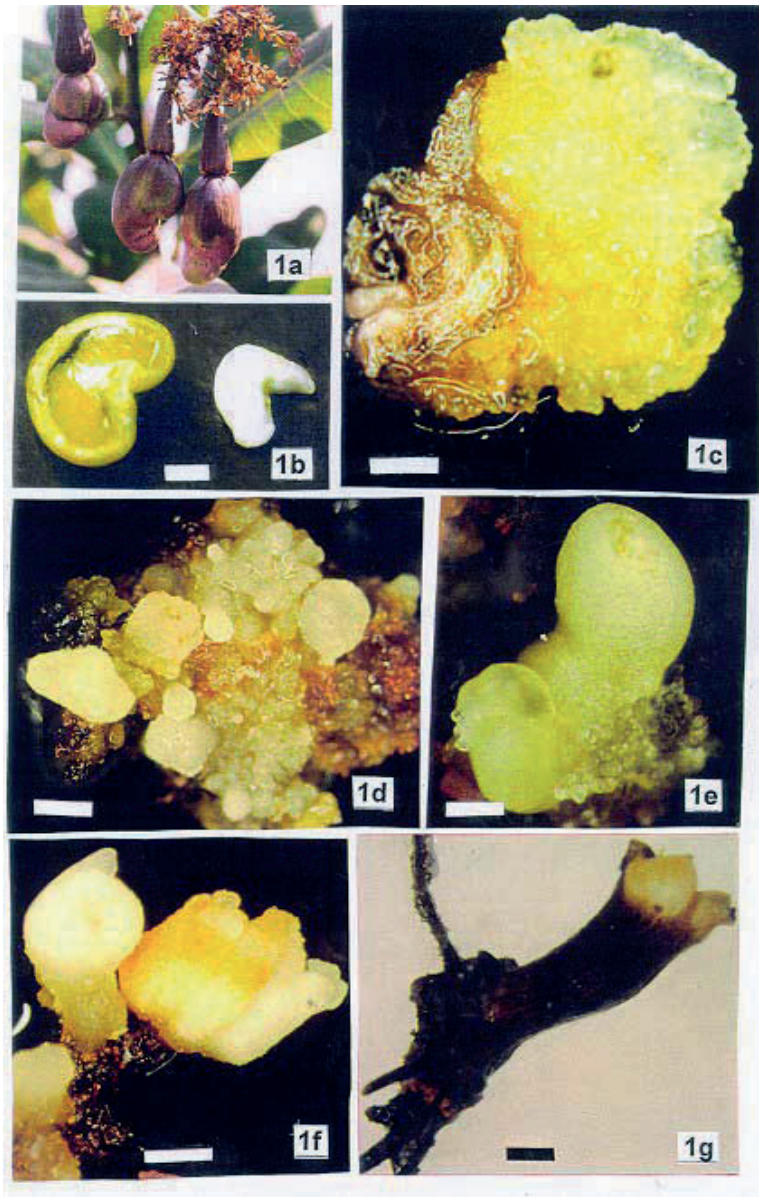

Figure 1: Somatic embryogenesisfrom nucellus in cashew
1a. Immature fruits used for experiments
1b. Immature fruit dissected to show intact white ovule (bar = 6 mm)
1c. Yellowish proliferating callus formed from nucellar tissue 3 weeks after inoculation, on MS + 2,4-D 5 µM + GA_3 15 µM + BA 5 µM (bar = 6 mm)
1d. Embryogenic mass differentiated into somatic embryos on MS + 2,4-D 10 µM + GA_3 15 µM + CH 0.05% + CW 10% v/v (bar = 1 mm)
1e. Torpedo stage somatic embryo (bar = 1mm)
1f. Cotyledonary stage somatic embryo (bar = 2 mm)
1g. Germinated somatic embryo (on MS + AC 0.5%) (bar = 10 mm)

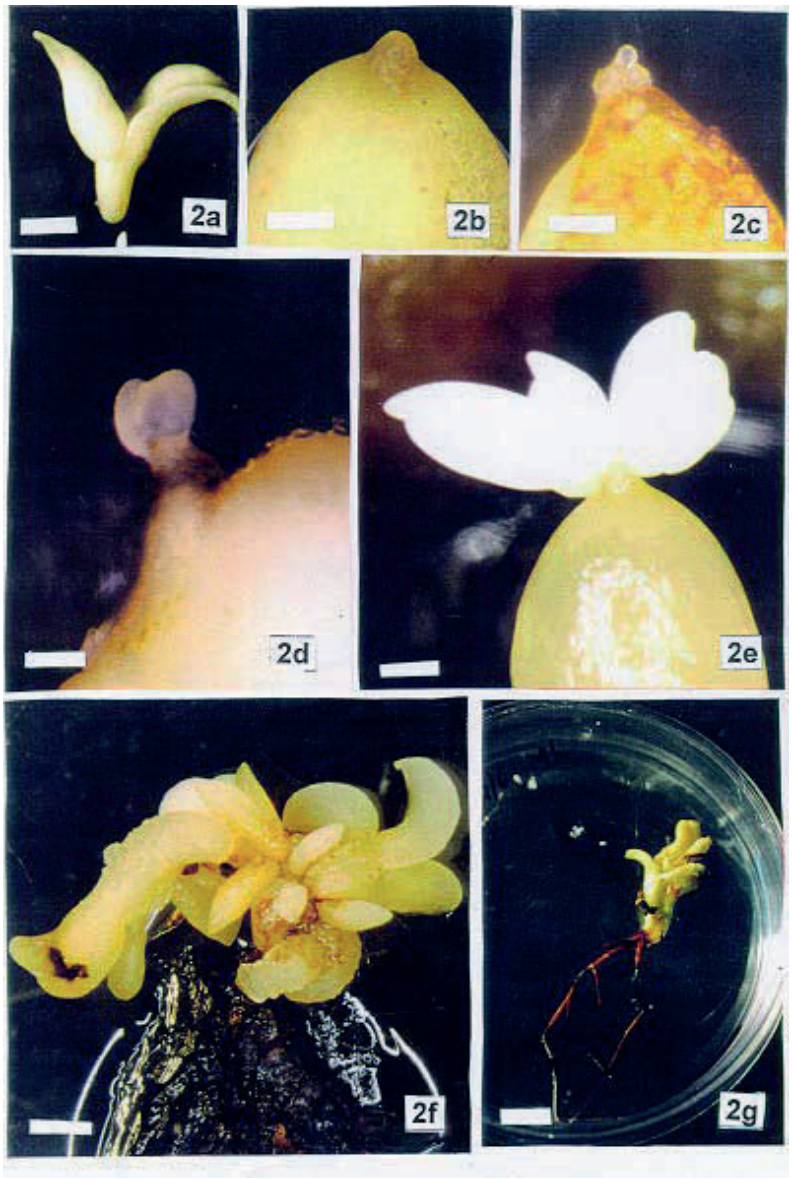

Figure 2: Somatic embryogenesis from immature zygotic embryo in cashew
2a. Immature zygotic embryo explant (IZE) (bar = 10 mm)
2b. Initiation of somatic embryogenesis from radicle tip of IZE (bar = 0.5 mm)
2c. Globular somatic embryo at radicle tip (bar = 0.5 mm)
2d. Heart stage somatic embryo at radicle tip (bar = 0.5 mm)
2e. Cotyledonary stage somatic embryo at radicle tip (bar = 0.5 mm)
2f. Cluster of asynchronously developing somatic embryos (bar = 10 mm)
2g. Somatic embryo germinated on MS + AC 0.5% (bar = 10 mm)

SOMATIC EMBRYOGENESIS PROTOCOL: COFFEE (*COFFEA ARABICA* L. AND *C. CANEPHORA* P.)

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1. INTRODUCTION

Coffee is one of the most important agricultural products on the world market and is the most widely grown tropical tree crop. The economies of 50 countries in America, Africa and Asia depend on this crop, which is grown on 11.2 x 10^6 ha. Some 125 million people depend on coffee production, including 25 million producers. Although coffee is the world's most heavily traded commodity apart from oil, it has been overproduced for several years. The very low prices resulting from overproduction have drastic socio-economic consequences in producing countries. The coffee consumed worldwide primarily comes from two cultivated species: *Coffea arabica* and *Coffea canephora*, which account for 75 and 25% of the world market respectively. The coffees produced by the two species are commercially known as "arabica" and "robusta".

C. arabica is grown in high-altitude tropical regions (500 to 2 500 metres). It is the only tetraploid (2n = 4x = 44 chromosomes) and autogamous species of the *Coffea* genus, which contains 80 species. *C. arabica* is well suited to high-altitude regions, and produces high quality coffee with a low caffeine content. However, this species is susceptible to the main diseases such as leaf rust (*Hemileia vastatrix* Berk & Br.) and coffee berry disease (*Colletotrichum coffeanum* (CBD) and pests such as root knot nematodes.

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C. canephora is a diploid (2n = 2x = 22 chromosomes), allogamous species that produces coffee with a higher caffeine content, which is poor in quality when compared to the quality of *C. arabica*. It is grown in low-altitude tropical regions (0 - 100 m). With respect to genetic improvement, it is a very worthwhile source of genes for resistance to rust, CBD and nematodes. Given its allogamy, the *C. canephora* species comprises genetically heterogeneous individuals. The progenies of cross fertilization therefore have substantial genetic variability.

Horticultural vegetative propagation through cuttings is still not available for *C. arabica*, probably due to the greater difficulty in achieving satisfactory multiplication rates. Moreover, the difficulties in transporting cuttings and the risk of disease propagation have prohibited the use of cuttings on a commercial level. Likewise, male sterility is still not available for the propagation of heterozygous materials. Arabica varieties are sold in seed form after a relatively lengthy pedigree selection process, taking at least 20 years. Micropropagation techniques may be applicable for mass production of selected *C. canephora* clones, and for interspecific hybrids such as Arabusta, but they are of particular interest in the case of *C. arabica*, for which F1 hybrid superiority over the varieties has been largely demonstrated in Kenya, Ethiopia and Central America. Among these techniques, somatic embryogenesis has the greatest multiplication potential, enables numerous technical simplifications, and should consequently entail the lowest production costs (Berthouly and Etienne, 1999). The use of somatic embryogenesis in coffee is not limited to mass propagation of heterozygous structures. For breeders, it is a veritable tool for rapid dissemination of genetic progress, it is also the basis for genetic modification, and enables cell and embryo culture in bioreactors.

2. INDUCTION OF EMBRYOGENIC TISSUE

The most widely used explant used in coffee is a leaf fragment Young leaves are collected from selected mother plants in the field. These leaves still have to be tender in order to be reactive, and perfectly healthy. Particular attention has to be paid to mother plant upkeep, to improve the physiological and sanitary status of the plant and thereby increase the reactivity of the plant material and limit losses due to contamination after

in vitro introduction. The chosen leaves are washed beforehand in tapwater. They are then disinfected by immersion in 10% calcium hypochlorite solution containing 1% Tween 80 for 20 min followed by 8% calcium hypochlorite solution for 10 min and rinse 4 times with sterile water. The recipient containing the leaves is then transferred to the laminar flow hood for explant preparation. Explants measuring 1 square cm are cut without the main vein, and place on the culture medium underside upwards.

Somatic embryogenesis in several *Coffea* species and genotypes is well documented (see review: Berthouly and Etienne, 1999). Two types of processes have generally been described using leaf sections as explants:

1. 'Low frequency': somatic embryos are obtained quickly (70 days approximately) on only one medium without producing callus. Generally, a small number of somatic embryos (a few to 100 per explant) are obtained.
2. 'High frequency using two media': an induction medium for primary callogenesis, and a secondary regeneration medium to produce embryogenic friable callus regenerating several hundred thousand somatic embryos per gram of callus (Söndahl and Sharp, 1977). The high frequency procedure (from leaf explants to somatic embryos) takes about 7-8 months for *C. canephora* and Arabusta, and 9-10 months for *C. arabica*. This process enables the use of a liquid medium for both embryogenic tissue proliferation, and the regeneration phase, and it was consequently preferred for scale-up and development of mass propagation procedures.

In this section, we only describe the high-frequency somatic embryogenesis process developed in the last 10 years for low-cost mass production of coffee *in vitro* plantlets. In order to meet this dual objective, the process developed on coffee includes two original stages: i) a regeneration stage taking place completely in liquid medium using a temporary immersion bioreactor and ii) direct sowing in the nursery of mature embryos produced in the bioreactor**.** It takes 5 culture stages to produce directly acclimatizable somatic embryos, each with a specific

medium (cf Table 1). These media were first described by Van Boxtel and Berthouly in 1996.

To initiate the culture, the sterilized explant is cultured on a callus inducing medium ('C' : Table 1), underside upwards. This medium consists of MS half strength salts (Murashige and Skoog, 1962) supplemented with 400 mg/l malt extract, 10 mg/l thiamine, 1 mg/l nicotinic acid, 1 mg/l pyridoxine, 100 mg/l myoinositol, 1 mg/l glycine, 100 mg/l casein hydrolysate, 88mM sucrose, 2.3µM 2,4-dichlorophenoxyacetic acid (2,4-D), 4.92µM indole-3-butyric acid (IBA), 9.8µM isopentenyladenine (2iP) and 2.5 g/l Phytagel. The pH is adjusted to 5.6 with 1 N NaOH before autoclaving for 20 min at 121°C. Primary callus is produced on the leaf explant by culturing for one month in a 60 x 15 plastic Petri dish containing 12 ml culture medium. Callus induction is achieved at 26°C in the dark. There is systematic production of compact white primary callus around the edge of the explant (scar tissue) and on the main veins. Losses through contamination could amount to several dozen percent and are directly linked to the phytosanitary condition of the mother plant.

After culturing for one month, all the explants are transferred to embryogenic callus production medium ('ECP': Table 1) which is also made up of half-strength MS salts supplemented with 800 mg/l malt extract, 20 mg/l thiamine, 200 mg/l myoinositol, 20 mg/l glycine, 40 mg/l cysteine, 60 mg/l adenine sulphate, 200 mg/l casein hydrolysate. Growth regulators are replaced by 4.5µM 2,4-D and 17.7 µM benzylaminopurine (BA). Sucrose, pH and phytagel are not changed. Two to three explants are transferred to Gerber jars containing 20 ml 'ECP' medium and seal with plastic caps. When transfer after 4 weeks on the 'ECP' medium, the calli and explants gradually turn brown, and after 13-16 weeks yellow friable calli form on the same medium (Fig. 1a). They appear indifferently on several types of primary callus, but it is compact, spongy, little or highly developed. They grow for 3-4 weeks. Without subcultures in either solid or liquid medium the calli lose their yellow colour and regeneration capacity (Berthouly and Michaux-Ferrière, 1996). Embryogenic tissues can be harvested two or three times from the same explant

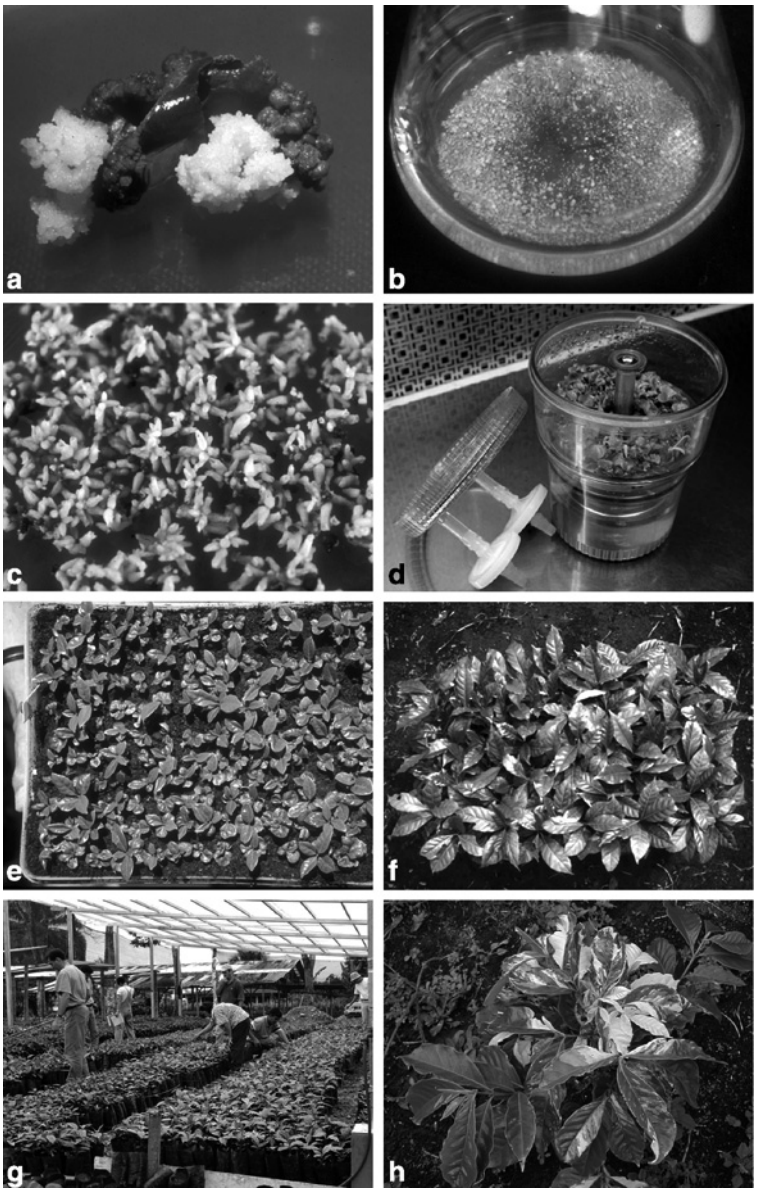

Figure 1. Somatic embryogenesis in coffee: (a) Growth of embryogenic calli on a browned primary callus. (b) Proliferation of embryogenic tissues in liquid medium. (c) Torpedo-shaped embryos produced in a temporary immersion bioreactor. (d) Mature cotyledonary somatic embryos in a RITA temporary immersion bioreactor. (e) Conversion into plantlets of cotyledonary embryos 4 weeks after direct sowing in nursery. (f) Aspect of plantlets ready to be transferred in 1-litre plastic nursery bags, 16 weeks after direct sowing. (g) Growth in nursery of somatic embryo-derived plantlets. (h) Aspect of 'variagata' phenotypic variant.

provided the base of the callus is not harvested. These calli contain embryogenic cells. When placed under the appropriate culture conditions (in medium with only BA as cytokinin), each cell is capable of producing a somatic embryo. The ability of explants to produce embryogenic calli depends on the physiological status of the leaf, the genotype, and the concentration of plant growth regulator in the medium (Berthouly and Etienne, 1999). Cultures are stored on shelves in the dark at 26±1°C throughout this period.

3. MAINTENANCE OF EMBRYOGENIC TISSUE

Maintenance of embryogenic tissues is not efficient on semi-solid medium but it can be achieved by establishing a cell suspension. A cell suspension (Fig. 1b) is obtained by transferring friable embryogenic tissues to the callus maintenance medium ('CP': Table 1). The 'CP' liquid medium consists of half-strength MS salts supplemented with 200 mg/l malt extract, 5 mg/l thiamine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 50 mg/l myoinositol, 10 mg/l cysteine, 100 mg/l casein hydrolysate. The growth regulators are 4.5 µM 2,4-D and 4.7 µM kinetin. Sucrose concentration is lowered to 44 mM. The resulting cell suspension cultures are cultured in 250 ml Erlenmeyer flasks with a cellulose stopper at 26°C on a rotary shaker at 100 rpm. Stable embryogenic cell suspension cultures are obtained 2-3 months after initiation in a liquid medium. Using a high culture density (over 20 g/l) strongly governs cell proliferation – to the detriment of embryo regeneration – hence the success of this phase. For subculturing in a 250 ml Erlenmeyer containing 50 ml liquid medium, only aggregates smaller than 1 mm in diameter are collected using a narrow mouth pipette (Falcon). The cell suspension cultures are maintained by subculture at 6-week interval. The culture media are totally renewed every two weeks. Cell suspensions are kept in the dark. Stable embryogenic cell suspension cultures of several coffees species and genotypes have been obtained using this procedure. It is preferable not to keep suspensions for more than 6 months, to avoid the risk of somaclonal variations (Etienne and Bertrand, 2003).

4. EMBRYO PRODUCTION IN BIOREACTORS

A temporary immersion culture system (Teisson and Alvard, 1995; Fig. 1d) was used for the entire regeneration phase using embryogenic suspensions. The RITA bioreactor (CIRAD, France) is a 1-litre unit, which comprises two compartments, an upper one with the plant material and a lower one with the medium. The overpressure applied in the lower compartment (1 litre of sterile air.min^{-1} in the vessel) pushes the medium into the upper one. Plant material is immersed as long as overpressure is applied. During the immersion period, air is bubbled through the medium, gently agitating the tissues and renewing the head space atmosphere inside the culture vessel, with the overpressure escaping through outlets on the top of the apparatus.

For embryo production, two hundred milligrams of embryogenic masses from a cell suspension are placed in the 1l-RITA bioreactor along with 200 ml regeneration medium ('R': Table 1). The 'R' medium is derived from the 'C' medium except that casein hydrolisate is increased to 400 mg/l, myoinositol to 200 mg/l and glycine to 2 mg/l. Moreover, 10 mg/l cysteine and 40 mg/l adenine sulfate are added along with 17.8 µM BA. The nylon screen separating the two bioreactors compartments on which the plant material is cultured has a mesh of 400µm. The liquid culture medium is changed every two months. Complete torpedo-shaped development (Fig. 1c) is achieved after 4 months in this medium, with an immersion rate of 1 min twice a day. We recently observed that increasing the frequency for short immersions (1 min) stimulate somatic embryo formation and quality in *C. arabica*, without inducing hyperhydricity (Etienne and Berthouly, 2002). On the other hand, increasing immersion times by 5 min or more led to a considerable reduction in somatic embryo production and in their quality, becoming more critical as the immersion frequencies increased. At the end of the developmental phase, each bioreactor contains around 8 000 torpedo-shaped somatic embryos. The RITA bioreactors are placed at 26°C, with a 12h/12h photoperiod and 50µmol m^{-2} s^{-1} photosynthetic photon flux density.

5. EMBRYO MATURATION

To enhance embryo maturation, the biomass of each bioreactor is divided to achieve a density of approximately 1500-2000 embryos per RITA

bioreactor. Maturation is triggered by applying an embryo maturation medium ('M' : Table 1) for 2 months at an immersion rate of 1 min twice a day; this medium is renewed once a month. The 'M' medium consists of MS salts supplemented with 10 mg/l thiamine, 100 mg/l myoinositol, 1.3µM BA, and sucrose is increased to 117 mM. At the end of that period, M medium enriched with sucrose (234 mM) is applied for 2 weeks before direct sowing in the nursery**.** The high sucrose content in the medium enables the formation of reserves (primarily starch) for use during germination in the greenhouse. The density used proves to be optimum for effectively promoting elongation of the embryonic axis (increasing from 5 to 10 mm long), and the development and opening of the cotyledons (Barry-Etienne et al., 1999). In addition, the physical constraints associated with the high densities used during embryo development and maturation favour the synchronized development of embryos with uniform morphology (86% had reached the mature stage and possessed a pair of open cotyledons and a well-developed chorophyllous embryonic axis at the end of the maturation phase (Barry-Etienne et al., 2002). The embryos become chlorophyllous during this period.

6. GERMINATION AND TRANSFER TO SOIL BY DIRECT SOWING

Sowing conditions for direct transfer of somatic embryos to soil are fixed as follows: light and moisture conditions are identical to those previously defined for acclimatization of fully developed *in vitro* plantlets. The first four weeks of this phase are the most critical. In fact, mature somatic embryos, taken from an aseptic controlled environment including temperature, light and high relative humidity, are extremely fragile. The embryos photosynthesize very little and do not have roots, hence they require intensive care during this stage. The following precautions should be taken: i) protect the sowed embryos from the rain and from bright light; ii) maintain a constant temperature (21-25°C) and high relative humidity to prevent the plants drying out.

To maintain high relative humidity during the first month, the plants are watered two or three times a day inside an acclimatization tunnel using an automatic misting type system (or a clean portable pump). Each week, one side of the tunnel is lifted, beginning with the short and then the long

Table 1: Composition of the different media used for the different stages of somatic embryogenesis in coffee.

	Callus induction medum (C) (mg/l)	Embryogenic callus production medium (ECP) (mg/l)	Callus maintenance medium (CP) (mg/l)	Regeneration medium (R) (mg/l)	Embryo maturation medium (M) (mg/l)
	(except when otherwise stated)				
Basic medium	MS/2	MS/2	MS/2	MS/2	MS
Casein hydrolysate	100	200	100	400	-
Malt extract	400	800	200	400	-
Thiamine	10	20	5	10	10
Nicotinic acid	1	-	0.5	1	-
Pyridoxine	1	-	0.5	1	
Myoinositol	100	200	50	200	100
Glycine	1	20	-	2	-
Cysteine	-	40	10	10	-
Adenine sulfate	-	60	-	40	-
2,4-D	2.26 μM	4.52 μM	4.52 μM	-	-
IBA	4.92 μM	-	-	-	-
2iP	9.84 μM	-	-	-	-
BAP	-	17.76 μM	-	17.76 μM	1.33 μM
KIN	-	-	4.65 μM	-	-
Sucrose	88 mM	88 mM	44 mM	88 mM	117 mM
PH	5.6	5.6	5.6	5.6	5.6
Phytagel	2.5 g/l	2.5 g/l	-	-	-

sides, eventually removing all the sides after a month. The embryos can withstand lower relative humidity and higher temperatures. After the first 4 weeks, it is sufficient to keep the soil moist. As with the relative humidity, *in vitro* plantlet hardening with respect to light is very gradual. Initially, during acclimatization, the light received should be 25 to 30% of natural sunlight. By the time of planting, it should be 100%. To prevent the leaves from being scorched, the shift from 30 to 100% of natural light should be gradual, using different shading devices (black canvas, banana leaves, palm leaves).

Mature embryos are sown vertically on top of the substrate (2 parts soil, 1 part sand, 1 part coffee pulp) and sterilise with chemical treatment (Dazomet (DMTT), Union carbide). The somatic embryo culture density in plastic boxes (l.w.h = 30.21.10 cm) is approximately 3600/m^2. The cultures are placed under a transparent roof, which provide 50% shade and water for 2 min twice a day. A nutritive solution can also be applied, for instance a liquid medium containing semi-dissolved Murashige and Skoog's mineral salts (MS/2) or any well diluted leaf fertilizer (Bayfolan, 2.5 ml/l). Fertilization is provided every two days during this period.

Under these conditions, roots are systematically produced in the second and third weeks, 95% embryos germinate and 81% regenerate whole plantlets (Fig. 1e). A degree of heterogeneity in plantlet development is seen in the plastic boxes; this is closely linked to initial morphological variability, more specifically in terms of embryo cotyledon area.

For growth and hardening in the nursery, the plantlets are transferred 20 weeks after direct sowing to a substrate comprising soil and coffee pulp (3/1, v/v) in a 1-litre plastic nursery bag. The plantlets are cultivated under a transparent roof (Fig. 1g) and watere twice a week until they reache the required size for planting out in the field (30-40 cm).

7. SOMACLONAL VARIATION

The variants aside, there are no differences in the main agronomic characteristics (plant morphology, mineral and biochemical composition of the seed, cup quality) among trees produced from embryogenic

suspensions and seeds or microcuttings (Etienne and Bertrand, 2001). Identification of variants is based on morphological observation in the nursery or in the field. Plants regenerated *in vitro* that has different morphological traits of the initial clone when acclimatized in the nursery or planted in the field are referred as somaclonal variants. These somaclonal variants are identified according to height, morphology, leaf shape, productivity, fruit shape, leaf density, stomatal density and guard cell chloroplast number. Seven types of phenotypic variants are characterized. Based on the vigour and productivity of the regenerated plants, it is possible to class the variants in order of severity of physiological disorders: "colour of juvenile leaves", "Giant", "Dwarf", "Thick leaf (“Bullata”)", "Variegata" (Fig. 1h), "Angustifolia", "Multi-stem". The "Dwarf", "Angustifolia" and "Multi–stem" types are the most frequent among produced plants (1.4, 4.8 and 2.9%, respectively). The frequency of variants increases exponentially with the age (t) of the embryogenic suspension, in accordance with a function of the type Freq = 0.99 $e^{0.267t}$. For all genotypes, the degree of somaclonal variation is low (1.3%) in plants produced from embryogenic callus or 3-month-old suspensions. Thereafter, the frequency of variants increases significantly, reaching 6, 10 and 25% in plants produced from suspensions aged 6, 9 and 12 months, respectively. Strong differences are found between genotypes with the 12-month-old suspensions only. The suspension age and genotype also affect the type of variants produced. The severity of somaclonal variations increases with suspension age and the ‘Dwarf’, ‘Angustifolia’ and ‘multi-stem’ variants are successively represented. For all genotypes combined, the "Angustifolia" variation is the most common. The other variations are specific to certain genotypes or distributed randomly among the genotypes. Apart from the ‘Dwarf’ phenotype, it is possible to detect most of the variants at the end of the nursery stage. Dwarf variants can be seen after one month after planting out.

8. CONCLUDING REMARKS

Abundant literature exists on the development of somatic embryogenesis in coffee, and more particularly on optimizing growth regulator applications during the initial stages of embryogenic tissue production. In that respect, it can be considered that coffee plants are not particularly recalcitrant to somatic embryogenesis. However, one of the consequences is the multiplicity of protocols published to date by many teams. This

fragmentation probably slowed down the development of this technology for commercial application, by not leading the scientific community to work on a joint protocol. Protocols leading to direct embryogenesis should be reserved for the management of genetic resources or embryo production for physiological studies. Only protocols leading to indirect embryogenesis are appropriate for mass production of elite planting materials.

We now have reliable information, on a relatively large scale, on how *C. arabica* (Etienne and Bertrand, 2001, 2003) and *C. canephora* (Ducos et al., 2003; Bertrand et al., 2002) plants obtained by somatic embryogenesis perform in the field. The results show that only the propagation of *C. arabica* entails any risks of somaclonal variations and that culture conditions, particularly the amplification phase in cell suspension form, clearly affects the number of variants observed. In the future, it will be essential to take into account data on conformity in the field for any optimization of the somatic embryogenesis procedure.

Embryo production in liquid medium, and more recently the possibility of directly sowing somatic embryos in the nursery, have led on to pilot production of performant arabica F1 hybrids in various countries, showing promise for commercial production in the short term. The purpose of current research on the procedure is primarily to reduce production costs, in order to be competitive with plants traditionally produced from seed and sold for less (USD 0.25-0.30/plant). In addition, as planting densities are very high for *C. arabica* dwarf varieties (between 5 000 and 10 000 trees/ha) planting costs are substantial. To achieve this objective, research is focusing on optimizing the acclimatization/nursery phases and on improving the efficiency of various stages in the procedure, facilitated by a scale-up.

9. REFERENCES

Barry-Etienne, D., B. Bertrand, N. Vásquez & H. Etienne, 1999. Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and the regeneration of plants. Plant Cell Rep. 19:111-117.

Barry-Etienne, D., B. Bertrand, A. Schlonvoïgt & H. Etienne, 2002. The morphological variability within a population of coffee somatic embryos produced in a bioreactor affects the regeneration and the development of plants in the nursery. Plant Cell Tiss. Org. Cult. 68 :153-162.

Berthouly, M. & H. Etienne, 1999. Somatic embryogenesis of Coffee. In: Jain, S.M.; Gupta, P.K.; Newton, R.J., eds. Somatic embryogenesis in woody plants, vol.5, pp. 259-288. Kluwer Academic Publishers, London.

Berthouly, M. & N. Michaux-Ferrière, 1996. High frequency somatic embryogenesis in *Coffea canephora*: induction conditions and histological evolution. Plant Cell Tiss. Org. Cult. 44:169-176.

Bertrand B., F. Anzueto, M.X. Moran, A.B. Eskes & H. Etienne, 2002. Creation and distribtuion of a rootstock variety (*Coffea canephora*) by somatic embryogenesis. In D. Bery (ed.), Research and coffee growing. Pp. 95-107. Cirad, France, ISSN : 1254-7670.

Ducos, J.P., R. Alenton, J.F. Reano, C. Kanchanomai, A. Deshayes & V. Pétiard, 2003. Agronomic performance of *Coffea canephora* P. trees derived from large-scale somatic embryo production in liquid medium. Euphytica. 131: 215-223.

Etienne, H., F. Anthony, S. Dussert, S. Fernandez, P. Lashermes & B. Bertrand, 2002. Biotechnological applications for the improvement of coffee (*Coffea arabica* L.). In Vitro Cell. Dev. Biol.-Plant. 38:129-138.

Etienne, H. & M. Berthouly, 2002. Temporary immersion systems in plant micropropagation (review). Plant Cell Tiss. Org. Cult. 69:215-231.

Etienne, H. & B. Bertrand, 2001. Trueness-to-type and agronomic characteristics of *Coffea arabica* trees micropropagated by the embryogenic cell suspension technique. Tree Physiol. 21: 1031-1038.

Etienne, H. & B. Bertrand, 2003. Somaclonal variation in *Coffea arabica*: effects of genotype and embryogenic cell suspension age on frequency and phenotype of variants. Tree Physiol. 23: 419-426.

Murashige, T. & F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissues cultures. Physiol. Plant. 15:473-497.

Söndahl, M.R. & W.R Sharp, 1977. High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z. Pflanzenphysiol. 81:395-408.

Teisson, C. & D. Alvard, 1995. A new concept of plant in vitro cultivation liquid medium: Temporary immersion. In: M. Terzi et al. (eds.) Current Issues in Plant Molecular and Cellular Biology (pp 105-110). Kluwer Academic Publishers, Dordrecht, The Netherlands.

Van Boxtel, J. & M. Berthouly, 1996. High frequency somatic embryogenesis from coffee leaves. Plant Cell Tiss. Org. Cult. 44:7-17.

PROTOCOLS FOR SOMATIC EMBRYOGENESIS AND PLANTLET FORMATION FROM THREE EXPLANTS IN TEA (*CAMELLIA SINENSIS (L.) O. KUNTZE*)

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1. INTRODUCTION

Tea plant production via micropropagation is technically possible but its commercial utility in tea industry is severely hampered because of the cost factor. Tea producers still rely on conventional methods of propagation using either single leaf node cuttings or seeds. Clonal variability of rooting response is also a big problem with micropropagated shoots. Another major disadvantage of micropropagation is that the resulting plants will only have adventitious roots and hence are highly susceptible to drought. On the other hand, with the increase in world demand for high quality tea, the demand for clonal planting material is also increasing. Somatic embryogenesis as a tool for *in vitro* propagation and genetic improvement could play a more significant role in the production of tea. Besides, the recent surge of biotechnological advances like gene cloning and gene transfer offer great promise for rapid improvement of genotypes with desirable traits and integrate well with the technique of somatic embryogenesis. Although somatic embryogenesis has been fully exploited in herbaceous species there remain difficulties with woody species like tea. Unlike micropropagated shoots, the plants derived from somatic embryos have both root and shoot together. Furthermore, the production cost via somatic embryogenesis is potentially cheaper than the micro-cuttings especially if bioreactors and automation are used in the production process. This gives them a decided advantage over the

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plants generated from micropropagation or conventional propagation, particularly in situations where moisture stress limits growth and production. Here we describe three promising protocols for direct somatic embryo production without callus formation from three explants types: nodal buds, immature embryos and mature seed cotyledons (Akula and Akula 1999).

2. INDUCTION OF SOMATIC EMBRYOS FROM NODAL BUDS OF TEA

2.1. Establishment of tea cultures from field material

Contamination due to endemic bacteria and fungi is a major problem in establishing axenic cultures from field grown tea explants. These explants often release large quantities of polyphenols into the medium within a few hours of culturing, which results in the death of the culture. We carried out extensive experimentation to control the browning and blackening problem. We found that a series of washes with fungicide, strong reducing agents (citric acid and ascorbic acid) and antibiotics followed by bleach were highly effective in overcoming this problem. The detailed protocol for the initiation of tea cultures from nodal explants harvested from field grown plants is given below:

A. Explant:

1. Collect the actively growing young shoot tips with 8-10 nodes from tea bushes by cutting with a sharp scalpel and immediately dip the cut edge into a jar containing water.
2. Bring the shoot tips to the laboratory, chop off the leaves (with petioles) and dip the defoliated shoot tips into the water.

B. Sterilzation:

1. Wash under running tap water with circular airflow for 1h.
2. Incubate in fungicide (0.1% Benlet in distilled water, DW) for 1h under vacuum.
3. Wash again thoroughly with tap water for 10-15 min to remove excess fungicide.
4. Rinse with 70% ethanol for 1 min and finally rinse with sterile double distilled water (SDW).
5. Treat with commercial bleach with 1% available chlorine for 20 minutes while continuing to stir.
6. Wash with SDW at least 10 times or till water remains clear.

7. Treat with filter sterilised solution containing antibiotic (0.1% cefotaxime) and antioxidants (equal volumes of 1.5% ascorbic acid and citric acid) for 4 h on shaker at 100 rpm.
8. Gently wash with SDW.
9. Inoculate 8-10 mm long nodal cutting with a single axillary bud on bud sprouting medium (BSM). Refer to Table 1. Incubate in the dark for four weeks, with regular sub-culturing every 36 hrs during the first two weeks to fresh medium of the same composition and thereafter. Weekly sub-culturing is recommended until the axillary bud starts sprouting and medium remained clear.
10. Excise the sprouting axillary bud with minimal mother tissue attached. Transfer it to the shoot bud culture maintenance medium (BMM)[table 1] and incubate until the explant is 15-20 mm long with 2-4 leaves.

2.2 Induction of somatic embryogenesis:

1. Excise single nodal cuttings (5-8 mm) from in vitro shoot cultures. Transfer then to pre-induction medium (PIM) table 1] and incubate for 6-8 wk and observe for nodal bud multiplication.
2. Select the cultures with active multiple shoot formation (de novo). Transfer them to a fresh medium of the same composition (PIM) and incubate for 16 wks without sub-culturing.
3. Observe the emergence of brown, obovate, translucent and expanded structures, which reach sizes up to 12-15 mm in diameter. Non-responding cultures normally tend to degenerate.
4. Transfer the expanded and swollen cultures onto embryo induction medium (EIM) [table 1].
5. Within 4 weeks of the culture period, yellowish globular clumps of primary somatic embryos emerge out of the primary culture. These globular embryos originate directly from the base of the axillary buds and eventually separate from the original explant on their own. From a single original explant we could harvest 6-15 somatic embryos every 4-week period by regular subculture to a fresh medium of same composition (Figure1a).

3. SOMATIC EMBRYOS FROM IMMATURE ZYGOTIC EMBRYOS OF TEA- ONE STEP PROTOCOL

1. Harvest immature green fruits (20-25 days after pollination) of tea from the tea bushes.
2. Remove the pericarp and select uniform seeds.

3. Sterilize the seeds under a sterile hood with commercial bleach (sodium hypochlorite having 1% of available chlorine) for 15 m. followed by several washes in sterile water.
4. Excise the immature zygotic embryos from the pool of liquid endosperm after dissecting the seeds aseptically.
5. Select the zygotic embryos ranging size between 3-6 mm and transfer to Pre-induction medium (PIM) [table 1].
6. Incubate for 8-16 wk. Proliferation of somatic embryos start appearing within 8 weeks of culture, 40% explants respond by producing primary somatic embryos. More explants respond with the length of incubation period. The overall efficiency of the induction and the site of response on the explant are greatly influenced by the age of maturity of the zygotic embryo (Figure 1b).

4. SOMATIC EMBRYOGENESIS FROM COTYLEDONS OF MATURE SEEDS

1. Harvest fresh mature green fruits of uniform size (>13 mm diameter) from tea bushes.
2. Remove the seed coat and rinse with 70% alcohol for 3-5 min followed by sterilization with commercial bleach (with 1% available chlorine) for 10 min.
3. Wash thoroughly with SDW 3 times, for duration of 5 min each time.
4. Cut the cotyledons into thin slices (1mm thickness) after excising embryonic axis and transfer to pre-induction medium (PIM) [table 1]
5. Incubate the cultures in dark for 90 days without any subculturing.
6. After this long incubation period in the dark, the slices will start swelling and turn to a yellowish brown color. At this stage, select the swollen and yellowish cultures and transfer then to fresh medium of the same composition and incubate at 16-h photoperiod (30 micro mole/$m^{-2}s^{-1)}$). Transfer to fresh medium regularly after every 4 weeks.
7. After 12 weeks, observe the formation of clusters of compact, greenish somatic embryos from the cut edges of the cotyledonary pieces.
8. The somatic embryos obtained by this method look hard, and are tightly attached to the original explant. The embryos need to be separated from the mother explant and transferred to embryo development medium (EDM) [table 1].

9. Transfer to a fresh medium of the same composition every 6 wk-interval. After 2-3 transfers, observe the formation of globular yellowish secondary embryos (Figure 1c).

5. MATURATION AND GERMINATION OF SOMATIC EMBRYOS

The maturation and germination of somatic embryos in tea is very asynchronous and not controllable. More than 60% of the somatic embryos incubated on REM with (0.005 mg l^{-1} IBA + 0.01 mg l^{-1} BA) or without hormones [table 1] show the tendency of repetitive embryogenesis by budding off new globular somatic embryos. Low percent of embryos (20-30%) convert directly into emblings with distinct roots and shoots. Thus, within a culture plate, all stages of developing (globular, heart, torpedo and cotyledon) somatic embryos can be found at any one time (figure 1d). Two to five percent of the cultures show abnormal developments such as thick, short and stout roots without shoot formation.

6. REPETITIVE SOMATIC EMBRYOGENESIS AND PLANT RECOVERY USING TEMPORARY IMMERSION SYSTEM

All 3 methods described above irrespective of explant type, will be able to produce repetitive or secondary embryogenesis upon transfer of uniformly sized (2-3mm) somatic embryos on to a petri plate contained embryo multiplication medium (REM) [table1]. However the efficiency of multiplication is varied from one explant to another. In most cases, no callus formation occurs. However, after 6 cycles of production, a few percent of embryos tend to show callus formation

To improve the multiplication rate and to synchronize the process of plant recovery, a novel method of temporary immersion system was used. A modified TIS was constructed as described by Alvard et al (1993). The two chambers of an autoclavable 250-ml filter unit (Nalgene, Nalge Co.) were connected by insertion of a glass tube into the base of the support plate normally used to hold membrane filters. Air hoses were connected to the two chambers, and the flow of air was kept sterile by fitting 0.22-µm hydrophobic filter units (Millepore). Liquid medium (100 ml) was placed in the lower chamber and somatic embryos in the upper chamber. Air pumped into the lower chamber displaced the liquid medium through the glass

tube to the upper chamber, immersing the somatic embryos. Air pumped to the upper chamber forced the medium to return to the lower chamber. An electrical current from the printer port of a computer was used to operate solenoids that controlled airflow from an aquarium pump to either the upper or lower chambers. A computer program written in Turbo Basic version 1.0 (Borland International Inc.) was used to control the electrical current to the printer port. This program required three pieces of data, a 'pump up' time (airflows to the lower chamber), a 'pump down' time (airflows to the upper chamber), and an 'idle time' (no airflow). 'Pump up' and 'Pump down' times were always set at one minute each while the 'idle time' was varied. The program was then set to run in a continuous cycle through the three different stages. In this way embryos were completely immersed for one minute at set intervals.

Uniform sized (2-3 mm) globular-stage somatic embryos were cultured in TIS chambers containing 100 ml of liquid medium. Somatic embryos were immersed for 1-2 minute in liquid medium once every six hour for 8-weeks. We could obtained 24-fold increase in the number of globular stage somatic embryos, which then were subjected to consecutive treatment of dehydration, re-hydration and starvation as summarized in figure 2. Gradual dehydration was investigated by reducing the immersion frequency either 7 days or 14 days, instead of immersing the embryos every 6-h interval for 1 week, after which immersion ceased. After completion of a 4-week period without immersion (starvation), the number of plantlets (fully formed emblings with a tap root and distinct shoot formation bearing at least one pair of leaves) and the number of embryos undergoing maturation (torpedo to cotyledonary stage embryos) were recovered (figure 2 e).

7. ACCLIMATIZATION

1. Deflask the plantlets with strong taproots and 4-6 leaves. Wash in distilled water and transfer to small pots (5x12 cm) filled with pre-sterilised potting mix (sand: peat: vermiculite, 1:2:1).
2. Transfer the pots to a greenhouse at 80-95% humidity under low light (15-M photon.m^{-2}.s^{-1} over 16-hr) and incubate for 8-10 days. Next, move the pots to normal greenhouse conditions for further weaning.
3. In a 5-6 week period, formation of 1-3 new leaves occurs (figure 2 f) and is ready for transfer to a bigger pot (14x16 cm). Maintain this in the greenhouse until it is ready (16-20 weeks) for final transfer to the field.

8 CONCLUSIONS

The present protocol is efficient and highly reproducible for the production of somatic embryos and subsequent plant recovery. Our methods described here are very simple and rapid with minimal use of growth regulators to avoid any abnormalities related with cytokinin effect as found in pea (Van Doorne et al., 1995). The novel application of TIS in tea somatic embryo production and plant recovery is very promising. Current methods of tea somatic embryo production on agar nutrient plates are less efficient, thus making it impossible to adopt this technology for commercial purpose. In TIS, the multiplying tea somatic embryos are uniformly immersed in liquid medium, which results in synchronised development and more recovery of plantlets. TIS system saves labour, avoids contamination with several fold increase in production and thus making it more attractive for commercial applications. Production can be scaled up easily by adding more and more flasks to the existing system. However, more challenges are ahead, particularly in generating quality clonal stock in a more predictable way. Further experiments focussed on the use of TIS and the influence of various growth factors like ABA and cytokinins on the maturation of tea somatic embryos would give us better insight in understanding the developmental process of tea somatic embryos. Evaluation of various stage specific regulatory genes coupled with the functional genomic approaches may provide us with wide array of molecular tools, and is also a good option in unravelling the overall process of somatic embryogenesis in tea.

10. REFERENCES

Akula A and Akula C (1999) Somatic embryogenesis in Tea (*Camellia sinensis* (L.) O. Kuntze) In: *Somatic embryogenesis in woody plants*. Vol. **5**, pp 239-257, (eds. Jain S M, Gupta Pramod, Newton R.J.) Kluwer Academic Publishers. Dordrecht /Boston /London.

Alvard D, Cote F, Teisson C (1993) Comparison of methods of liquid medium culture for banana micropropagation. Effect of temporary immersion of explants. *Plant Cell Tissue Organ Cult.* **32**:33-37

Jefferson R A 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biological Report*. **5**:387-405.

Van Doorne L E, Marshall G and Kirkwood R C. 1995. Somatic embryogenesis in pea (*Pisum sativum* L.); effect of explant, genotype and culture conditions. *Ann Appl Biol*.**126**:169-179

Table 1: Media details

Medium Composition	**BSM $mg^{-1}L$**	**BMM $mg^{-1}L$**	**PIM $mg^{-1}L$**	**EIM $mg^{-1}L$**	**EDM $mg^{-1}L$**	**REM $mg^{-1}L$**
Ammonium Nitrate	1650	1650	1650	825	825	825
Boric Acid	6.2	6.2	6.2	6.2	6.2	6.2
Calcium Chloride Anhydrous	332.2	332.2	332.2	332.2	332.2	332.2
Cobalt Chloride. $6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025
Cupric Sulfate $5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025
Na_2-EDTA	37.26	37.26	37.26	37.26	37.26	37.26
Ferrous Sulfate $7H_2O$	27.8	27.8	27.8	27.8	27.8	27.8
Magnesium Sulfate	180.7	180.7	180.7	90.35	90.35	90.35
Manganese Sulfate H_2O	16.9	16.9	16.9	16.9	16.9	16.9
Molybdic Acid, Sodium Salt $2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25
Potassium Iodide	0.83	0.83	0.83	0.83	0.83	0.83
Potassium Nitrate	1900	1900	1900	950	950	950
Potassium Phosphate Monobasic	170	170	170	85	170	170
Zinc Sulfate $7H_2O$	8.6	8.6	8.6	8.6	8.6	8.6
Adenine Hemi sulfate	-	-	100	-	-	-
L-Glutamic Acid	-	-	100	-	-	-
Casein hydrolysate	0.00	0.00	0.00	0.00	500.0	500.0
Myo-inositol	100	100	100	100	100	100
Nicotinic Acid	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine. Hcl	0.5	0.5	0.5	0.5	0.5	0.5
Thiamine. HCl	0.1	0.1	0.1	0.1	0.1	0.1
Glycine	2.0	2.0	2.0	2.0	2.0	2.0
Gibberlic Acid	0.0	5.0	0.0	0.0	0.0	0.0
6-Benzylaminopurine (BA)	0.0	0.0	0.5	0.0	0.0	0.01
Indole-3-butyric Acid (IBA)	0.0	0.0	0.1	0.0	0.0	0.005
Sucrose ($g^{-1}L$)	0.00	30.00	30.00	30.00	30.00	30.00
Agar- Bi-Tek ($g^{-1}L$)	7	7	7	7	7	7
pH	5.75	5.75	5.75	5.75	5.75	5.75

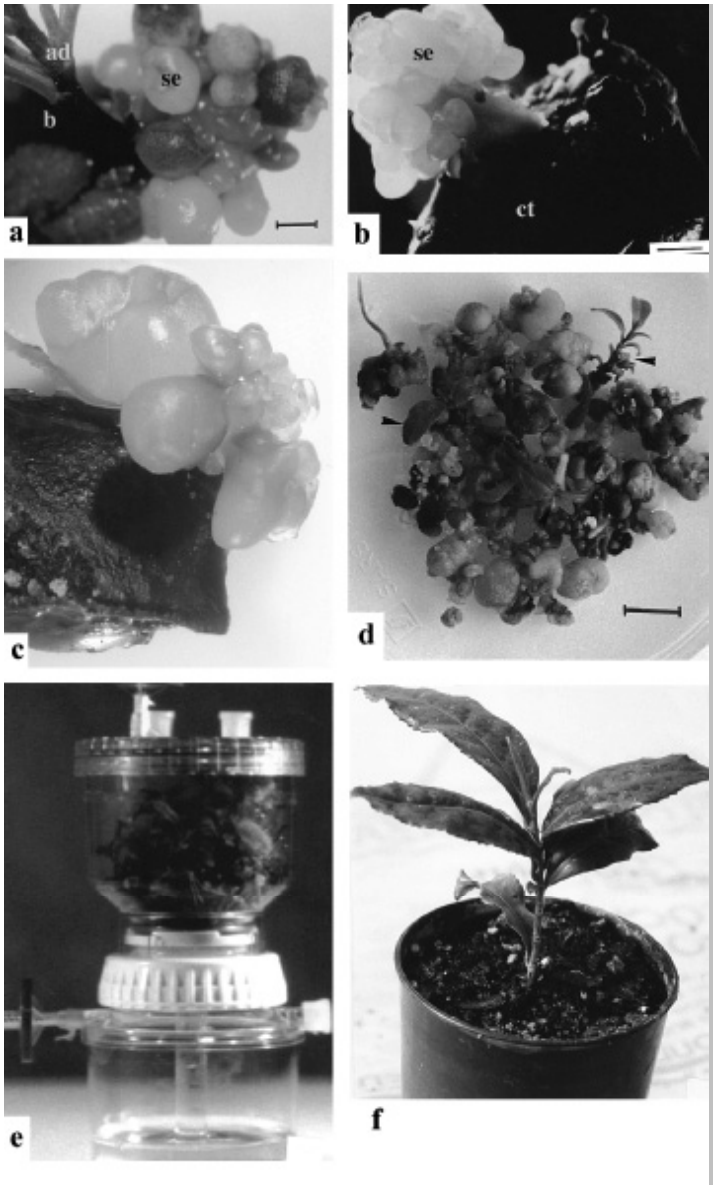

Figure1. Induction of somatic embryogenesis and subsequent plant recovery from three explants **a**) Proliferating somatic embryos (se) from nodal cuttings, ad-adventitious shoots **b**) Occurrence of somatic embryogenesis from immature zygotic embryo, ct-cotyledon **c**) Emerging somatic embryos from the cotyledon of mature seed **d**) A single culture showing all stages of maturation of somatic embryos and fully formed plantlets **e**) TIS apparatus showing upper jar filled with emblings of tea **f**) Fully acclimatizes plantlet ready for field transfer

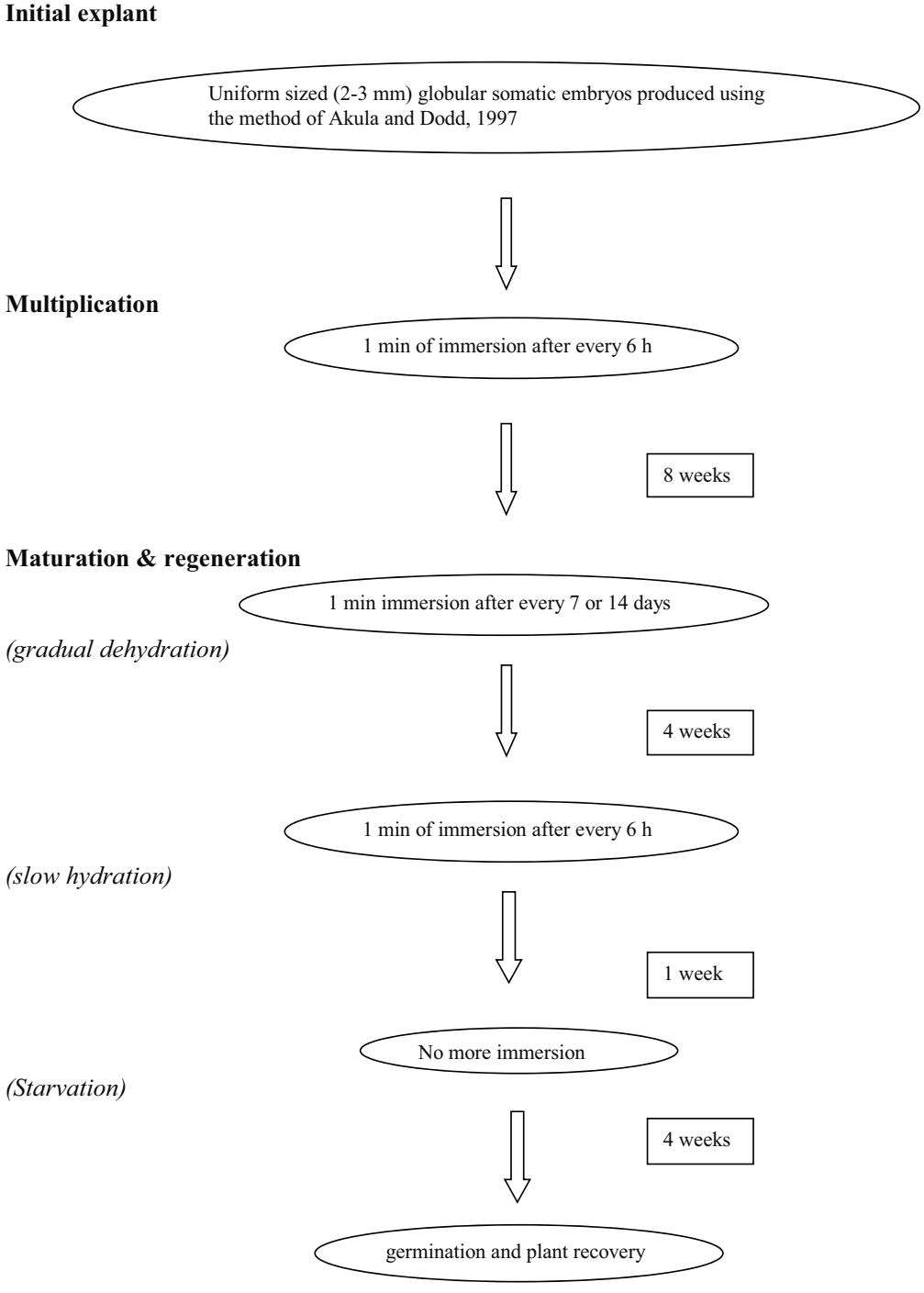

Figure 2: Repetitive embryogenesis and plant recovery- using TIS in tea

PROTOCOL OF SOMATIC EMBRYOGENESIS FROM *CITRUS* SPP. ANTHER CULTURE.

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1. INTRODUCTION

Citrus is most widely cultivated fruit crop worldwide and its annual production was 104.5 million metric tons during 2002 (FAOSTAT, Database). The most commercially important *Citrus* species are oranges (*C. sinensis* L. Osbeck) and tangerines (*C. unshiu* Marc., *C. nobilis* Lour., *C. deliciosa* Ten., *C. reticulata* Blanco and their hybrids) with more than 80%, followed by lemons (*C. limon* L. Burm. f.), limes (*C. aurantifolia* Christm. Swing.) and grapefruits (*C. paradisi* Macf.) in almost equal proportions.

Citrus breeding is focused on obtaining new scions and rootstocks resistant or tolerant to biotic and abiotic stresses. Regarding the new varieties, important goals are: an increased yield, a longer ripening season, regular fruit bearing, seedlessness, improved external and internal quality of the fruits, and a shorter juvenile non-fruiting period.

Citrus breeding is based on conventional methods (hybridization, selection, mutation) as well as biotechnologies such as *in vitro* tissue culture, regeneration from protoplasts, somatic hybridization, *in vitro* mutant selection, genetic transformation and haploid production.

Efficient procedures of regeneration of *Citrus in vitro* are nedeed, together with the modern techniques of cellular and molecular biology, for value added improvement, in a short time. Somatic embryogenesis is an efficient method of plant regeneration. In citrus, somatic embryogenic callus is valuable for propagation or genetic improvement. In fact, it can also be used for somatic hybridization by protoplast

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fusion, genetic transformation, synthetic seed production, elimination of pathogens and *in vitro* germplasm storage.

Anther culture is the most commonly used method to produce haploids and doubled-haploids (Jain et al., 1997). However, it could also be employed in obtaining somatic embryos and the regeneration of many woody plants, e.g. *Vitis* (Mauro et al., 1986; Cersosimo et al., 1990; Mozsar and Sule, 1994; Torregrosa L, 1998; Perrin et al., 2001). By anther culture service tree (*Sorbus domestica* L.), somatic embryos were produced even though the conversion into plantlets was not obtained (Arrillaga et al., 1995). Similar results were obtained in *Prunus persica* L. (Hammerschlag, 1983) and *Feijoa sellowiana* Berg. (Canhoto and Cruz, 1993).

2. EMBRYOGENIC CULTURE INITIATION

In vitro anther culture is affected by numerous factors: genotype, pre-treatment applied to anthers or to floral buds, pollen developmental stage, donor plant conditions, culture media composition, and growth chamber conditions.

2.1. Explant-type, Pre-treatment and Sterilization

Materials and equipment required: sterile distilled water**,** 70% (v/v) ethyl alcohol, 20% v/v commercial bleach, 1% acetocarmine in 45% acetic acid, 1 mg/ml of 4`,6-diamidino-2-phenylindole dihydrochloride (DAPI), stereo microscope, light microscope, fluorescent microscope, flow hood, sterilized filter paper, Petri dishes, forceps, culture media (see Table 1).
Floral buds are collected from the plant growing in the field during March- May, depending on the season and on the genotype.
Pollen developmental stage is determined by staining one or more anthers per floral bud size with acetocarmine, or DAPI staining.

Acetic-carmine method:

1. Anthers are squashed in acetocarmine stain (1% acetocarmine in 45% acetic acid) for observation under an optical microscope to determine the pollen developmental stage.

DAPI staining:

1. Anthers are squashed in a few drops of DAPI solution (1 mg/ml) and observed under a fluorescent microscope to identify the pollen developmental stage.

A correlation between flower size and pollen uninucleate stage (the most responsive stage for anther culture) was established. Only flower buds of this size (3.5-6.0 mm long for mandarin, Fig.1A, and clementine, and 6.5-10.0 mm long for sour orange, lemon and grapefruit) containing microspores at the uninucleate stage are selected for anther culture. However, several researchers have observed different developmental stages within a single anther, and between different anthers of the same flower bud in *Citrus* and in *Poncirus* as well as in many other genera (Hidaka et al., 1979; 1981; Chen, 1985; Vasil, 1967; Shull and Menzel, 1977).

Anther culture technique:

1. After pre-treatment (4°C for 10-15 days, in the dark), the buds are surface sterilized in the laminar flow hood by immersion for 3 min in 70% (v/v) ethyl alcohol, followed by immersion in sodium hypochlorite solution (about 1.5% active chlorine in water) containing a few drops of Tween 20 for 15-20 min, and finally rinsed three times for five minutes each with sterile distilled water.
2. Petals are aseptically removed with forceps, and anthers (Fig. 1B) are carefully dissected and placed onto the culture medium. Anthers from 3-4 flower buds (60-80 anthers) are placed in each Petri dish (60 mm diameter) containing 10 ml solid medium.
3. Wrap Petri dishes with Parafilm and incubate at 27$\pm$1°C for fifteen days in the dark, and then place under cool white fluorescent lamps (Philips TLM 30W/84) with a photosynthetic photon flux density of 35 μmol m^{-2} s^{-1} and a 16 h photoperiod.
4. Every 2nd week, cultures are observed for ten months,.
5. Embryogenic calli are transferred to the multiplication medium.
6. Embryoids are isolated and placed in the germination medium.
7. Subculturing is done every month.
8. Characterization of regenerants.
9. *In vivo* acclimation.

2.2. Culture Medium Composition

The composition of the media employed is shown in Table 1. All growth regulators are added before autoclaving. The pH of media was adjusted to 5.8, with 1N KOH before autoclaving at 110 kPa, 121 °C for 20 min; solidification of media was achieved by 0.8-0.75 % w/v washed purified agar (Sigma, A 8678).

The basal medium used in *Citrus* anther culture for somatic embryo induction is Nitsch and Nitsch (1969) and for maintaining callus and for embryo germination is Murashige and Skoog, (MS) (1962).

2.3. Initiation

After 2-3 months anthers start to produce calli or embryoids (Fig. 1C). The average frequency of callus induction in *Citrus* is about 19% for mandarin, 59% for sour orange, 70% for grapefruit. Most of the calli are non-morphogenic (Fig. 1D), but many of them appear highly embryogenic (Fig. 1E) and they maintain their embryogenic potential for a long time. The embryogenic potential of mandarin is however higher than other genotypes: more than 100 embryo are obtained from one responsive anther after seven subcultures of embryogenic calli.

The morphogenic calli appear friable and white. The embryogenic calli differentiate into a clump of embryoids (Fig. 1F).
Green, compact and non-morphogenic calli emerging from anthers were also observed in *Poncirus*, *C. clementina* and *C. limon*.

2.4. Culture Maintenance

The highly embryogenic haploid callus is multiplied in MS medium supplemented with 5% sucrose, 0.02 mg l^{-1} NAA and 0.8% agar, maintaining its embryogenic potential for several years.

1. Separate the white, translucent, embryogenic tissue from the green or white non-embryogenic callus.
2. Subculture the embryogenic tissues from individual anthers separately into M2 maintaining medium (Table 1).
3. Keep the stock culture lines in the dark at 27±1 °C, with 5-6 week subcultures.

2.5. Embryo Development

Plantlet formation from cultured anthers may occur either directly through embryogenesis or indirectly through embryogenesis from anther-derived callus.

The well-structured embryoids develop normally like zygotic embryos, through the globular, the heart, the torpedo and the cotyledonary stages (Fig. 1G; Fig. 1H). Secondary embryogenesis (direct and indirect) has been observed, more frequently in the root region of the embryos (Fig. 1I).
As the embryos appear, they are germinated in Petri dishes with M3 (germination medium, table 1), and they are later transferred to Magenta boxes (Sigma V8505) or to test tubes (Fig. 2A).

Somatic embryos vigorously germinate *in vitro* with a conversion rate of 80-89% depending on the genotype (higher for sour orange).

2.6. Embryo teratology

Often teratomatal structures and morphological anomalies, cotyledonary-fused (Fig. 2B), pluricotyledonary (Fig. 2C) or fascinated and thickened embryoids are observed. Sometimes pseudobulbils, with or without callus, are produced in *Citrus* anther culture (Fig. 2D; Fig. 2E).

2.7. Embryo Germination, Plant Recovery and Hardening

1. Collect cotyledonary somatic embryos from embryogenic calli grown on medium M2.
2. Leave embryos to germinate on M3 medium (Table 1). Incubate embryos in the light at 27 $\pm$ 1 °C (with a 16-hour photoperiod).
3. Transfer plantlets to Magenta boxes (Sigma V8505) or to test tubes.
4. After 3-4 months, when the plantlets are about 4-5 cm high, wash them with sterile water to remove the medium from their roots and then transfer to the greenhouse in Jiffy pots (Fig. 2F) or in pots containing sterile peat moss, sand and soil in the ratio of 1:1:1, for the hardening phase.

5. To avoid dehydration, during the first 40-50 days, cover the plantlets with a polythene bag and seal with an elastic band. Reduce the humidity by gradually increasing the sizes of the holes in the bag.

2.8. Plant characterization

2.8.1. **Ploidy of regenerated plants**

Haploid, and especially diploid, trioploid, aneuploid and mixoploid calli and plantlets have been produced from *Citrus* and its relatives by anther culture. Non-haploids may arise from: a) somatic tissue of anther walls, b) the fusion of nuclei, c) endomitosis within the pollen grain, and d) irregular microspores formed by meiotic irregularities (D'Amato, 1977; Sangwan-Norrel, 1983; Sunderland and Dunwell, 1977; Narayanaswamy and George, 1982).

The chormosome number has been counted in root tip cells from regenerated embryos and plantlets, using the standard Feulgen technique (Lillie, 1951).

Chromosome counts carried out on root apices of embryos and of plantlets obtained from *in vitro* anther culture of sour orange, mandarin and grapefruit showed the diploid set of chromosomes (2n=2x=18) (Germanà et al., 1994; Germanà 2003a).

2.8.1.1. **Cytological characterization of regenerants**

1. Pre-treat somatic embryos with 0.05% colchicine for 2 hours at room temperature.
2. Fix material in 3:1, v/v ethanol: glacial acetic acid overnight. Store fixed samples in EtOH at 4 °C until viewing.
3. Macerate tissue sample in 1N HCl for 5-10 min at 60°C..
4. After the maceration, stain with Schiff's reagent.
5. Observe samples under a light microscope.

2.8.1.2. Flow cytometry analysis

1. Chop with a razor blade a cotiledonary embryo obtained from anther culture with a young leaf of the mother plant to release nuclei from the cells in isolation buffer (Partec).
2. Filter through a 30 mm nylon net in order to remove cell debris.
3. Mix the suspension of nuclei with a 2.5 fold volume of staining solution
4. Determine relative DNA content of the sample by a Partec (Münster, Germany) flow cytometer.

The presence on the DNA histogram of only one peak demonstrates the diploid status of regenerants.

2.8.1.3. **Isozyme analyses**

Because of the spontaneous diploidization of the haploid calluses, cytological analysis cannot always distinguish androgenic from somatic regenerants. Isozyme analyses have been employed to identify the origin of calluses and plantlets obtained from anther culture (Germanà et al., 1991; 1994; 2000a, b; Germanà and Reforgiato, 1997; Germanà, 2003a). Isozyme techniques allow us to distinguish between androgenetic and somatic tissue when the enzyme is heterozygotic in the diploid condition of the donor plant and the regenerants are missing an allele.

The crude extracts of embryoids and plantlets obtained are analysed using two enzyme systems: phosphoglucoisomerase (PGI) and phosphoglucomutase (PGM), as reported by Grosser et al. (1988). Numbering for isozymes (PGI-1) and lettering for different allozymes are the same as used by Torres et al. (1978).

C. aurantium and *C. reticulata* are heterozygous for PGI-1 and PGM. According to Torres et al. (1978), the heterozygous sour orange parent is FS (F= allele specifies fast migration toward the anode enzyme; S= allele specifies a slowly migrating enzyme) in PGM, and WS (W= allele specifies an enzyme migrating faster than F; S allele specifies a slowly migrating enzyme) in PGI. The heterozygous mandarin parent is FI (F= allele specifies fast migration toward the anode enzyme; I= intermediate) in PGM, and WS (W= allele which specifies an enzyme migrating faster than F; S= allele specifies a slowly migrating enzyme) in PGI. For analysis

of calli and leaves obtained from anther culture, the presence of a single band can be regarded as homozygous state. In the experiments with sour orange and mandarin anther culture, both enzyme systems of all examined samples confirmed the somatic nature of regenerants because of the contemporary presence of the alleles and the genetic similarity between the mother plants and the regenerants (Fig. 2G; Fig. 2H).

Materials and equipment required for electrophoretic separation of active enzymes from plant tissue: Distilled water, Vacuum apparatus, Refrigerator to house all gel units, Freezer (-10 to –20°C) for storing the stains and enzymes, Power supplies: BioRad Model 3000xi Computer Controlled Power Supply, Hot plate to heat starch solutions, pH Meter for preparing buffers and staining solutions, Balances, Gel apparati, Suction and volumetric flasks.

Plant material: Young, tender, fast growing leaves or root of the mother plants and embryogenic or non embryogenic callus o***r*** leaves regenerated from anther culture.

Stock solutions

L-Histidine buffer: L-Histidine 20.186 gr/l; Citric Acid 8.25 gr/l; Bring to volume with distilled water.
Tris HCl 1M: Trizma base 121 g/l; Dissolve in distilled water; pH 8 with HCl.; Bring to volume with distilled water.
Starch gel electrophoresis

Gel preparation

1. Mix in a suction flask the Histidine buffer (16 ml Histidine stock to 400 ml of distilled water) and the Starch hydrolized (40 g).
2. Heat the solution to boiling.
3. Immediately apply vacuum to remove excess gases. Continue to heat solution to boiling.
4. Pour gel into the tray.
5. Cover the gel with a glass plate to compress the partially set gel to a uniform thickness.
6. Allow to cool overnight.

Extraction

1. Macerate a small amount of tissue (~100 mg) directly into a filter paper little square (wicks) (3 MM Whatman chromatography).

Gel loading

1. Remove glass plate.
2. Trim the gel 4 cm from the cathodal end.
3. Separate the gel to facilitate the wicks insertion.
4. Insert a plastic straw at the anodal and cathodal end of the gel to introduce torsion.
5. Cover the gel with inert material and put the gel tray in contact with the buffer.

Electric field conditions: Costant mA 45; Time: 3 hours.

Gel slicing

1. Accomplish the longitudinal slicing using a taut, fine stainless or spring steel wire.
2. Move the wire slowly through the gel .
3. Place the slices in separate stains.

Staining solutions

Glucose Phosphate Isomerase (PGI)

- Tris HCl 1 M pH 8 5 ml
- $MgCl_2.6H_2O$ 50 mg
- Fructose-6-P 50 mg
- $NADP^+$ 5 mg
- MTT 10 mg
- PMS 2 mg
- Glucose-6-P DH 20 units Add just before incubating.
- Distilled water 50 ml

Procedure

Incubate the gel in the dark at 25°C for about 2 hours.
Stop reaction and fix in 10% glycerol.

Phosphoglucomutase

- Tris HCl 1 M pH 8 5 ml
- $MgCl_2.6H_2O$ 50 mg
- Glucose-1-P 250 mg
- $NADP^+$ 10 mg
- MTT 20 mg
- PMS 2 mg
- Glucose-6-P DH 20 units Add just before incubating
- Distilled water 50 ml

Procedure
Incubate the gel in the dark at 25°C overnight.
Stop reaction and fix in 10% glycerol.

3. APPLICATIONS

Embryogenic calli are essential for crop genetic improvement through biotechnology. Particularly, in citrus breeding, somatic calli have high value since they can be used for protoplast fusion (Grosser et al., 2000).

Somatic embryos are also particularly interesting because of their use as artificial seeds, for clonal propagation, *in vitro* germplasm preservation, genetic transformation, efficient plant breeding and for basic research in plant physiology and biochemistry (Senaratna, 1999).

3.1 Somatic embryo encapsulation

Synthetic seed technology may be of great value in breeding programs, allowing propagation of many elite genotypes in a short time. It has been developed using somatic embryos and/or other micropropagules like axillary shoot buds, apical shoot tips. They can be converted into plantlets under *in vitro* or *ex vitro* conditions with the advantages of the micropropagation added with the advantages of handling and storage of natural seeds. Furthermore it can be useful in conservation of endangered plants or to facilitate exchange of axenic material between laboratories (Pattnaik and Chand, 2000).

The advantages of synthetic seeds include ease of transport, longer periods of viability and the clonal nature of the plants obtained. Mycorrhizas, pesticides and fertilizer can be incorporated into the capsules to enhance germination rates and seedling growth.

Synthetic seeds have been produced by coating embryoids obtained by anther culture of mandarin Avana with calcium-sodium alginate gel beds (Fig. 3A; Fig. 3B) (Germanà et al., 1999). When embryos were sown non-encapsulated, encapsulated with a hormone-free artificial endosperm or encapsulated with an artificial endosperm added with giberellic acid (1 μM), percentages of conversion on medium culture were 15.0, 26.7 and 50.0 respectively. Encaspulated embryos were stored

at 4°C for 1 month without loss of vitality. Embryos sown on perlite showed a conversion rate of 25% if encapsulated or 5% if not.

Artificial endosperm: ½ MS basal medium added with 0.25 g/ l of malt extract, 20 g/l sucrose, and 4.5 g/l of galactose and 1µM of GA_3.
Na-alginate (medium viscosity) solution: 2.5% w/v
$CaCl_2$ solution: 1.1% w/v.

Procedure:

1. Mix embryos in 3% Na-alginate solution
2. Drop individually into calcium chloride
3. Wash with artificial endosperm
4. Storage at 4°C in Petri dishes wrapped with Parafilm

4. CONCLUSIONS

Efficient methods of regeneration *in vitro* as the somatic embryogenic cultures are the basis for modern plant biotechnology involving the possibility of applying innovative, rapid and efficient methods of plant propagation and improvement.

Anther culture represent in *Citrus*, a method to reach very quickly the homozygousity (Germanà, 1997; Germanà, 2003b; Germanà and Chiancone, 2003), as well as an efficient tool to obtain a source of highly embryogenic somatic calli (Germanà, 2003a). These technologies and the response to them are genotype-dependent and a number of investigations have to be carried out to improve the knowledge and the understanding of these processes.

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Photos of encaspulation end synthetic seed conversionhave been taken at the Dipartimento di Arboricoltura e Protezione delle Piante (Università di Perugia).

4. REFERENCES

Arrillaga I., Lerma V. Pérez-Bermudez P., Segura J. Callus and somatic embryogenesis from cultured anthers of service tree (*Sorbus domestica* L.). Hortscience 1995; 30: 1078-1079.

Canhoto J.M., Cruz G.S. Induction of pollen callus in anther cultures of *Feijoa sellowiana* Berg. *(Myrtaceae).* Plant Cell Reports 1993; 13: 45-48.

Cersosimo A., Crespan M., Paludetti G., Altamura M.M. Embryogenesis, organogenesis and plant regeneration from anther culture in *Vitis.* Acta Horticulturae. 1990; 280: 307-314.

Chen Z. A study on induction of plants from *Citrus* pollen. Fruit Var J 1985; 44-50

D'Amato F. Cytogenetics of differentiation in tissue and cell cultures, In: Reinert J., Bajaj Y.P.S. (eds), Applied and fundamental aspects of plant cell, tissue, and organ culture, 1977, pp. 343-356. Springer-Verlag, Berlin Heidelberg, New York.

Deng X.X., Deng Z.A., Xiao S.Y., Zhang W.C. Pollen derived plantlets from anther culture of Ichang papeda hybrids No.14 and Trifoliate orange. Proc. Intl. Soc. Citricult. 1992; 1:190-192.

Germanà M.A. Haploidy in *Citrus*. In: Jain S.M., Sopory S.K., Veilleux R.E. (eds), *In Vitro* Haploid Production In Higher Plants, 1997, Vol. 5, pp. 195-217. Kluwer Academic Publisher, Dordrecht, The Netherlands, London.

Germanà M.A. Somatic embryogenesis and plant regeneration from anther culture of *Citrus aurantium* and *Citrus reticulata.* Biologia, Bratislava. 2003a; 58/4:843-850.

Germanà M.A. Haploids and doubled haploids in *Citrus* spp. In: Maluszynski M., Kasha K.J.,Forster B.P., Szarejko I. Doubled haploid production in Crop Plants. A manual. 2003b, pp. 303-307 Kluwer Academic Publishers.

Germanà M.A., Crescimanno F.G. De Pasquale F., Wang Y.Y. Androgenesis in 5 cultivars of *Citrus limon* L. Burm. f. Acta Horticulturae 1991; 300: 315-324.

Germanà M.A., Wang Y.Y., Barbagallo M.G., Iannolino G., Crescimanno F.G. Recovery of haploid and diploid plantlets from anther culture of *Citrus clementina* Hort. ex Tan. and *Citrus reticulata* Blanco. Journal of Horticultural Science 1994; 69 (3): 473-480.

Germanà M.A., Reforgiato G. Haploid embryos regeneration from anther culture of 'Mapo' tangelo (*Citrus deliciosa* x *C. paradisi*). Advances in Horticultural Science 1997; 11: 147-152.

Germanà M.A., Piccioni E., Standardi A. Effects of encapsulation on *Citrus reticulata* Blanco somatic embryos conversion. Plant Cell Tissue Organ Cult 1999; 55, 235-237.

Germanà M.A., Crescimanno F.G., Motisi A. (2000a) Factors affecting androgenesis in *Citrus clementina* Hort. ex Tan. Advances in Horticultural Science 14: 43-51.

Germanà M.A., Crescimanno F.G., Reforgiato G., Russo M.P. Preliminary characterization of several doubled haploids of *Citrus clementina* cv. Nules. *Proceedings of the First International Symposium on Citrus Biotechnology*. Goren, R. and Goldschmidt, E.E. (eds) Eilat, Israel, Acta Horticulturae 535, 2000b, pp. 183-190.

Germanà M.A., Chiancone B. Improvement of *Citrus clementina* Hort. ex Tan. microspore-derived embryoid induction and regeneration. Plant Cell Report 2003; 22: 181-187.

Grosser J.W., Gmitter F.G., Chandler J.L. Jr. Intergeneric somatic hybrid plants from sexually incompatible woody species: *Citrus sinensis* and *Severina disticha.* Theor Appl Genet 1988; 75**:** 397-401.

Grosser J W, Ollitrault P, Olivares-Fuster O. Somatic hybridization in citrus: An effective tool to facilitate variety improvement. In Vitro Cellular and Developmental Biol-Plant 2000. 36 (6): 434-449.

Hammerschlag F.A. 1983. Factors influencing the frequency of callus formation among cultured peach anthers. Hortscience 18 (2): 210-211.

Hidaka T., Yamada Y., Shichijo T. *In vitro* differentiation of haploid plants by anther culture in *Poncirus trifoliata* (L.) Raf. Japan. J. Breed. 1979; 29: 248-254.

Jain S.M., Sopory S.K., Veilleux R.E. (eds), *In Vitro* Haploid Production In Higher Plants, 1997, Vol. 5, Kluwer Academic Publisher, Dordrecht, The Netherlands, London.

Lillie R.D. Simplification of the manufacture of Schiff reagent for use in histochemical procedures. Stain Tech 1951; 26: 163-165.

Mauro M. C., Nef C., Fallot J. Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet-Sauvignon. Plant Cell Reports 1986; 5: 377-380.

Mozsar J., Sule S. A rapid method for somatic embryogenesis and plant regeneration from cultured anthers of *Vitis riparia*. Vitis, 1994; 33: 245-246.

Murashige T., Skoog F.A. Revised medium for rapid growth and bioassays with tobacco tissue cultures. 1962; Physiol. Plant. 15: 473-497.

Narayanaswamy S., George L. Anther culture. In: Johri, B.M. (ed.), Experimental embryology of vascular plants. Springer-Verlag, Berlin. 1982 pp. 79-103.

Nitsch J.P., Nitsch C. Haploid plants from pollen grains. Science 1969; 163: 85-87.

Pattnaik S., Chand P. K. Morphogenic response of the alginate –encapsulated axillary buds from *in vitro* shoot cultures of six mulberries. Plant Cell Tissue Organ Cult 2000; 60, 177-185.

Perrin M., Martin D., Joly D., Demangeat G., This P., Masson, J.E. Medium-dependent response of grapevine somatic embryogenic cells. Plant Science 2001; 161:107-116.

Sangwan-Norreell, B.S. Male gametophyte nuclear DNA content evolution during androgenic induction in *Datura innoxia*. Z. Pflanzenphysiol. 1983; 111: 47-54 .

Senaratna T., Dixon K., Bunn E., Touchell D. Smoke saturated water promotes somatic embryogenesis in geranium. Plant Growth Regulators 1999; 28:95-99

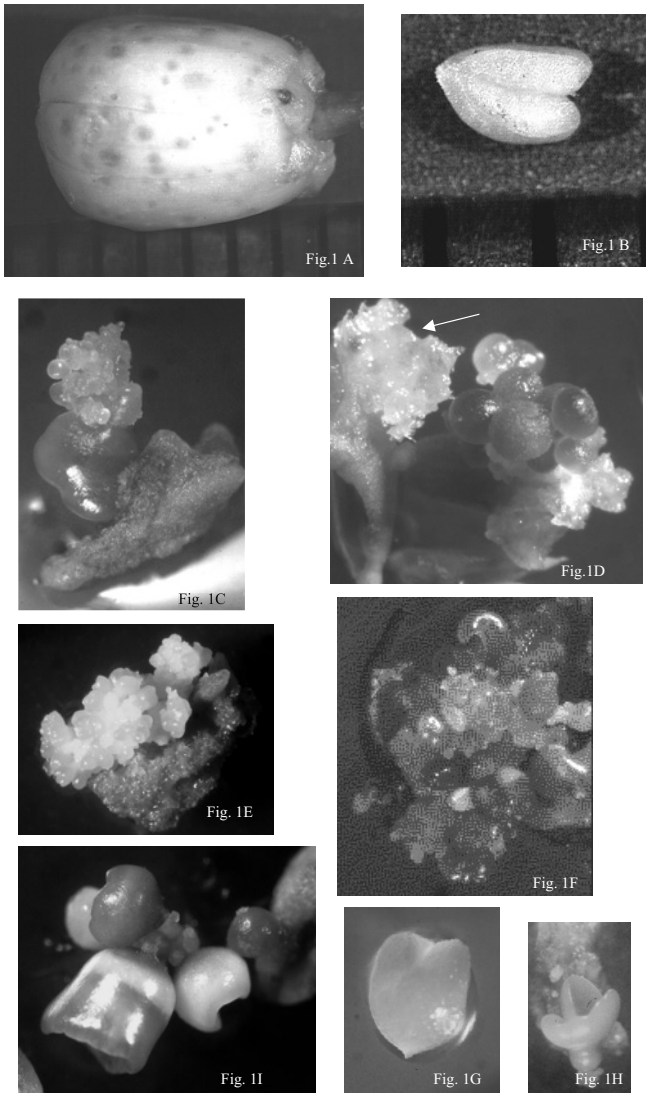
Shull J.K., Menzel M.Y. A study of the reliability of synchrony in the development of pollen mother cells of *Lilium longiflorum* at the first meiotic prophase. Am. J. Bot. 1977; 64: 670-679.

Sunderland N., Dunwell J.M. Anther and pollen culture, In: Street, H. E. (ed), Plant Tissue and Cell Culture, Blackwell, Oxford. 1977, pp 223-265.

Torregrosa, L. A simple and efficient method to obtain stable embryogenic cultures from anthers of Vitis vinifera L. Vitis 1998; 37**:** 91-92.

Torres, A.M., Soost, R.K., Diedenhofen, U. Leaf isozymes as genetic markers in *Citrus*. Amer. J. Bot. 1978; 65: 869-81.

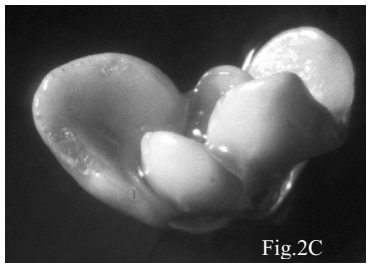
Vasil, I.K. Physiology and cytology of anther development. Biol. Rev. 1967; 42: 327-373.



(1A). Flower bud (6 mm) of mandarin Avana containing anthers with microspores at the uninucleate stage. (1B). Anthers of mandarin Avana containing microspores at the uninucleate stage before culture. (1C). Morphogenic, friable callus emerging after four months of culture from anthers of mandarin Avana. (1D). Non-morphogenic callus (arrow) from mandarin anther culture. (1E) Somatic embryogensis from sour orange AA CNR 10 anther culture. (1F). Cluster of embryoids in different stages of development emerging from a single MTC N.L.19 anther in culture. (1G) and (1H). Different developmental stages of somatic embryos. (1I). Secondary embryogenesis in sour orange AA CNR 23.


Fig.2A


Fig.2B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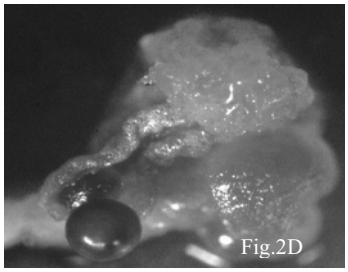
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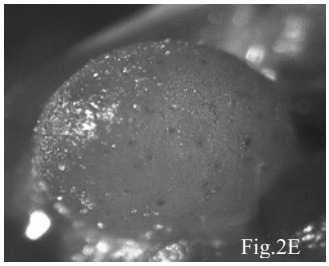
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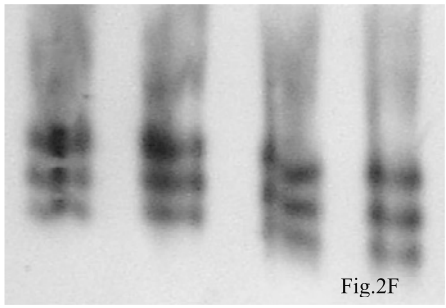
Fig.2F

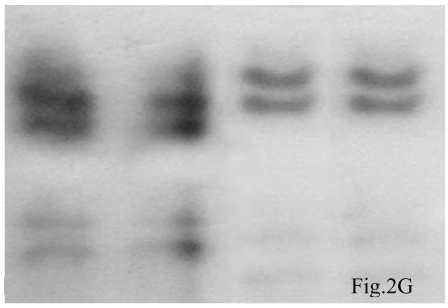
Fig.2G

(2A) Plantlet of MTC NL19 regenerated from anther culture. (2B). Cotyledonary-fused embryos in sour orange anther culture (2C). Pluricotyledonary embryoids from AA CNR 23 anther culture. (2D) and (2E) Pseudobulbils in grapefruit Star Ruby anther culture. (2G) and (2H) Isozyme pattern of PGI (Fig. 2G) and PGM (Fig.2H) of sour orange: the first and the third lanes on the left are respectively the zymograms of the A.A. CNR 10 and 23 mother plants; the other ones are those of the regenerants. (2F) Plants of mandarin MTC NL19 transferred to *ex vitro* conditions.


Fig.3A

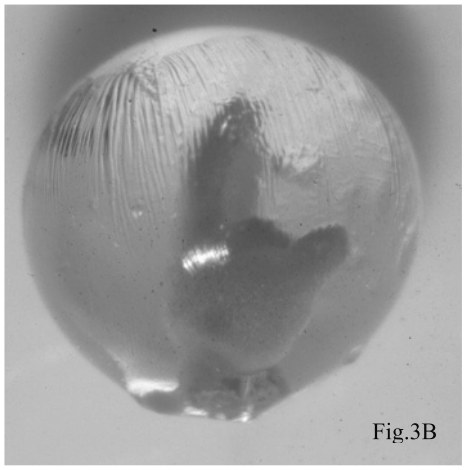
Fig.3B

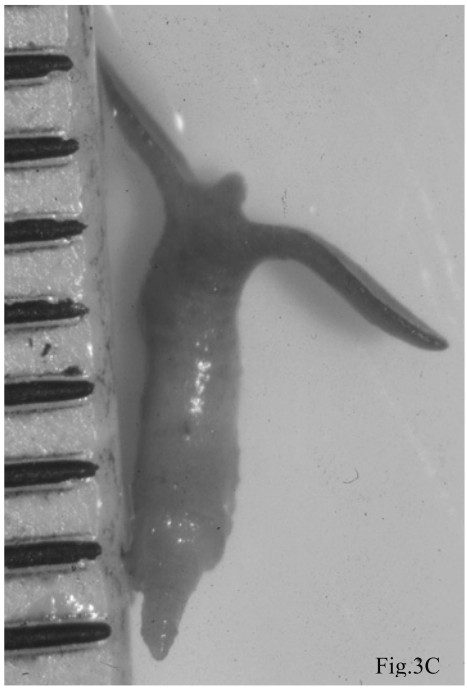
Fig.3C

(3A) Encaspulated somatic embryos of mandarin Avana regenerated from anther culture (3B). Encaspulated Avana somatic embryo conversion.

Table 1: Composition of *Citrus* anther culture media for somatic embryogenesis. Concentrations in mg l^{-1}.

Constituent	Induction Medium **M1**	Maintaining Medium **M2**	Germination Medium **M3**
Inorganic macro elements			
KH_2PO_4	68	170	170
KNO_3	950	1900	1900
NH_4NO_3	720	1650	1650
$MgSO_4 \cdot 7H_2O$	185	370	370
$CaCl_2 \cdot 2H_2O$	166	440	440
Inorganic micro elements			
$MnSO_4 \cdot H_2O$	-	-	-
$MnSO_4 \cdot 4H_2O$	25	22.3	22.3
H_3BO_3	10	6.2	6.2
KI	-	0.83	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$CoCl_2 \cdot 6H_2O$		0.025	0.025
$ZnSO_4 \cdot 7H_2O$	10-	8.6	8.6
Iron source			
$FeSO_4 \cdot 7H_2O$	27.8	27.8	27.8
$Na_2EDTA.2\ H_2O$	37.3	37.3	37.3
Vitamins			
Myo inositol	100	100	100
Glycine	2	2	2
Nicotinic Acid	5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5
Thiamine HCl	0.5	0.1	0.1
Biotin	0.05	-	
Folic acid	0.5	-	-
Ascorbic acid	500	-	-
Other substances			
Coconut water (Sigma, Chem.)	5%	-	-
Casein	400	-	-
Glutamine	200	-	-
Sucrose	34 000* 68 000** 102 000***	50 000	30 000
Growth regulators			
NAA	0.2	0.02	0.01
Kinetin	1.0	-	-
6-BA	0.5	-	-
ZEA	0.5	-	-
GA_3	0.5	-	1.0
Agar (washed from Sigma)	8,000	8,000	7,500
pH	5.8	5.8	5.8

Response to sucrose concentration is genotype-dependent: *AA CNR 23; ** AA CNR 10 and Mandarin Avana; *** Mandarin MTC

Abbreviations:; GA_3 - gibberellic acid; MS = Murashige and Skoog (1962); N6= Chu (1978), NAA = □-naphthaleneacetic acid; 6-BA = 6-benzylaminopurine; ZEA = zeatin.
MTC= Mandarino Tardivo di Ciaculli; A.A CNR= sour oranges selected by National Research Council.

INTEGRATED SYSTEM FOR PROPAGATION OF *THEOBROMA CACAO L.*

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1. INTRODUCTION

Theobroma cacao L. is a tropical tree, with origins in the Amazon basin, currently cultivated throughout the tropics to supply the global demand for cocoa, the main ingredient for the manufacture of chocolate. A high degree of yield variation is a general characteristic of cacao plantations worldwide, due in part to the predominant use of seed propagation, in this mostly self-incompatible and highly heterozygous tree crop (Figueira and Janick, 1995). A recent study of the early yield of five high producing cacao families grown in full sun in Puerto Rico indicated that 2 to 3% of the trees in a population accounted for more than 60% of the yield (Irizarry and Rivera, 1999).

Improvements made by plant breeders are often rapidly lost, as farmers propagate plants through seeds, and segregation results in a highly heterozygous population of plants. Vegetative propagation systems provides a means to capture such additive genetic gain, and has been used to some degree with cocoa with the development of grafting, and rooted cutting techniques. Modern biotechnology offers a suite of new approaches to speed up the development and deployment of genetically improved genotypes. Recently, research conducted at Plant DNA

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 209–227.

Technology, (Sondahl et al., 1994) CIRAD, Montpellier, France (Alemanno et al., 1996a, b, c; Alemanno et al., 1997); Nestles Inc., Tours, France; Almirante Cacao Research Station, Bahia, Brazil (Lambert et al., 2000); and at The Pennsylvania State University (Antúnez de Mayolo et al., 2003; Li et al., 1998; Maximova et al., 2002; Traore et al., 2003), has led to the development of efficient methods for somatic embryogenesis of cacao. The main advantages of tissue culture methods include the possibility of rapidly generating asexually propagated, uniform plants with highly valued genetic traits. Additionally, for cacao, somatic embryogenesis offers a system for clonal production of orthotropic plants with normal dimorphic architecture and taproot formation. The production and testing of disease free materials and germplasm conservation via cryo-preservation are other important potential contributions of plant tissue culture to the improvement, preservation and distribution of cacao germplasm, which is currently preserved only in living collections in the tropics.

Building on work of others, our group has optimized a protocol for cacao somatic embryogenesis using floral staminode and petal base explants. This system is relatively expensive, and thus its utility limited primarily as a research tool. To address this limitation, we have incorporated three propagation steps that increase the multiplication rate and thus reduce the cost of production of orthotropic plants. These steps include secondary embryogenesis (Maximova et al., 2002), micropropagation (Traore et al., 2003) and production of orthotropic rooted cuttings in non-sterile greenhouse or field conditions (Guiltinan et al., 2000). The sequential application of these systems could provide a low cost, rapid clonal multiplication system. This chapter will give detailed protocols for each of these methods. Updated and further information including PDF versions of our publications containing detailed photographs of the protocols can be found at our website (guiltinanlab@cas.psu.edu). Instructions for joining an email discussion group on this topic are also available at the web site. While we have extensively tested and verified these protocols in the lab and greenhouse, they are a work in progress; new adaptations will be posted at the web site. Furthermore, field-testing of plants produced by this method is underway to assess the long-term sustainability of these plants in field production conditions.

2. PRIMARY SOMATIC EMBRYOGENESIS FROM CACAO FLOWER EXPLANTS

2.1. Culture Conditions

Growth chambers: 25º-30ºC, cool white fluorescent lights with an intensity of 50-100 µmol*m^{-2}*s^{-1} measured at shelf height, 16/8 hour photoperiod (light/dark). Greenhouse conditions are maintained at: 60-65% humidity, 30ºC day, 24ºC night (+/-approx. 5ºC), 50% shade under full sun (250-500 µmol*m^{-2}*$s^{-1)}$, supplemental HID 400W sodium halide lights are utilized to supplement during low light periods and to extend photoperiod to 14 hours in short daylight periods of the year. Gentle horizontal airflow is applied continuously.

2.2. Simplified protocol for collection and surface-sterilization of flower buds from greenhouse or field near to the dissection site

Immature flower buds are collected early in the morning (between 8 and 9 a.m.) in clean containers containing cold water (Fig. 1). Prior to collection, observe and evaluate the growth and development of flower buds and select unopened flowers of medium-large size (relative to the size of the flowers on an individual tree). Flowers in advanced developmental stages or harvested in the afternoon often open during surface sterilization, contributing to contamination of the tissue culture explants. Surface-sterilize the flowers in a 1% (v/v) Bleach solution (Clorox—6% sodium hypochlorite) using sterile water in a sterile plastic or glass container (Fig. 1). Perform the following steps of this protocol inside sterile tissue culture transfer hood. Decant the cold water from the container and transfer all the flower buds into new container with Bleach solution. Immerse flowers in the Bleach solution and sterilize for 20 min. During the sterilization step assure contact of the solution with the flower buds. This can be achieved by gently inverting the tube a few times every 5 minutes. After 20 min, completely remove the Bleach solution and add 50 ml room temperature (RT) autoclaved water to rinse the flower buds. Invert tubes several times and decant water. Repeat the rinse with fresh water 2 more times. Decant the last rinse and remove as much water as possible. With sterile forceps

transfer flowers to a sterile Petri dish inside the transfer hood. Close the Petri dish to prevent desiccation of the flower buds and place in the hood until dissection as soon as possible (not longer than the end of the day).

2.3. Alternative protocol for collection, sterilization and transportation of material from field to the dissection site locations

If the flowers will not be dissected the same day, the flowers can be treated in the field with bleach then shipped in cold conditions. After arrival at a suitable laboratory within 3 days, the material is re-sterilized and dissected for culture induction.

Field Sterilization: Follow with the standard sterilization protocol but use water at 4ºC and maintain at 4ºC (keep on ice) throughout the procedure. For this pre-sterilization, filter sterilized tap water can be used without negative effect on the culture. After rinsing the flowers with 4ºC cold water three times decant the water and add 50 ml of 4ºC DKW basal, sugar and hormone media free medium (Table 1). Gently invert the tubes few times to wet the flowers. Pour out the liquid from the tube and drain for 5 seconds. Hold the tube with the sterile flowers horizontally and disperse the flowers across the tube so none of the flowers are bathed in any residual water. If possible performed the sterilization inside sterile tissue culture transfer hood. If the procedure is done on open air at the field site, repeating the procedure in sterile conditions prior to dissection may be necessary.

Shipping: Place the tubes with flowers in a horizontal position in a plastic bag. In the bottom of an insulated container place a bag of crushed ice and layer with few layers of paper towels or other absorbent material. Place the bag with the flower on the top of the paper towels and cover with more paper towels on the sides and the top. Place more bags with crushed ice on the sides and the top of the flowers keeping them separated from the ice with the paper towels. The temperature in the transportation container should be approximately 1ºC in the beginning and should not increase above 16-17ºC over the course of 3 days. After a maximum of 3 days the flowers are dissected following the standard protocol.

2.4. Dissection of flower buds and callus induction

Transfer 4-10 sterile flower buds to a sterile Petri dish. It is important not to transfer excess water from the tube when moving the flower buds to the new dish. The number of buds transferred depends on a personal preference and one's tissue culture skills. Prepare in batches that will take no longer than 5 min. to avoid desiccation of the cut surfaces. Slice the flower buds across at a position 1/3 of the flower length from the base using a sterile scalpel blade. A number 11 scalpel blade is recommended. Extract staminodes and petal tissues together through the opening at the cut end using sharp sterile forceps. Separate and discard the attached stamens and pistil. The two most commonly used explants for embryogenesis are staminodes and petal bases. Transfer staminodes and petal base explants into a Petri dish containing 25-30 ml of PCG medium (Li et al., 1998) (Table 1). Separate any fused staminodes and petal base explants and distribute explants evenly on the entire surface the medium (up to 50 explants per dish), assuring good contact of the explants with the medium without immersion. Separate the petal base explants and the staminodes into different Petri dishes to evaluate the regeneration potential of the individual tissues. Seal the Petri dishes with parafilm and maintain cultures in the dark at 25-30ºC for 14 days (cardboard box in a growth chamber works well). Follow with transfer of the explants to a Petri dish containing 25 - 30 ml of SCG medium (Maximova et al., 2002) (Table 1). Seal the dishes and maintain cultures in the dark for another 14 days. Callus formation should be apparent by the end of this culture period. At this step, good contact of the explants with the medium without immersion is important.

2.5. Somatic embryo induction and maintenance

After the first 4 weeks, transfer all explants to Petri dishes containing 30 ml of ED medium (Li et al., 1998)(Table 1). Incubate in the dark for 14 days. At this step the size of the individual explants will increase and calli will develop (Fig. 1). Two morphologically distinct types of callus are produced as a result of the induction. The first type, consist of elongated cells and appears white under a dissecting microscope. Somatic embryos almost never develop from this type of cell clusters. The second type consists of round cells and appears light to dark brown and friable. These cell clusters

were often found in association with somatic embryos (Maximova et al., 2002). Subculture explants onto fresh ED medium every 14 days, and maintain cultures in the dark. As the explants increase in size, reduce their number per plate to 20-25. During the ED culture period, large numbers of somatic embryos develop. The initiation of new embryos continues for up to 10 months and embryos at different developmental stages (globular, heart torpedo and mature) are present on individual explants at the same time. Cultures can be maintained on ED medium for up to one year. This is not a synchronous system; new embryos will continue to develop for about 8-10 months with maximum production at 24 weeks post culture initiation. At each subculture mature individual embryos could be selected and transferred to conversion medium (see section on conversion).

3. INDUCTION OF SECONDARY EMBRYOGENESIS

Secondary embryogenesis is used to increase greatly the number and quality of embryos produced (Maximova et al., 2002). For secondary embryogenesis, select recently matured, primary embryos with developed cotyledons (Fig. 1). Cotyledons with high embryogenic potential are light yellow or sometimes pink in color. Avoid using very young cotyledons, which are transparent/white or old, thickened hairy cotyledons with long, visible trichomes. The optimal time frame for secondary embryo initiation is 21 to 26 weeks after primary culture initiation (Maximova et al., 2002). Separate the cotyledons from the embryo hypocotyl and slice with a sharp scalpel blade (#10 or 11 is recommended) into 4 by 4 mm pieces. Culture the explants in a Petri dish containing 25 - 30 ml of SCG medium (Table 1). At this step good contact of the explants with the medium without immersion is very important. Seal the dishes and maintain cultures in the dark for 14 days. Callus formation should be apparent by the end of this culture period. Transfer cotyledon explants to Petri dishes containing 30 ml of ED medium and culture in the dark for 14 days. Subculture explants onto fresh ED medium every 14 days, and maintain cultures in the dark. Secondary embryos should form within 2 to 3 months after culture initiation with no callus or minimal callus development. The embryos produced by secondary embryogenesis are of better quality and have more synchronized development than the primary embryos (Maximova et al.,

2002). Maintain the individual embryos in the dark with a subculture interval of 14 days on ED medium until they reach maturity and are ready for conversion.

4. SOMATIC EMBRYO CONVERSION AND PLANT ESTABLISHMENT

Select mature somatic embryos (up to 2 cm in length) with distinctive cotyledons and an extended axis. Place the embryos on PEC medium (Traore, 2000) (Table 1) at a 90º angle, root tip first, up to the base of the cotyledons at a density of 6 to 10 embryos per Petri dish. For this step use deep Petri dishes (100 x 20 mm) with 30 ml PEC medium. Seal the culture dishes with parafilm and maintain cultures under light (16/8h photoperiod) at 25-30ºC. Subculture embryos to fresh PEC medium every 30 days until shoots with one or two leaves develop. Transfer shoot-producing embryos with 2 leaves of at least 1 cm in length and developed primary root into Magenta GA3 vessels or glass jars containing 143 ml of RD medium (Traore, 2000)(Table 1). Place the embryos root tip first, at a density of 4 to 5 embryos per vessel. Maintain cultures under light with a 16/8h photoperiod for 30 to 90 days. At this stage young plants are sensitive to ethylene buildup, which could cause leaf drop. The recommendation is to use magenta GA3 vessels with vented lids (Sigma Chemical, St. Louis, MO Cat. #C3430) and to seal the container with Petri seal (Diversified Biotech, Boston, MA) to prevent contamination. Transfer embryos to fresh RD medium every 2 months. Before transferring gently break up the new media with sterile forceps then insert plants into medium. To preserve the fragile roots, hold the plant with one pair of forceps, press the plant into the fresh media gently inserting the roots into the media. Occasionally, plantlets produce abnormal shoots, which continue to produce cotyledon like leaves with long stems/internodes. To promote normal development of these plantlets, prune (decapitate) with a sharp scalpel by cutting the apical shoot approximately 0.5 cm above the first node. If the shoot is extraordinarily long, decapitate between any of the lower nodes, assuring the presence of at least one node for adventitious shoot proliferation (Traore et al., 2003).

Plants that develop at least 4-5 leaves (each greater than 2 cm long) and healthy primary and secondary roots (more than 2 cm long) are ready for acclimation in the greenhouse (Fig. 1). Transfer these plants into D40 Deepots (plastic, 62.5mm x 250mm; Hummert, Earth City, MO, USA) or bags containing autoclaved, pre-moistened soil with good drainage or pure sand. Saturate the soil mixture/sand with water prior to planting. Observe the plants every day during the first week and water carefully or as needed. If sand is used, it is best to set up a drip irrigation system programmed to water the plants 5 or more times a day. However, it is important not to water excessively, since at this stage the plants are very sensitive to over watering, which reduces oxygen access to the roots. Immediately after transplanting, it is necessary to start application of diluted fertilizer with each watering. Our fertilizer of choice is 1/10 Hoaglund's solution (Table 1), however, other fertilizers may be employed. The young plants are placed on a misting bench with 100% humidity and 70% shade (approximately 100 $\mu mol*m^{-2}*s^{-1}$), and are misted for 10 seconds every 15 minutes. After 4 weeks they are transferred and maintained to maturity at 60-65% humidity with 50% shade (250-500 $\mu mol*m^{-2}*s^{-1}$). By the end of the acclimatization period the plants should reach approximately 5-10 cm in height. At this point, the rate of growth should be similar to a seed grown plant. The acclimatization and/establishment periods vary for individual plants dependent on the quality of the embryos and the condition of the plantlets (number of roots, leaves and height) at the time of the transfer to the greenhouse. Plantlets produced by this method have been grown to maturity in greenhouse conditions (Fig. 1). Bare rooted plantlets up to 12 inches in length have been transported to field conditions and also grown to maturity (Fig. 1).

5. MICROPROPAGATION OF SOMATIC EMBRYO-DERIVED PLANTS

Somatic embryos can be micropropagated *in vitro* to increase the number of plants of an individual clone. This system was previously described in details (Traore et al., 2003).

5.1. Harvest of micro cuttings and root induction

For apical and nodal stem micro-cuttings, select healthy somatic embryo-derived plantlets grown on RD medium with developed roots and leaves. Plantlets ready for micropropagation should have green healthy leaves at least 3 cm long. Young purple leaves often drop after the micropropagation, reducing the rooting ability of the shoots. Identify a cutting point at an internode approximately 1 to 2 cm below the apex and the first 2 or 3 leaves of the shoot. If the shoots have more than 5 or 6 nodes and leaves, nodal micro cuttings can also be harvested and rooted successfully. Using a sterile scalpel, trim only the green leaves above the cutting point to1/3 of their size. Holding the tip of the shoot with sterile forceps cut the stem at the selected point/s. (see section below for further instructions on culturing the remaining stock plantlet). Transfer micro-cuttings into a Magenta box or glass jar containing 50 ml or less of root induction (RI) medium (Table 1) (Traore et al., 2003). The depth of the medium in the container needs to be 1 cm or more. Place the micro cutting vertically with the basal cut end immersed 3 to 5 mm into the medium. Root induction period varies from 48h to 7 days depending on the genotype. Incubate cultures under light (16/8 h photoperiod). Longer exposure to IBA could cause formation of excessive callus and/or a large number of roots with stunted growth, although some genotypes may require an extended root induction period.

5.2. Root development, maintenance and plant acclimation

After the root induction period, transfer explants into a Magenta box or glass jar containing 140-150 ml of RD medium (Table 1). Assure that the basal ends of the micro-cuttings are always immersed in the medium. Incubate under light (16/8 h photoperiod) and transfer explants as necessary every 2 months into fresh RD medium. Roots emerge and grow 2-3 cm within 30 - 40 days after root initiation. Some plants fully develop within the two-month period and can be transferred to the greenhouse. For acclimation of these plants follow the protocol described above (Sec. 4). Plants produced via orthotropic micro cuttings have a tendency to jorquette prematurely when established in the greenhouse. If this is undesirable the jorquette height can be corrected by removing/decapitation of the apical,

jorquette bud/s as soon as it appears. Following removal, an axillary orthotropic shoot from below the cut will be released and will grow to achieve a standard height (1 – 1.5 m) prior to the appearance of the next jorquette (Bertrand and Agbodjan, 1989).

5.3. Stock plant maintenance for continuous micro cutting production

Prepare fresh RD medium and pour 140-150 ml into Magenta GA3vessels or glass jars. Before transferring decapitated stock plantlets, gently break the surface of the medium with sterile forceps. Pick up the decapitated plantlets by the stems with sterile forceps and slowly pull out of the old medium. Place on the surface of the fresh RD medium. Hold the roots close to the tip with second pair of sterile forceps and insert into the fresh medium until the roots are entirely immersed into the medium. Maintain cultures under light (16/8h photoperiod) at 25 -30°C for 14 days. New axillary shoots develop and are ready for harvest in 6-8 weeks after previous harvest. When the shoots grow to approximately 3 cm in height, harvest the cuttings and proceed using the above-described protocol for rooting. It is important to harvest the new shoots regularly to prevent the re-establishment of apical dominance and to promote axillary bud proliferation. It is not unusual that after decapitation one or more newly developed shoots grow faster and re-establish themselves as dominant shoots.

6. PRODUCTION AND ROOTING OF ORTHOTROPIC CUTTINGS UNDER GREENHOUSE CONDITIONS

Once orthotrpic plants have been acclimated and are growing well in the greenhouse, a further amplification step can be applied using single node stem segments to produce rooted cuttings. Plantlets produced with this method grow similar to seedlings, with a orthotropic growth phase followed by jorquetting and transition to plagiotropic adult growth phase. This step can be performed at low cost, greatly reducing the overall

expense of an integrated orthotropic propagation system starting from somatic embryos. These plants can be used as self-rooted stock, for budwood or as root stock as required.

6.1. Stock plant establishment

Juvenile somatic embryo-derived plants at sapling stage (at or near jorquette height) should be used as "bentwood" stock plants for generation of orthotropic cuttings for rooting. Prior to bending the stock trees, prune the jorquette branches down to only 1-2 cm of their length. This promotes further the release of dormant axillary orthotropic meristems leading to greater orthotropic shoot production. With the pots remaining upright, arch the plants over so that the main trunk is at a horizontal position and secure the shoots with a metal hook or rope. The stock plants can be planted and established directly in the ground with the same success. Many orthotropic shoots should begin to proliferate in 1 – 2 weeks along the topside of the trunk length near the basal, curving end. Two to three months after bending, these orthotropic shoots should achieve 0.7 – 1.0 m in height and be ready for harvest.

Alternatively stock plants for orthotropic shoots can be established without bending the stems. For this method newly acclimatized SE-derived plants are grown in the greenhouse in D40 Deepots (plastic, 62.5mm x 250mm; Hummert, Earth City, MO, USA) for approximately 6 months until they are 3-4 feet tall, but without jorquette. At that time excise the apical shoot leaving short stump with only 4-6 leaves. The excised shoot can be segmented into 4 cm single leaf cuttings for rooting as described above. New axillary shoots are released from the remaining stock plant and cuttings are produced and rooted following the above-described protocol. The advantage of this method compared to the "bentwood" garden is that the area for stock plant maintenance is reduced. However, since the stock plants initially have fewer axillary buds, our observations are that they produce fewer shoots (data not published).

6.2. Rooted cutting production

Excise the shoots at the base of the stem, close to the trunk of the stock plant. After the first and the consequent harvests new shoots are generated at or near the cut. Hence the stock plants can be harvested repeatedly. Excise hardened, semi-hardened and dark green orthotropic shoots early in the morning while leaf water potential is still high (Fig. 2). Cuttings from shoots, at all developmental stages root well, but the semi-hardwood cuttings showing beginnings of characteristic browning of the stem have demonstrated the highest rooting potential (Guiltinan et al., 2000). Prune all leaves to 1/3 of the length. Make single leaf cuttings with stems approximately 4 cm long. Stems should be cut such that 0.5 – 1 cm remains above the node/leaf axils and 2 – 3 cm of the stem remains below the node. If the internodes are shorter than 2-3 cm, still make the cuttings 4 cm long, but remove the lower leaves completely. Dip the base of the stems in rooting solution for 5 seconds (Fig. 2). The quick dip method as described by Evans has been quite successful for the propagation of single-leaf cuttings under our conditions (Evans, 1951). Rooting solution consists of a 1:1 mixture of □-Naphthalene acetic acid (NAA) and □ - Indole-3-butyric acid (IBA) at a total combined concentration of 4 g/L. The hormones are dissolved in 50% ethanol and the solution should be prepared fresh prior to treatment. After hormone treatment, insert the cutting in to a 0.5 cm wide and 3 cm deep opening in wet sand or rooting media of choice (Fig. 2). Gently press the sand around the stem of the cutting. Any well draining growth media could be used for this rooting method, however fine play sand or a 1:1 mix of sand and soil has demonstrated excellent results in greenhouse conditions. Place the new cuttings under intermittent mist (10 sec. every 10 or 15 min.) or a fog for 4-6 weeks. Relative humidity should be maintained at or near 100%, keeping the leaves wet without inundating the cuttings. Light intensity should remain approximately 100 μmol * m^{-2} * sec. $^{-1}$ PAR, or 85% shade. During the misting period water the cuttings with fertilizer every 3-4 days. A Hoaglund's fertilizer at 150ppm nitrogen works well. The first roots will appear at approximately 3 weeks after hormone treatment. By the end of the 6-week misting period all cuttings with rooting potential should form roots. When using play sand as a rooting media and applying the described conditions we routinely produce rooted cuttings with 90-95% success (Carter Miller, data not published).

6.3. Acclimation, establishment, and general cultural management for plants produced by rooted cuttings methods

Any rooted cutting with 2 or more roots and a growing axillary bud may be transplanted to plastic bags or pots with soil (Fig. 2). After transplanting, the plants are removed from the mist and should remain under 50% shade, and RH of 60-65%. Water and fertilizer are applied as needed. Best results are observed when drip irrigation system is used and the plants are fertilized 5 or more times daily. It is important when watering not to damage the new tender shoots. After the first flush has hardened the plants can be transferred to the greenhouse, shade house or field with increased light and lower RH conditions. If cuttings jorquette prematurely, the jorquette bud may be removed as soon as it appears similar to method applied for plants produced via micropropagation.

7. CONCLUDING REMARKS

Since the early work of cacao researchers in the late 1800s, propagation systems have been an important tool, enabling the multiplication of wild or breeding genotypes for distribution, germplasm collection and for replicate performance trials. Rooted cutting and grafting have been used throughout the world for propagation of cacao, however to date, a large percentage of production stocks are still grown from seed. In the 50s and 60s plant tissue culture methods were developed for the propagation of a wide variety of species, but were not applied to cacao until the late 70s, and then, with very limited success.

The protocol presented here is the current optimization of the various experiments we and others have performed on cacao somatic embryogenesis (see introduction). In addition, we also present a number of related propagation systems (Guiltinan et al., 2000). Although the somatic embryogenesis technology has been successfully utilized in multiple research laboratories around the world these methods should be regarded as a work in progress. To date, embryos have been produced from more than 100 different *Theobroma cacao* genotypes at multiple locations including laboratories in Ghana, Ivory Coast, Brazil, Malaysia, Puerto Rico and

others.

However, the initial protocol was developed and optimized for Scavina-6 genotype only (Li et al, 1998, Maximova et al., 2002). We have observed that there are genotypic variations observed in the response to the protocol. In most cases genotypes with Scavina-6 parentage respond very well and produce a large number of embryos. Other genotypes produce very few embryos during primary embryogenesis, but the number dramatically increases during secondary embryogenesis. Finally, there are genotypes that need further optimization of the conditions to produce embryos. While these methods are potentially very powerful, we have estimated that by using them it is possible to produce 800,000 plants from a single flower in two years, it should be noted that this is new technology that has yet to be fully tested in the field. Although our early field tests in Saint Lucia, Puerto Rico and Brazil are promising, the use of plants for large scale propagation and production must clearly await full validation of the methods through large scale field testing over multiple harvests and in several environments and with multiple genotypes. Our group and others are beginning to establish such tests. We invite our partners, the readers of this manuscript, to work together to set up additional field tests to help validate this system.

ACKNOWLEGEMENTS

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8. REFERENCES

Alemanno, L., M. Berthouly, and N. Michaux-Ferriere, 1996a, Embryogenese somatique du cacaoyer a partir de pieces florales: Plantations, recherche, developpemant, p. 225-233.

Alemanno, L., M. Berthouly, and N. Michaux-Ferriere, 1996b, Histology of somatic embryogenesis from floral tissues cocoa: Plant Cell, Tissue and Organ Culture, v. 46, p. 187-194.

Alemanno, L., M. Berthouly, and N. Michaux-Ferriere, 1996c, Somatic embryogenesis of cocoa from floral parts: Plantations, Recherche, Developpement, v. Juillet-Aout.

Alemanno, L., M. Berthouly, and N. Michaux-Ferriere, 1997, A comparison between *Theobroma cacao* L. zygotic embryogenesis and somatic embryogenesis from floral explants: In Vitro Cell Dev Biol-Plant, v. 33, p. 163-172.

Antúnez de Mayolo, G., S. N. Maximova, S. Pishak, and M. J. Guiltinan, 2003, Moxalactam as a counter-selection antibiotic for Agrobacterium-mediated transformation and its positive effects on *Theobroma cacao* somatic embryogenesis: Plant Sci, v. 164, p. 607-615.

Bertrand, B., and A. K. Agbodjan, 1989, Propagation of cocoa by orthotropic cuttings. Initial results and prospects: The Cafe Cacao, v. 33, p. 147-156.

Evans, H., 1951, Investigations on the propagation of cacao: Tropical Agriculture, v. 28, p. 147-203.

Figueira, A., and J. Janick, 1995, Somatic embryogenesis in cacao (*Theobroma cacao* L.), *in* S. Jain, P. Gupta, and R. Newton, eds., Somatic Embryogenesis in Woody Plants, v. 2: Netherlands, Klumer Academic Publishers, p. 291-310.

Guiltinan, M. J., C. Miller, A. Traore, and S. Maximova, 2000, Greenhouse and field evaluation of orthotropic cacao plants produced via somatic embryogenesis, micro and macropropagation: 13th International Cocoa Research Conference.

Irizarry, H., and E. Rivera, 1999, Early yield of five cacao families at three locations in Puerto Rico: J. Agric. Univ. P.R., v. 82, p. 167-176.

Lambert, S. V., M. Guiltinan, S. Maximova, and W. M. Aitken, 2000, *Ex vitro* propagation via mini-cuttings of acclimatized cocoa plants issued from somatic embryogenesis: 13th International Cocoa Research Conference.

Li, Z., A. Traore, S. Maximova, and M. J. Guiltinan, 1998, Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao L.*) using thidiazuron: In Vitro Cell. Dev. Biol. - Plant, v. 34, p. 293-299.

Maximova, S. N., L. Alemanno, A. Young, N. Ferriere, A. Traore, and M. Guiltinan, 2002, Efficiency, genotypic variability, and cellular origin of primary and secondary somatic embryogenesis of *Theobroma cacao* L.: In Vitro Cell. Dev. Biol. - Plant, v. 38, p. 252-259.

Sondahl, M. R., T. B. Sereduk, Z. Chen, C. M. Bellato, S. J. Liu, and C. H. Bragin, 1994 Somatic embryogenesis and plant regeneration of cacao. US Patent #5.312.801.

Traore, A., 2000, Somatic embryogenesis, embryo conversion, micropropagation and factors affecting genetic transformation of *Theobroma cacao L*.: PhD thesis, The Pennsylvania State University, University Park.

Traore, A., S. N. Maximova, and M. J. Guiltinan, 2003, Micropropagation of *Theobroma cacao* using somatic embryo-derived plants: In Vitro Cell. Dev. Biol. - Plant, v. 39, p. 332-337.

Table 1. Composition of media used for cacao somatic embryogenesis.

Medium components	PCG mg/L	SCG mg/L	ED mg/L	PEC mg/L	RI mg/L	RD mg/L	Hoagland's Fertilizer mg/L
NH_4NO_3	1416	400	1416	1416	708	708	-
$Ca(NO_3)_2.4H_2O$	1969	-	1969	1969	984.5	984.5	-
$CaCl_2.2H_2O$	149	-	149	149	74.5	74.5	-
$CaCl_2$	-	72.5	-	-	-	-	-
$Ca(NO_3)_2$	-	386	-	-	-	-	47
K_2SO_4	1559	990	1559	1559	779.5	779.5	-
$MgSO_4.7H_2O$	740	-	740	740	370	370	12
$MgSO_4$	-	180.7	-	-	-	-	-
KH_2PO_4	265	170	265	265	132.5	132.5	-
$(NH_4)_2SO_4$	-	-	-	-	-	-	1.65
$NH_4N_2PO_4$	-	-	-	-	-	-	8.6
$Zn(NO_3)_2.6H_2O$	17	-	17	17	8.5	8.5	-
$MnSO_4.H_2O$	33.4	22.3	33.4	33.4	16.7	16.7	0.03
$CuSO_4.5H_2O$	0.25	0.25	0.25	0.25	0.125	0.125	$1.25X10^{-2}$
H_3BO_3	4.8	6.2	4.8	4.8	2.4	2.4	0.15
$Na_2MoO_4.2H_2O$	0.39	0.25	0.39	0.39	0.195	0.195	-
$FeSO_4.7H_2O$	33.8	27.8	33.8	33.8	16.9	16.9	-
Na_2-EDTA	45.4	37.3	45.4	45.4	22.7	22.7	-
KCL	-	-	-	-	-	-	0.37
$ZnSO_4.7H_2O$	-	8.6	-	-	-	-	0.06
$(NH_4)_6MO_7O_{24}$	-	-	-	-	-	-	0.06
Fe-EDTA	-	-	-	-	-	-	1.84
myo-Inositol	200	100	100	100	-	-	-
Glutamine	250	-	-	-	-	-	-
Thiamine-HCL	2	10	2	2	-	-	-
Nicotinic acid	1	1	1	1	-	-	-
Glycine	2	-	2	2.187	-	-	-
Pyridoxine	-	1	-	-	-	-	-
Arginine	-	-	-	0.435	-	-	-
Leucine	-	-	-	0.328	-	-	-
Lysine	-	-	-	0.456	-	-	-
Tryptophane	-	-	-	0.511	-	-	-
KNO_3	-	-	-	300	300	300	30
Glucose	20,000	20,000	1000	20,000	10,000	10,000	-
Sucrose	-	-	30,000	10,000	5000	5000	-
2,4-Dichloro-phenoxyacetic Acid	9.05uM	9.05uM	-	-	-	-	-
6-Benzylamino-purine	-	.22uM	-	-	-	-	-
Indole-3-Butyric Acid	-	-	-	-	12.4uM	-	-
Thidiazuron	.023uM	-	-	-	-	-	-
Phytagel	2000	2200	2000	1750	1750	1750	-

All chemicals are purchased from Sigma Chemical Co., St. Louis, Mo.
Abbreviations: PCG – Primary Callus Growth; SCG – Secondary Callus Growth; ED – Embryo Development; PEC – Primary Embryo Conversion; SEC – Secondary Embryo Conversion; RI – Root Induction; RD – Root Development.

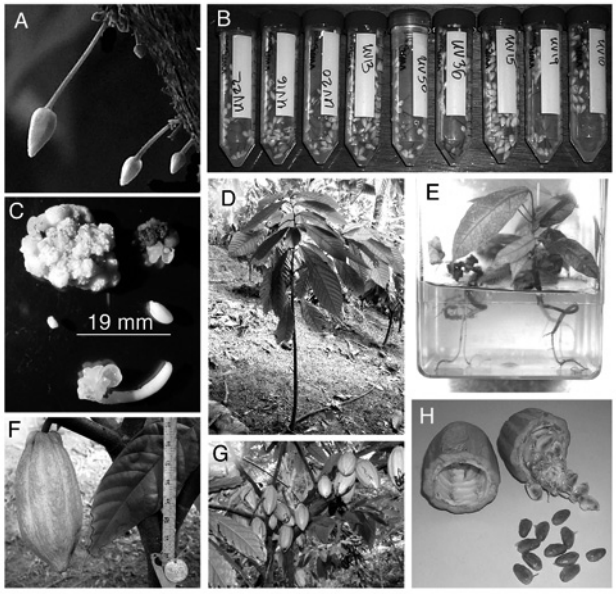

Figure 1. Production of cacao somatic embryos. A, Immature flowers prior to collection. B, Immature flowers in sterilization and shipping containers. C, Stages of somatic embryogenesis, top left, unproductive white callus, top right, dark callus with developing somatic embryo, middle left, globular somatic embryo, middle right, torpedo somatic embryo, bottom, mature cacao somatic embryo. D, Somatic embryo-derived plant in the field. E, Somatic embryo plantlet after conversion ready for acclimation in greenhouse. F-H, Fruit and seeds from somatic embryo derived plants grown in field conditions.

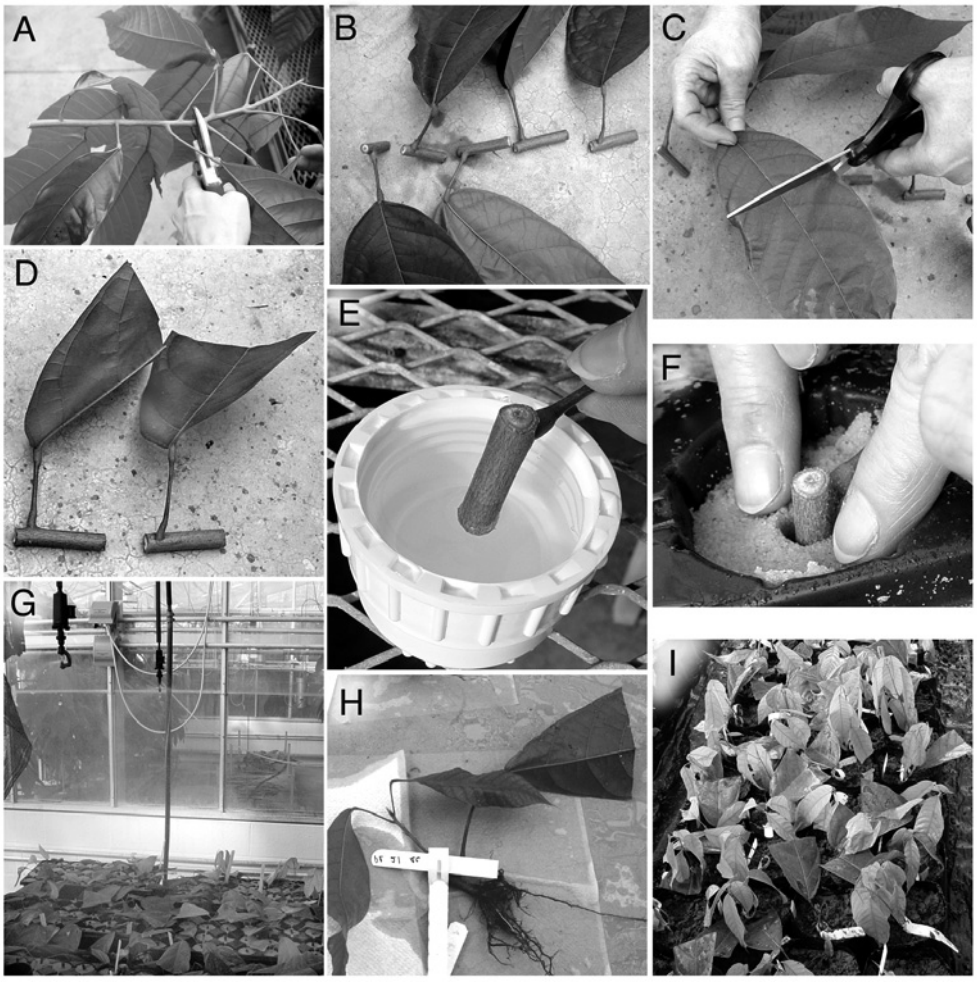

Figure 2. Production of single node rooted cutting from somatic embryo derived cacao plantlets. A, Selection of semi-hardwood stem sections (left) discard still fully green segments (right). B, Single node cuttings from stem sections 6-10 mm diameter. C, Cutting of leaves to 1/3 of original size. D, Single node segments with trimmed leaves. E, Dipping of stem segment in liquid root induction hormone solution. F, Placing a cutting in sand rooting media. G, Misting of cuttings in greenhouse. H, Finished rooted cutting ready for planting. I, Bed of rooted cuttings in containers in shade house prior to field planting.

MANGO (*MANGIFERA INDICA* L.)

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1. INTRODUCTION

The mango (*Mangifera indica* L. Family-Anacardiaceae), one of the most important fruit crops of the world, is also acknowledged as the King of Fruits of India (Majumdar and Sharma, 1990). Since time immemorial the mangoes have been the favourite of kings and commoners alike in India for their luscious taste and captivating flavour. The mangoes are woody trees of the tropics, which can attain a height of 40 m or more with large canopy (Singh, 1990). They grow well on a well-drained alluvial or lateritic soil with a range of pH 5.5 to 7.5, and a water table below 180 cm around the year.

Mango cultivars fall into two broad categories: monoembryonic and polyembryonic. In India about thousand mango cultivars are known to exist. Almost all the commercial cultivars of *M. indica* are monoembryonic, whereas, the polyembryonic cultivars are about a dozen only and are much inferior in fruit size and quality (Mathews and Litz, 1992).

The mangoes have a natural distribution throughout South-East Asia. However, they are grown in all tropical and sub-tropical regions of the world (Litz *et al*., 1995). Polyembyronic cultivars are predominately grown mainly in South-East Asia, Central America, Haiti, and USA, Australia, and South Africa; whereas, monoembryonic cultivars are in India, South America, Africa and Florida (USA) (Mathews and Litz, 1992). Major mango producing countries are India, Mexico, Pakistan, Thailand, China, Brazil, Philippines and Indonesia. World production of the mango fruits is approximately 15.7 million tonnes/annum and

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out of which India contributes 9.5 million tonnes/annum (Kendurkar *et al*., 1995).

Mango fruits have been an esteemed item of diet and are put to multifarious uses right from the first stage of development to maturity and ripening stage. The unripe mangoes are rich in vitamin C and the ripe fruits are rich in provitamin A and contain moderate levels of vitamin C (Lakshminarayana, 1980). Besides fruits, various other parts of the tree e.g. wood, bark, dried flowers, leaves, etc. are also put to several domestic and industrial uses.

Since mango is a cross-pollinated crop, enormous variations exist in the seedlings raised even from the fruits of a single tree. Mango breeding is impractical due to long juvenile period (6-8 years approximately), alternate bearing, large number of flowers within a panicle, disproportionate number of staminate flowers, a high frequency of premature flower and fruit drop, and inefficiency of fruit set (Majumdar and Sharma, 1990; Mathews and Litz, 1992).

Mango cultivars are extremely heterogenous due to lack of systematic breeding efforts and allogamous and allotetraploid nature (Mathews and Litz, 1992). The seedling trees produce heavy crops but the fruit size and quality are, in general, much inferior. The monoembryonic cultivars do not come true- to -type from seeds and therefore, are vegetatively propagated. The conventional methods are labour intensive and time consuming.

In this chapter we have described protocols for (a) plant generation via somatic embryogenesis from nucellus (b) plant regeneration from protoplasts and (c) plant regeneration from encapsulated somatic embryos of two important varieties of mango-Amrapali and Chausa (Ara *et al*., 1999, 2000 a, b).

The cultivar Amrapali is a hybrid scion cultivar, which is distinctly a dwarf variety, highly regular and prolific in fruit bearing and has excellent fruit quality. The tree flowers in the first week of March. Anthesis and fruit set occur simultaneously which extend up to 2nd or 3rd week of March. The fruitlets develop and grow about 1 cm long during first week of April. The size of fruitlets increases continuously.

The fruitlets from end of 2nd week to middle of 4th week of April are used for experiments.

The cultivar Samar Behisht Chausa is a superior chance seedling variety of North India (Singh, 1990). This mango variety is one of the sweetest mango varieties and it lacks acidity. The tree flowers in the last week of February. Anthesis and fruit set extend up to 1st or 2nd week of March. The fruitlets for experiments are collected from 2nd to 3rd week of April.

2. MATERIALS

1. Sodium hypochlorite (0.1% v/v**),** Tween 20 detergent, 2-3% Cetrimide solution, Sterile double distilled water, tap water, Hg Cl_2 solution (0.05% w/v)
2. Flow hood, sterile petri plates, pipettes, test tubes, forceps, scalpel, Erlenmeyer flasks, aluminium foil, cotton plugs
3. Immature fruitlets
4. Media (see Tables 1-3)

Basic medium composition is listed in Table 1.Required modifications for each culture stage is listed in Tables 2 and.3. Mix all the ingredients accordingly and bring the media to its volume, adjust the pH to 5.80 ± 0.05,add gelling reagent, heat up to boiling to melt the gelling reagent prior to autoclaving at 108kPa, 121° C for 15 min. Pour 8-10 ml medium in test tubes (150 x25 mm) or 15-20 ml / petri plates (100x17 mm).

3. METHOD

A. Somatic embryogenesis from nucellus

The regeneration method can be divided into seven steps viz (i) explant sterilization and initiation of cultures, (ii) initiation of embryogenic callus, (iii) culture maintenance (iv) embryo development ,(v) embryo maturation (vi) embryo germination and conversion into plantlets and (vii) acclimatization and field transfer.

(i) Explant Sterilization and Initiation of Cultures

In vitro culture establishment of mango, like other woody tree species, is very difficult primarily because of fungal and bacterial contamination. Hence, it requires a very careful sterilization before culture. A method of surface sterilization of the mango fruitlets according to Litz *et al*. (1982), Ara (1998) and Ara *et al.* (2000 a) is given below:

1. Wash the fresh immature mango fruitlets in running tap water for at least 20 min.
2. Surface sterilize the washed fruitlets by dipping them in a solution containing 0.1% (v/v) sodium hypochlorite, 2-3 drops of tween-20 per 250 ml water, and 2-3% (v/v) cetrimide solution prepared in tap water for 15-20 min and shaking continuously.
3. Wash the fruitlets again under running tap water for 20-30 min in order to remove the chemicals.
4. Cut the surface sterilized fruitlets into two equal halves under aseptic conditions inside laminar flow hood with the help of sterilized stainless steel scalpel and take out the ovules and collect them in a 150 ml flask containing 25 ml sterilized double distilled water
5. Rinse them in 0.05% (w/v) $HgCl_2$ solution for 3 min and wash thoroughly with sterilized double distilled water.
6. Dissect the ovules carefully, discard the zygotic embryo from each ovule and use the ovular half possessing only nucellar tissue for initiation of culture.
7. Place the intact nucellus explants in such a way that the nucellus tissue is in contact with the surface of the induction medium (modified MS medium, Tables 1&2) for callus initiation or detach fleshy layer of the nucellus from inner side of the ovular half to get the excised nucellus explant (Fig 1A) and place them in culture vessels containing the induction medium(modified MS medium) for callus initiation (Tables 1&2).
8. Incubate the cultures in the dark at 25 ± 2°C.
9. Transfer the cultures of intact nucellus explants to fresh medium of same composition at one or two day intervals in the first week of inoculation and later at 7 to 10 day intervals to avoid necrosis

of explants due to phenolic leaching. When excised nucellus explants are used, transfer explants to a fresh medium at 7-10 day regular intervals to overcome phenolic leaching.

(ii) Initiation of Proembryogenic Callus:

Callus initiation from the excised and intact nucellus tissues starts in the 3 rd week of inoculation on modified MS medium (Table 2) and continues up to 5th week.The initial calli are moist and dark brown or black. Induction of pale yellow proembryogenic calli (PEC) occurs only from the moist-friable calli.

Transfer about 10 mg (fresh weight) PEC into test tube containing induction medium (Tables 1&2) and keep the cultures for the next three weeks in the dark at 25±2°C for initial proliferation of embryogenic callus.

(iii) Culture Maintenance

After 8 weeks of culture initiation, transfer the explants showing PEC induction to medium containing B5 macrosalts with full strength MS iron-EDTA and L-glutamine (Tables 1&2) and keep in darkness at 25±2°C for three weeks to promote further proliferation of PEC.

Mango embryogenic cultures can be maintained either as relatively slowly proliferating cultures on semi-solid medium or as rapidly proliferating suspension cultures on a rotary shaker at 100-120 rpm. A regular pattern of subculturing of the PEC on the medium having B5 macrosalts (full strength) with 2,4-D (1.0 mg/l) alone or with Kinetin (1 mg /l) is necessary for long-term maintenance of embryogenic potentiality of the PEC. In failing to do so, the cultures can rapidly turn dark brown and lose their morphogenic potential regardless of state of medium.

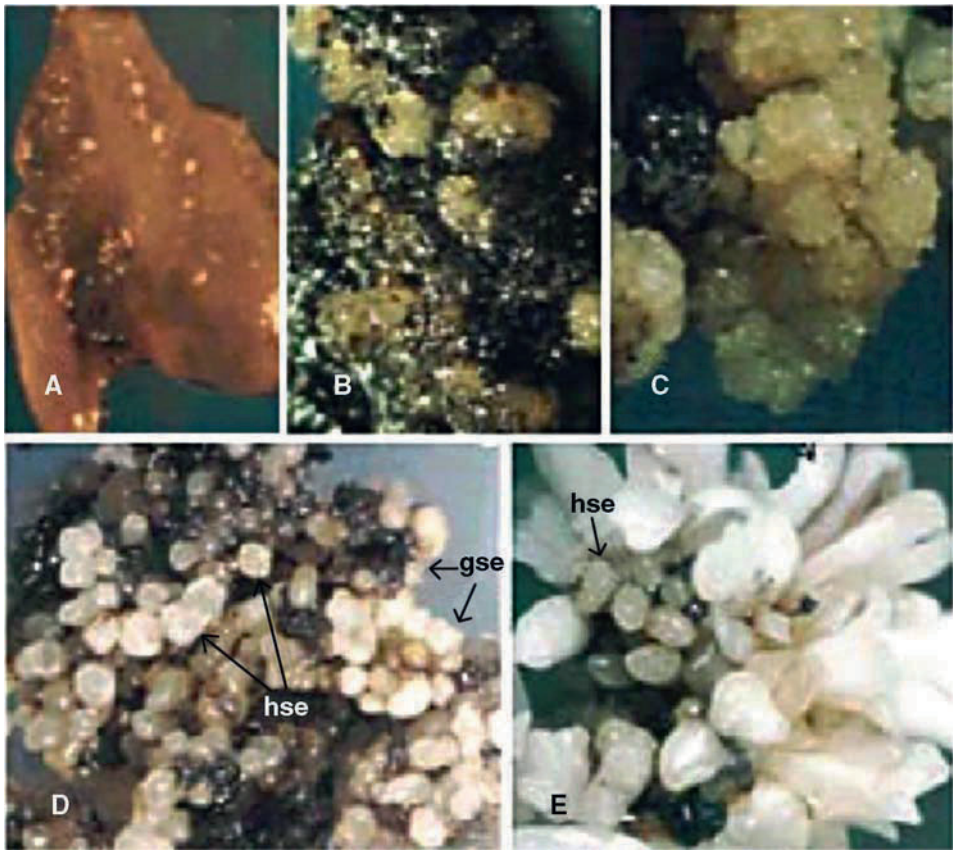

Figure- I Stages of Somatic Embryogenesis in Mango*(Mangifera indica* L.). A. Excised nucellus as an explant. B. Showing initiation of proembryogenic calli (yellow) attached with excised nucellus. C. Proliferating proembryogenic calli D. Globular and heart stage somatic embryos produced from the embryogenic calli. E. Cotyledonary embryos.

Abbreviations: cse = cotyledonary stage somatic embryos ; gse = globular somatic embryos ; hse = heart- stage somatic embryos; pec= proembryogenic callus

(iv) Embryo Development:

The completion of somatic embryogenesis occurs only on 2,4-D free embryo development medium (Table 2). The presence of 2,4-D inhibits the progression of embryo development beyond heart-stage.

After 8 –10 weeks, transfer about 10 mg PEC (cream or pale yellow coloured, compact or irregular shaped) into test tubes containing somatic embryo development medium (Table 2) and keep in dark at 25±2° C for four weeks for differentiation of somatic embryos (Fig 1 D).

(v) Embryo Maturation:

For maturation transfer immature,transparent to translucent globular to late heart / early cotyledonary stage somatic embryos to semi-solid embryo maturation medium (Table 2) and keep in the dark at 25±2°C. After 3 weeks when the cotyledons of somatic embryos become opaque white (Fig 1 E) transfer them to 16 h photoperiod (60 μE $m^{-2}s^{-1}$ light intensity provided by cool white fluorescent tubes, Philips) at 25 ± 2°C.

Addition of 0.001 or 0.005 mg/l ABA (abscisic acid) in combination with 10% (v/v) coconut water can increase the percentage of maturation.

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(vi) Embryo Germination and Conversion into Plantlets:

Transfer 9-week-old cotyledonary stage somatic embryos with opaque, white and thick cotyledons measuring 8 mm or more and having indistinct hypocotyl/short hypocotyl to 150 ml Erlenmeyer flasks containing 15-20 ml liquid germination medium (Table 2) and expose the cultures to 16h photoperiod (60 μE $m^{-2}s^{-1}$ light intensity) at 25 ± 2°C.

After 4 weeks transfer the germinating embryos, showing emergence of tap root to liquid medium of the same composition but without GA_3 to stimulate growth of root and shoot since the medium containing GA_3 inhibits their growth.

(vii) Acclimatization and Field Transfer: .

Transfer well developed plantlets (after 6-8 weeks of germination) with long roots to plastic pots containing a mixture of sand and soil in

a 3:1 ratio and keep in artificial light (60 μE m^{-2} s^{-1},same at which the somatic embryos are matured and germinated) at a temperature ranging between 25-30°C. Initially cover the plantlets with polythene bags to maintain high humidity over (90%) and irrigate regularly with tap water. After 20 days, transfer the pots to sunlight initially for a short duration (30 min); gradually, increase the time and remove the polythene bags. Finally transfer the plants, 3 –4 month old to garden soil and keep in sunlight.

B.Somatic Embryogenesis and Plant Regeneration from Protoplasts:

The plant protoplasts (naked plant cells obtained after dissolving the cell wall) offer exciting possibilities in the fields of somatic cell genetics and crop improvement. In culture, the isolated protoplasts often perform better than single cells and therefore serve as an excellent starting material for cell cloning and development of mutant lines. The ability of these naked plant cells to fuse with each other irrespective of their origin has opened a novel approach in raising new hybrids and cybrids (Bhojwani and Razdan, 1996). A method of protoplast isolation, culture and regeneration in mango cv. Amrapali is described below in four major steps:

1. Initiation and proliferation of proembryogenic masses

1. Remove the nucellus aseptically from the ovules of immature fruits (1.0 to 3.5 cm) and place on modified MS medium (Table 2) in test tubes (150x 25 mm) and maintain the cultures in complete darkness at 25±2 °C for callus initiation and induction of PEC.

2. After 8-10 weeks transfer the proembryogenic calli (cream or pale yellow coloured) to agar 0.8%(w/v) solidified 2,4-D free medium containing full strength B5 major salts, MS minor salts, iron-EDTA and organics, 400 mg/l L-glutamine and 6% (w/v) sucrose for proliferation.

3. After 3 weeks suspend about 10 mg PEC in liquid medium (35 ml in each Erlenmeyer flasks) of the same composition and maintain in

the dark on an orbital shaking incubator at 25±2° C at 100-120 rpm to establish cell suspension cultures.
Within 21-28 days of the initiation of suspension culture, the proliferating proembryogenic masses (PEMs), develops from the proliferating proembryogenic calli can be used for protoplast isolation.

Protoplast isolation and culture

1. Incubate approximately 1 g PEMs in 10 ml protoplast isolation medium (PIM, Table 3) containing 1.2% cellulase, 1.0% hemicellulase and 0.6% pectinase in 50 ml Erlenmeyer flasks in the dark at 25° C with gentle shaking (40-50 rpm). Filter-sterilize PIM and enzyme mixture solution with 0.45- μm millipore filter before use.

2. After 24 h incubation gently pass the mixture through two sterilized stainless steel sieves (80μm and 50μm in pore size) under aseptic conditions to remove large debris and undigested tissues. Centrifuge the resulting filterate at150 rpm for 3 min and discard the supernatant.

3. Suspend the pellet in PIM (2-3 ml) and centrifuge at 150 rpm for 3 min. Repeat this step three times to remove all traces the enzymes.

4. Resuspend the washed pellet in 1ml PIM, layer it on top of 3 ml sucrose pad [25% sucrose (w/v)] and centrifuge for 7 min at 150 rpm to collect the purified protoplasts.

5. Finally collect the isolated protoplasts with a Pasteur pipette and suspend them in PIM (10-15 ml) for getting purified protoplast suspension.
Determine the protoplast yield by counting the number of protoplasts per millilitre using a haemocytometer and assess the viability with fluorescein diacetate (FDA). To assess viability with FDA, mix equal volumes of 0.01% FDA solution (prepared in acetone) and protoplast suspension. After 5 min. examine the fluorescing protoplast under fluorescence microscope using a mercury vapour lamp/UV light. Count their number with haemocytometer and calculate yield and viability as the formulae given below:

$$\text{Yield/g PEMs} = \frac{\text{Total number of protoplasts from four 1 mm squares of haemocytometer}}{\text{Sample volume in 4 squares}} \times \text{Total volume of protoplast suspension}$$

$$\text{Total number of viable protoplast} = \frac{\text{Total no. of fluorescing protoplast from four 1 mm squares of haemocytometer}}{\text{Sample volume in 4 squares}} \times \text{Total volume of protoplast suspension}$$

$$\text{Viability (\%)} = \frac{\text{Total no. of protoplasts fluorescing with FDA}}{\text{Yield/g PEMS}} \times 100$$

6. Culture the freshly isolated protoplasts in liquid protoplast culture medium (PCM, Table 3) in 50 ml Erlenmeyer flasks each containing 5.5 ml PCM.
The optimum density of 5 x10^4cultured protoplasts per millilitre culture medium is required for highest frequency division (Ara *et al*., 2000b).

7.Keep the cultures in the dark at 25± 2 °C with gentle shaking (40-50 rpm, 5h/day) initially for 1 week in an orbital shaking incubator and then transfer to a static condition in the culture room at the same temperature in darkness.
Cell wall removal and regeneration of freshly isolated as well as cultured protoplasts can be confirmed with 0.01% calcoflour white. Prepare stock solution by dissolving 5mg calcofluor white in 5 ml PIM. Add 0.05 ml of this stock solution to 5 ml of protoplast suspension and after 5 min. wash it by repeated centrifugation and resuspension of the pellets in fresh PIM. Observe the fluorescing

protoplasts under fluorescence microscope using a mercury vapour lamp/UV light (Nagata and Takebe, 1970).

(iii) Callus proliferation, somatic embryogenesis and plant regeneration

1. Plate 1ml of suspension containing protoplast–derived microcalli on a thin layer of PCM (Table 3) gelled with 0.1% (w/v) phytagel supplemented with 2,4-D (1mg/l) in petri dishes (50x17mm) and keep in the dark for 3 weeks at 25±2° C.

2. After 3 weeks, subculture callus on 0.8% (w/v) agar gelled PCM without any growth regulator [approximately 10 mg callus per test tube (150x25 mm)] for differentiation of somatic embryos.

3. After 4 weeks transfer the somatic embryos (globular to early cotyledonary stage) to embryo maturation medium (Table 2) and keep in the dark at 25±2°C for maturation.

4. After 3 weeks, when the cotyledonary- stage somatic embryos are approximately 3 mm long, transfer the cultures to a 16-h photoperiod (30 μ.E m^{-2} s^{-1} light intensity provided by cool white fluorescent tubes, Philips) at the same temperature.

5. When the cotyledonary stage embryos are ≥ 1 cm long with opaque, white firm cotyledons *i.e*. after another 3 weeks, incubate them in 150 ml Erlenmeyer flasks containing 15-20 ml liquid germination medium (Table2) under 16-h photoperiod (60μE m^{-2} s^{-1} light intensity) at 25 ± 2° C.

6. After 4 weeks transfer to liquid medium of same composition but without GA_3 for root and shoot growth.

(iv) Establishment of plantlets in soil

Wash well developed plants and transfer to plastic pots containing a mixture of sand and soil in a 3:1 ratio and keep under artificial light (60μE m^{-2} s^{-1}intensity) at 25± 2°C. Cover the plants initially with

polythene bags to maintain humidity [>90%) and irrigate with tap water. After 20 days, transfer the pots to sunlight for 30 min. Gradually increase the exposure time and remove the polythene bags. After 3-4 months transfer the plants to garden soil in clay pots and keep in sunlight.

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C. Encapsulation

1. Select 6-week-old, 3-5 mm long immature cotyledonay stage somatic embryos with opaque white cotyledons (obtained from the protocol mentioned under section 3A) and use for encapsulation.

2. Prepare 2% alginic acid–sodium salt gel in a nutrient solution containing B_5 macrosalts, MS microsalts and MS organics (quarter strength each) and 100 mM
$CaCl_2$ solution in double distilled water and encapsulate the embryos individually under aseptic conditions following the procedure described by Redenbaugh and Walker (1990). Dip somatic embryos in alginic acid gel and drop them one by one with spatula in $CaCl_2$ solution. Allow them to remain in $CaCl_2$ solution for 40-45 min for hardening of beads.

3. Wash them with sterilized double distilled water.

4. Germinate the encapsulated somatic embryos in a medium containing half strength B5 macrosalts, full strength MS micro salts and organics, 3 %(w/v) sucrose, 0.6 % (w/v) agar and 1mg/l gibberellic acid. Abscisic acid (0.001 to 0.005 mg/l) can be used to delay germination (Ara *et.al*,1999).

5. Transfer the germinating somatic embryos to 150- ml Erlenmeyer flasks, each containing half strength B5 macrosalts with full strength MS microsalts, iron-EDTA and organics, 400 mg/l L-glutamine and 3%(w/v) sucrose.

6. Transfer the plantlets to soil as mentioned under section A.

CONCLUSION

Prior to development of our mango protocol for somatic embryogenesis and plant regeneration from nucellus, Litz group, USA had reported in several publications on mango (Litz et al 1982, Litz et al 1995 and Mathews and Litz, 1992) successful plant regeneration methods. However, their methods had following limitations:

(i) Cultivar dependent responses
(ii) Low frequency induction of somatic embryogenesis
(iii) Improper development and maturation of somatic embryos
(iv) Lower rate of somatic embryo germination and plant conversion
(v) Failure of survival of *in vitro* raised plants in soil

Our protocol has broadened the list of mono-embryonic elite mango cultivars that have responded to somatic embryogenesis at the high frequency rate.

There is an overall improvement in proper development and maturation of somatic embryos. With our protocol, higher rates of germination and conversion of somatic embryos has been achieved by changing the culture medium composition, physical state and nature of growth regulators used in the culture conditions. The survival rate of *in vitro* raised plants has also shown 40% improvement.

However this protocol still faces difficulties in getting high somatic embryogenic responses in different mango varieties, especially when leached poly phenols in the culture medium are not removed immediately. Owing to the availability of variable amount of 2,4-D to explant /callus in the culture medium, the developing somatic embryos demonstrate morphological variation at heart /cotyledonary developmental stages. During long-term maintenance of somatic embryogenic potential proembryogenic cells, cultures rapidly turn dark brown and lose their morphogenic potential if they are not subcultured regularly at a given time interval. The occurrence of apical necrosis in plants transferred to soil is also a problem in some cultivars.

Among mango growing countries, improvement in mango cultivars and its products would certainly enhance national earning by enhancing exports. Therefore, it is highly desirable to pay attention to improving protocols for large-scale plant production of the commercially important mango cultivars. For this it is necessary to remove phenolics from the culture medium. The conditions for proper development, maturation and conversion of somatic embryos have to be improved further. The possible cause of apical necrosis in the plantlets after transfer to soil has to be worked out so that the survival of plants is possible under field conditions.

REFERENCES

Ara,Hussain.(1998).Plant Regeneration from Somatic tissues, Protoplasts and Encapsulated Somatic embryos of Mango (*Mangifera indica* L.) Ph.D Thesis, Banaras Hindu University,Varanasi,India

Ara Hussain, Jaiswal, Uma and V.S.Jaiswal (1999).Germination and plantlet regeneration from encapsulated somatic embryos of mango (*Mangifera indica* L.).Plant Cell Rep. 19 (2):166-170.

Ara Hussain, Jaiswal,Uma and V.S.Jaiswal (2000 a) Somatic embryogenesis and plantlet regeneration in Amrapali and Chausa cultivars of mango *(Mangifera indica* L.).Current Science 78(2):164-169.

Ara,Hussain ,Jaiswal,Uma, and V.S.Jaiswal (2000 b) Plant regeneration from protoplasts of mango (*Mangifera indica* L.) through somatic embryogenesis .Plant Cell Rep. 19: (6) :622-627.

Bhojwani, S.S. and Razdan, M. K. (1996). Plant Tissue Culture: Theory and practice, A Revised Edition, Elsevier Science Publishers, Amsterdam.

Gamborg,O.L.,Miller, R.A. and Ojima,K.(1968).Nutrient requirements of suspension cultures of soybean root cells. Exp.Cell.Res., 50:151-158.

Kendurkar. S. V., Nadgauda, R. S., Phadke, C. H., Jana, M. M., Shirke, S. V. and Mascarenhas, A. F. (1995).Somatic embryogenesis in some woody angiosperms .In Somatic Embryogenesis in Woody Plants, Vol.I, S.M.Jain,P.Gupta and R.Newton (Eds), Kluwer Academic Publishers, The Netherlands,pp.49-79.

Lakshminarayana,S.(1980).Mango.In Tropical and Subtropical Fruits : Composition, Properties and Uses. S.Nagy and P.E.Shaw (Eds), AVI Publishing, Westport, Connecticut,pp.184-257.

Litz,R.E.,Knight,R.J. and Gazit,S.(1982) Somatic embryos from cultured ovules of polyembryonic *Mangifera indica* L.Plant Cell Rep.,1:264-266.

Litz,R.E.,Moon,P.A.,Mathews,H.,Jayasankar,S.,Monsalud,M.J.andPliego-Alfaro,F.(1995).Somatic Embryogenesis in Mango(*Mangifera indica* L.)In: Somatic Embryogenesis in Woody Plants,Vol.2, S.M.Jain, P.Gupta and R.Newton (Eds),Kluwer Academic Publishers, The Netherlands,pp.341-356.

Majumdar,P.K. and Sharma, D.K.(1990).Mango. In: Fruits:Tropical and Subtropical. T.K Bose and S.K.Mitra (Eds), Naya Prokash, Calcutta,pp. 1-62.

Mathews,H.and Litz,R.E.(1992).Mango. In: Biotechnology of Perennial Fruit Crops, Biotechnology in Agriculture, No.8, CAB International, F. A. Hammerschlag and R. E. Litz (Eds), University Press, Cambridge,U.K.,pp.433-448.

Murashige,T.and Skoog,F.1962.A revised medium for rapid growth and bioassays with tobacco tissue cultures.Physiol.Plan.,15:473-497.

Nagata T. and Takebe I.(1970).Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts.Planta (Berl.),92:301-308.

Redenbaugh,K. and Walker,K. (1990).Role of artificial seeds in alfalfa breeding. In: Plant Tissue Culture: Applications and Limitations, Developments in Crop Science, No.19.S.S.Bhojwani (Ed.), Elsevier Science Publishers, The Netherlands, pp. 102-135.

Singh, R. N.(1990).Mango. I.C. A. R , New Delhi, India.

Table 1. Chemical Composition of MS and Gamborg media

Macrosalts

Components	**Quantity in the medium (mg/l)**	
	MS (1962)	**Gamborg et al., (1968)**
Ammonium Nitrate [$NH_4 NO_3$]	1650	0
Ammonium Sulphate [$(NH_4)_2SO_4$]	0	134
Calcium Chloride[$Ca Cl_2.2 H_2O$]	440	150
Magnesium Sulphate[$Mg SO_4.7H_2O$]	370	246.5
Potassium dihydrogen orthophosphate[$KH_2 PO_4$]	170	0
Potassium Nitrate[KNO_3]	1900	2527.5
SodiumPotassium dihydrogen orthophosphate [$NaH_2PO_2.H_2O$]	0	150

Microsalts,Iron-EDTA and Organics (MS,1962)

Components **(i)Microsalts**	**Quantity in the medium (mg/l)**
Boric acid [H_3BO_3]	6.20
Cobaltous Chloride[$CoCl_2.6 H_2O$]	0.025
Copper Sulphate[$CuSO_4.5 H_2O$]	0.025
Manganese Sulphate[$MnSO_4.4 H_2O$]	22.3
Potassium Iodide[KI]	0.83
Sodium Molybdate[$Na_2MoO_4.2 H_2O$]	0.25
Zinc Sulphate[$ZnSO_4.7 H_2O$]	8.60
(ii) Iron-EDTA: Ferrous sulphate [$FeSO_4 7 H_2O$] Ethylenediaminetetraacetic acid disodium salt (Dihydrate)[$Na_2EDTA.2 H_2O$]	27.8 37.30
(iii) Organics:	
Myo-inositol (1,2,3,5/4,6-Hexahydroxy-cyclohexane)	100
Nicotinic acid (Niacin,Pyridine-3-carboxylic acid) Free acid	0.5
Pyridoxin-HCl (Vitamin B6)	0.5
Thiamine-HCl (Vitamin B1)	0.1

Table 2: Composition of media for different stages of somatic embryogenesis

Components		Quantity	in the	Medium *	
	A	**B**	**C**	**D**	**E**
Macrosalts	MS- HS	B5- FS	B5-FS	B5-FS	B5-HS
Microsalts	MS-FS	MS-FS	MS-FS	MS-FS	MS-FS
Iron-EDTA	MS-HS	MS-FS	MS-FS	MS-FS	MS-FS
Organics (vitamins and amino acids)	MS-FS	MS-FS	MS-FS	MS-FS	MS-FS
Ascorbic acid (mg/l)	100	0	0	0	0
L-glutamine (mg/l)	400	400	400	400	400
Sucrose (g/l)	60	60	60	60	30
Agar (g/l)	8	8	8	8	0
2,4-D (mg/l)	1.0	1.0	0	0	0
GA3 (mg/l)	0	0	0	0	1.0

Abbreviations: *A – Medium for callus initiation and production of PEC; B- medium for PEC proliferation /maintenance; C- medium for somatic embryo production; D- medium for somatic embryo maturation; E- medium for germination and conversion of somatic embryos.
B5-Gamborg *et al*.,(1968) ; MS-Murashige and Skoog (1962) ; FS- Full strength; HS- Half-strength

Table 3: Composition of culture medium for protoplast isolation and culture

Components	PIM	PCM
Macrosalts	B5-FS	B5-FS
Microsalts	MS-FS	MS-FS
Iron-EDTA	MS-FS	MS-FS
Organics	MS-FS	MS-FS
L-glutamine (mg/l)	400	400
Sucrose (g/l)	102.69	60
Mannitol (g/l)	72.87	60
Sorbitol (g/l)	18.22	0
Phytagel (g/l)	0	1

Abbreviations:PIM =Protoplast Isolation Medium, PCM= Protoplast Culture Medium,FS=Full strength, B5= Gamborg *et al*.,1968, MS= Murashige and Skoog , 1962

SOMATIC EMBRYOGENESIS INJACKFRUIT (*ARTOCARPUS HETEROPHYLLUS* LAM.)

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1. INTRODUCTION

Artocarpus heterophyllus (jackfruit, Fam. Moraceae) is a large, evergreen fruit tree with a dense crown, reaching a height of about 20 m. It is indigenous to Bangladesh, India, Myanmar, Sri Lanka, Thailand, Malaysia and Brazil and has been introduced into many other tropical countries. It is designated as multipurpose tree and has great economic importance for its fruits and timber. In South and South East Asia, ripe fruit is in great demand, particularly as a source of energy for villagers and working people. The fruiting perianths have a strong sweet, aromatic odour, fine texture and a rich appetizing taste. The perianths are rich in sugar; a fair amount of carotene is also present. They contain protein, fat, calcium, phosphorus and iron in quantities normally present in other fruits. Seeds and unripe fruits are mostly used as popular vegetable, which are starchy and contain fair amounts of protein, calcium and thiamin and have good pectin content. Leaves and remnants of the fruit are good source of nutrient fodder. The plant produces a moderately hard wood, which is widely used in many purposes such as construction of houses and furniture. The timber polishes well and does not warp and split. The wood is durable, seasons without any trouble and is not attacked by white ants or fungi (Anonymous, 1954).

Jackfruit is commonly grown from seeds; however, the seeds are recalcitrant and difficult to germinate even after a short period of storage (Singh, 1986; Sammadder, 1990) and also the species is predominantly cross-pollinated leading

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to high seedling variability. Because of seed propagation, the existing population of jackfruit comprises innumerable trees from each other in such fruit characters as shape, size and quality. Grafting is relatively difficult due to an abundance of sticky latex from cut surface (Rowe-Dulton, 1976; Singh, 1986).

Clonal propagation is suitable for production of true-to-parental type plants. The conventional method of clonal propagation in jackfruit is difficult. Therefore, *in vitro* clonal propagation method has been tried by many scientists (Amin, 1992; Amin and Jaiswal, 1993; Rahman, 1988a, 1988b; Rahman and Blake, 1988a, 1988b; Roy *et al.,* 1990, 1992, 2000). However, there is no report on its regeneration through somatic embryogenesis. Somatic embryogenesis offers a means of high frequency regeneration of specially selected genotypes. Embryogenic cell suspension culture has several advantages over other *in vitro* propagation methods in that it has potential high multiplication rates, potential for scale-up and delivery through bioreactor and synthetic seed technologies, amenability to cryogenic storage, and suitability for genetic transformation (Merkle and Trigiano, 1992; Percy *et al.*, 2000).

This chapter deals with latest protocol for establishing, maintaining, and maturing embryogenic tissue of jackfruit in order to produce high quality somatic seedlings.

2. EMBRYOGENIC CULTURE INITIATION

2.1 Explant

Fresh seeds are collected from ripe fruit of selected tree. The seeds are washed under running tap water to remove fruit pulp and slimy juice. They are then submerged in 70% ethanol, containing a few drops of tween 20 for 3 min, with continuous shaking. The ethanol is decanted and 1.5 % sodium hypochlorite solution with a few drops of tween 20 is added. The seeds are left in the solution for 20 min with frequent shaking. The liquid is decanted and the seeds are washed 3 times in autoclaved sterile distilled water. Outer seed coat (exine) of the seeds is removed with forceps. The decoated seeds are inoculated in agar gelled MS (Murashige and Skoog, 1962) basal medium without growth regulator and incubated at $24 \pm 2^{o}C$ in the dark for germination. Within 15 days seeds germinated and the epicotyls become 4-5 cm long. The cotyledons and epicotyls are excised and taken as explants for experiments.

2.2 Explants inoculation

To induce callus explants (cotyledons and epicotyls) are aseptically isolated and cut into pieces (0.5-0.7 cm long); and inoculate them into 100 ml conical flask containing 40 ml agar gelled (0.7%) MS (Murashige and Skoog, 1962) medium having (per liter) 100 mg meso-inositol, 0.5 mg nicotinic acid, 0.5 mg pyridoxin-HCl, 0.1 mg thiamine-HCl, 2 mg glycine, 3% sucrose, and 2.5 mgl^{-1} 2,4-D (2, 4-dichlorophenoxyacetic acid). The cultures are incubated at 24 ± 2°C in light (50 μmol m^{-2} s^{-1}).

2.3 Culture initiation

After 3 weeks, explants are dedifferentiated and large amount of callus is formed. Ninety per cent cultures induce callus in epicotyl segments while 75% cultures of cotyledon slices produce callus. However, the nature of callus induced in both the explants is yellowish white and semi friable. The callus tissues are sub-cultured every 3-week on to the fresh medium of same constituents.

After 3 subcultures, the nature of callus changes to friable and fast growing type and embryogenic callus comprises of small, compact cell clumps. The cell clumps are grouped in small or large aggregates. Cell aggregates resembled zygotic embryos at various stages of early development up to the globular stage (Fig. 1).

Embryogenic nature of culture was determined through observation in different stages of embryogenic development. Globular embryos (stage 1), characterized by a translucent head, are generally visible protruding from the surface of embryogenic cultures. Heart shaped (stage 2) somatic embryos have a smooth, opaque embryonal head with a little tapering base. Torpedo shaped (stage 3) embryos are also opaque and have little bifurcated cotyledonary initials starting to develop. Fully mature somatic embryos (stage 4) are characteristically white and have well-defined cotyledons with hypocotyls.

Table 1. Composition of media used for the different stages of somatic embryogenesis in jackfruit (*Artocarpus heterophyllus*)

Compounds[1]	Callus induction (mgl^{-1})	Embryogenic callus (mgl^{-1})	Embryo development (mgl^{-1})	Embryo germination (mgl^{-1})
NH_4NO_3	1650	1650	1650	1650
KNO_3	1900	1900	1900	1900
$CaCl_2$ .2 H_2O	440	440	440	440
$MgSO_4$ .7 H_2O	370	370	370	370
KH_2PO_4	170	170	170	170
KI	0.83	0.83	0.83	0.83
H3BO3	6.2	6.2	6.2	6.2
MnSO4. 4 H_2O	22.3	22.3	22.3	22.3
ZnSO4 . 7 H_2O	8.6	8.6	8.6	8.6
Na_2MoO_4. 2 H_2O	0.25	0.25	0.25	0.25
CuSO4. 5 H_2O	0.025	0.025	0.025	0.025
CoCl2. 6 H_2O	0.025	0.025	0.025	0.025
FeSO4. 7 H_2O	27.8	27.8	27.8	27.8
Na_2. EDTA. 2	37.3	37.3	37.3	37.3
H_2O	100	100	100	100
Meso-inositol	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5
Pyridoxine HCl	0.1	0.1	0.1	0.1
Thiamine HCl	2	2	2	2
Glycine	30000	30000	30000	30000
Sucrose	2.5	2.5	-	-
2,4-D	-	-	3.0	-
BA	-	-	1.0	-
IBA	-	-	300	-
Glutamine	-	-	-	1.0
GA3	-	-	-	10%
Coconut water	0.7%	0.7%	0.7%	0.7%
Difco Bacto-agar	5.8	5.8	5.8	5.8
pH				

[1]All macro- and micro elements are the ingredients of MS medium

2.4 Culture maintenance

The proliferating somatic embryogenic callus is regularly maintained by sub-culturing at 3-week interval on the same culture medium under similar culture conditions (MS medium containing 2.5 mgl^{-1} 2,4-D.). The callus mass having embryo primordia, which is to some extent beaded in nature, is selected. During each subculture, the callus should have a good contact with the culture medium; and embryogenic callus can be maintained for more than one year. The necessary contact with the medium is achieved by spreading the callus on the medium surface. After 3 subcultures, fresh callus is induced from new explant.

3. SOMATIC EMBRYO DEVELOPMENT

For the development of somatic embryos derived from embryogenic callus, pieces of 0.5 ± 0.05g callus are gently separated using forceps and transferred to 100 ml conical flasks containing 40 ml medium of MS salts containing (per liter) 100 mg meso-inositol, 0.5 mg nicotinic acid, 0.5 mg pyridoxin-HCl, 0.1 mg thiamine-HCl, 2mg glycine, 3% sucrose, 7.0 gl^{-1} agar, and 3 mgl^{-1} BA + 1 mgl^{-1} IBA. For optimal embryo development, it is important to separate callus into smaller pieces and spread them out on the medium surface. Every 3-week, developing somatic embryos are sub-cultured on the fresh medium. At the second subculture, globular and heart shaped somatic embryos are developed. The frequencies of heart shaped embryos vary depending on the explant-derived cultures. However, the embryos don't develop further even in following subcultures. In the culture medium, 300mgl^{-1} glutamine is added and that accelerates the embryo development (Figs 2 & 3). So, the medium determined for embryo development is MS + 3.0 mgl^{-1} BA (benzyl adenine) + 1.0 mgl^{-1} IBA (indole-3-butyric acid) + 300 mgl^{-1} glutamine. In this medium somatic embryos derived both from epicotyl and cotyledons are fully developed with normal cotyledons and a distinct hypocotyls (Fig. 4).

Table 2. Stages of jackfruit regeneration system and their specific medium additives, culture conditions, durations and associated responses

Stage	Medium additives[1] (mgl^{-1})	Light[2]	Duration[3] (wk)	Remarks
1.Callus induction	2,4-D (2.5)	Light	3	Yellowish white, semi friable callus formation
2. Embryogenic callus	2,4-D (2.5)	Light	9	Embryoge callus with large aggregates
3. Embryo development	BA(3.0), IBA(1.0), Glutanine (300)	Light	6	Somatic embryos develop
4. Embryo germination	GA_3 (1.0), CW (10%)	Light	5	Somatic embryos germinate
5. Somatic seedlings maturation	GA_3 (1.0), CW (10%)	Light	3	Somatic seedlings mature for transplantation

[1]Full-strength MS salts,
[2]Incubation at 16-h photoperiods (50 μmol m^{-2} s^{-1})
[3]Subculture at 3-wk intervals throughout culture duration

4. EMBRYO GERMINATION

High somatic embryo germination rate is species dependent and develop into somatic seedlings with a variety of treatments; even different nutrient supplements are often required for the different genotypes of the same species (Jain *et al.*, 1989). Well developed somatic embryos are isolated and placed in the agar gelled (0.7 %) MS medium supplemented with 3% sucrose + 1 mgl^{-1} GA_3 + 10% CW (coconut water) and incubated under fluorescent light in a 16/8 h light/dark cycle at 24 ± 1°C. Under this culture condition, somatic embryos become enlarged and turn green. Within 30-35 days, proper shoot and root systems develop and within 50 days somatic embryos develop into somatic seedlings, which are maintained *in vitro* until become 5-7 cm tall (Fig. 5); and are hardened and acclimatized.

5. ACCLIMATIZATION AND FIELD TRANSFER

The culture vessels containing plantlets are taken out from growth chamber and keep them at room temperature and normal day light for 10 days. The plantlets are removed carefully from the culture vessels, and agar adhered to the roots is gently removed under running tap water. Immediately, they are dipped in 5% solution of Bavistin (fungicide) for 5 min; transplant them in the cavity of polystyrene seedling trays (64 cavity each). The cavity is filled with autoclaved-mixture of compost and vermiculite (7:3). At the time of transplantation utmost care is taken to prevent root damage. The trays containing the plantlets are covered with transparent polyethylene sheet to maintain 80-85% relative humidity and placed under a double layer of 20% shade net in the greenhouse. Somatic seedlings are moistened twice a day. The polyethylene sheets are gradually removed to expose the plantlets to the outer environment; remove them completely after 20-25 d. Plantlets are regularly watered every 2 d depending on the weather and sprayed liquid fertilizer (10% solution of 4N: 4P: 2K). Within 3 months, plantlets are acclimatized and transferred to the nursery. After two months, the plantlets are transplanted into 10-liter polybags filled with compost and alluvial soil (2:3); and watered and fertilized as needed. After six months, plants are transplanted in the open field for proper growth (Fig. 6).

6. CONCLUSION AND FUTURE PROSPECTS

By implementing this protocol, somatic seedlings have been produced for the establishment of superior genotypes. Somatic embryogenesis has only been induced on cotyledon and epicotyl segments. Once initiated, the embryogenic cultures can be efficiently multiplied and maintained without loss of embryogenic potential by regular subculture of embryogenic callus on media supplemented with glutamine, low levels of a cytokinin and an auxin. The technique holds out the possibility of improving jackfruit through introgression of appropriate gene.

Nevertheless, several problems are needed to overcome before this technique can be used for large-scale plant multiplication. Although high yields of somatic embryos are obtained from cotyledon- and epicotyl derived calli, embryo development and germination rates are low, embryo-to-plant conversion is being limited by poor shoot development. So far, somatic embryogenesis in jackfruit originates from germinating seedlings. Somatic embryos have not yet been obtained from mature material of jackfruit. In order to realize the potential of this technique for clonal multiplication, further studies are necessary for this perennial fruit tree: firstly, to induce somatic embryo production from explants of mature selected trees; secondly, to achieve synchronized embryo development, and thirdly, to optimize medium composition so as to satisfy the better physiological requirements of embryos, thereby enabling more embryos to mature and enhancing conversion rate.

7. ACKNOWLEDGEMENT

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8. REFERENCES

Amin, M.N. 1992. *In vitro* enhanced proliferation of shoots and regeneration of plants from explants of jackfruit trees. Plant Tissue Cult. 2: 27-30.

Amin, M.N., Jaiswal, J.M. 1993. *In vitro* response of apical bud explants from mature trees of jackfruit (*Artocarpus heterophyllus)*. Plant Cell Tiss. Org. Cult. 33: 59-65.

Anonymous. 1954. The wealth of India, Raw Materials Vol-1: A. Dictionary of Indian Raw Materials and Industrial Products, pp. 444-453. Publications and Information Directorate, CSIR, New Delhi.

Jain, S.M., Dong, N., Newton, R.J. 1989. Somatic embryogenesis in slash pine (*Pinus elliottii*) from immature embryo cultured *in vitro.* Plant Sci. 65: 233-241.

Merkle, S.A., Trigiano, R.N. 1992. *In vitro* propagation of hardwoods. Applications of vegetative propagation in forestry. Proc. 1992 SRIEG Biennial Symposium on Forest Genetics: 1992, July 8-10, Huntsville, AL. New Orleans, LA USDA Forest Service General Technical Report SO-108: Southern Forest Experiment Station.

Murashige, T., .Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Percy, R.E., Klimaszewska, K., Cry, D.R. 2000. Evaluation of somatic embryogenesis for clonal propagation of western white pine. Can J. For. Res. 30: 1867-1876.

Rahman, M.A., Blake, J. 1988a. Factors affecting *in vitro* proliferation and rooting of shoots of jackfruit (*Artocarpus heterophyllus* Lam.). Plant Cell, Tissue and Organ Culture. 13: 179-188.

Rahman, M.A., Blake, J. 1988b. The effects of medium composition and culture conditions on *in vitro* rooting and *ex vitro* establishment of jackfruit (*Artocarpus heterophyllus* Lam.). Plant Cell Tissue and Organ Culture. 13: 189-200.

Rahman, M.A. 1988a. Effect of nutrients and IBA on the *in vitro* rooting and *ex vitro* establishment of jackfruit (*Artocarpus heterophyllus* Lam.). Bangladesh J. Bot. 17: 105-110.

Rahman, M.A. 1988b. Effects of nutrients on the growth and survival of *in vitro Artocarpus heterophyllus* Lam. plantlets after transfer to *ex vitro* conditions in the glass house. J. Hort. Sci. 63: 329-336.

Rowe-Dulton, P. 1976. *Artocarpus heterophyllus*-Jackfruit. In: Garner, R.J., Chaudhury, S.A. (ed.), The Propagation of Tropical Fruit Trees, pp. 269-290, FAO, CABI, London.

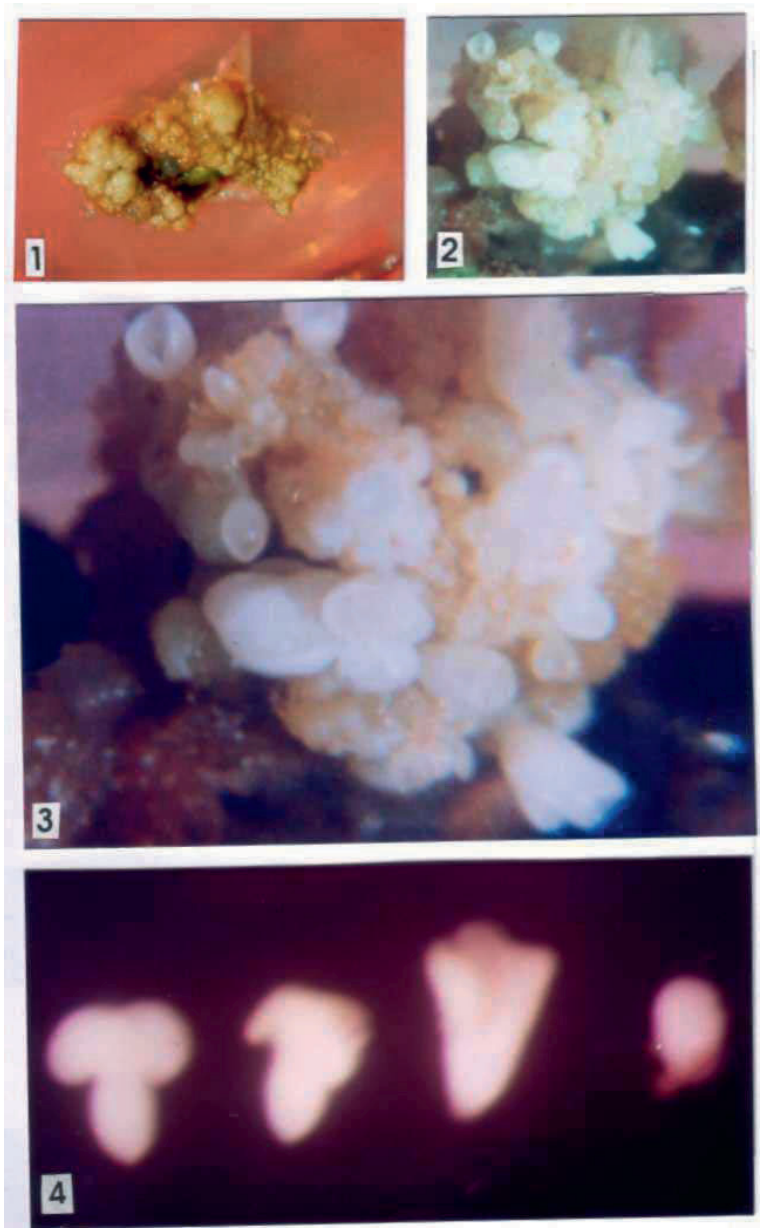
Roy, S.K.,. Rahman, S.L., Majumder,R. 1990. *In vitro* propagation of jackfruit (*Artocarpus heterophyllus* Lam.). J. Hort. Sci. 65: 355-358.

Roy, S.K., Islam, M.S. Sen, J., Hadiuzzaman, S. 1992. Effects of auxins, sucrose and agar concentrations on *in vitro* rooting of callus-induced microshoots of jackfruit (*Artocarpus heterophyllus* Lam.). Bangladesh J. Bot. 21: 93-98.

Roy, S.K., Roy, P.K., Sinha, P. Haque, M.S. 2000. Mass clonal propagation of *Artocarpus heterophyllus* through *in vitro* culture. In: Kubota, C. Chun, C. (ed.). Transplant Production in the 21st Century, pp. 219-225. Kluwer Academic Publishers. The Hague

Samaddar, H.N. 1990. Jackfruit. In: Bose, T.K., Mitra, S.K. (ed), Fruits: Tropical and Subtropical, pp. 638-639. Naya Prokash, Calcutta.

Singh, A. 1986. Fruit Physiology and Production. Kalyani Publishers, New Delhi.



Figures 1-4. Somatic embryogenesis from callus derived from epicotyl section of jackfruit. 1. Embryogenic callus obtained from seedling epicotyl cultured in MS + 2.5 mgl^{-1} 2,4-D + 3% sucrose. 2. Different stages of embryo formation from embryogenic callus cultured in MS + 3 mgl^{-1} BA + 1 mgl^{-1} IBA + 300 mgl^{-1} glutamine. 3. Same as mentioned in 2 but magnified. 4. Isolated different stages of somatic embryo.

Figures 5-6. Plants obtained via somatic embryogenesis. 5. Plantlet developed from germinating embryo cultured in MS + 1 mgl^{-1} GA_3 + 10% CW. 6. 3-year-old somatic plants in the field.

SOMATIC EMBRYOGENESIS IN INDIAN OLIVE (*ELAEOCARPUS ROBUSTUS* L)

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1. INTRODUCTION

Elaeocarpus robustus L. (Indian olive, Fam. Elaeocarpaceae) is a well-known evergreen fruit tree and 25 m tall. It is native to Bangladesh and India. The tree is of great economic importance for its fruits and timber. The importance of fleshy sour fruits having citric acid occupy an important position in tropical countries since they provide needed vitamin-C in diets. Its wood is important for timber uses as well as fuel (Anonymous, 1964; Drury, 1985; Das, 1987).

The fruit of Indian olive has several uses as food adjuncts for human being. The fleshy ripe fruit is delicious, which is eaten raw or cooked and pickled. The plant is also important for its therapeutic uses. Leaves are used in rheumatism, and as an antidote to poison (Caius, 1986) and are also considered as a cure for gonorrhoea (Drury, 1985). Fruit is tonic, emmenagogue, appetizer; useful in biliousness, liver complaints, scabies, burning of the eyes, carries of the teeth, toothache etc. (Kirtikar and Basu, 1881) and prescribed in dysentery and diarrhoea (Caius, 1986).

Elaeocarpus robustus produce fine textured, moderately hard and strong wood which takes good finish and fitting with good working properties. The swan wood has been used betterly in parquet flooring. It is also used as suitable wood in making small furniture and musical instruments. Wood has several important industrial uses as fuel and to prepare some form of essential equipments such as match splints and boxes, mathematical instruments, packing cases and boxes (Anonymous, 1964).

Indian olive is commonly grown from seeds, which are recalcitrant and difficult to germinate even after a short period of storage. The species is predominantly cross-pollinated leading to high seedling variability. Because of seed propagation, the plant qualities vary widely among the individuals. Clonal or vegetative propagation

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 257–266.

is important for reproduction and tree improvement of this species. The long reproductive cycle of this species is a serious constraint to tree improvement by conventional tree-breeding techniques. Clonal propagation by grafting is not successful in this plant. By organogenesis, Roy *et al.* (1998) reported i*n vitro* clonal propagation of Indian olive, however, there is no report on plant regeneration through somatic embryogenesis. In the last several years, rapid progress in plant regeneration and tree improvement by somatic embryogenesis has been achieved. This method has been reported in many important forest tree species (Jain, *et al.,* 1995). Somatic embryogenesis has a potential to produce a great number of complete plantlets in a short time. It may play a vital role in tree breeding programs and in production of transgenic trees obtained by recombinant DNA technology. Furthermore, embryogenic cultures can be used for cryogenic storage, when plant cells are exposed to ultra-low temperature and cellular metabolic activities are arrested. In the present chapter a protocol is described for propagation of Indian olive (*Elaeocarpus robustus*) through somatic embryogenesis.

2. EMBRYOGENIC CULTURE INITIATION

2.1 Explant

Fresh ripe fruits are collected from selected tree. The seeds are extracted by removing fruit pulp. The seeds are washed under running tap water to remove traces of fruit pulp; dip in 70% ethanol, containing a few drops of tween 20 for 5 min, with continuous shaking. The ethanol is decanted and 1.5% sodium hypochlorite solution with a few drops of tween 20 is added. The seeds are left in the solution for 20 min with frequent shaking. The solution is decanted and the seeds are washed 3 times in sterilized distilled water. Outer stony seed coat of the seed is removed by crushing it with a hammer and inner embryo is rescued with forceps. For germination the rescued embryos are inoculated in agar gelled MS (Murashige and Skoog, 1962) basal medium without growth regulator and incubate at 24 ± 2^{o}C in the dark. Within 20 days, embryos germinate. The cotyledons and epicotyls are excised and used as explants.

2.2 Explants inoculation

Explants (cotyledons and epicotyls) are aseptically isolated, cut into pieces (0.5-0.7 cm long) and inoculate them into 100 ml conical flask containing 40 ml agar gelled (0.7%) medium of MS salts with (per liter) 100 mg meso-inositol, 0.5 mg nicotinic acid, 0.5 mg pyridoxin-HCL, 0.1 mg thiamine-HCL, 2 mg glycine and 3% sucrose, supplemented with (1) 2.5 mgl^{-1} 2,4-D and (2) 2 mgl^{-1} BA + 0.5 mgl^{-1} NAA. The cultures are incubated at 24 ± 2^{o}C in light (50 µmol m^{-2} s^{-1}).

2.3 Culture initiation

2.3.1. Epicotyl

Within 3-4 weeks, the epicotyl explant cultured in 2,4-D, dedifferentiated and a large amount of callus is formed. Nearly hundred percent cultures induced callus in epicotyl segments (Fig. 1). The nature of callus induced is friable and creamy white. In the second experiment, when epicotyl segments are cultured in MS + 2 mgl^{-1} BA + 0.5 mgl^{-1} NAA, small callus develops at the cut ends of the explant.

2.3.2 Cotyledon

In the same nutrient medium (MS + 2.5 mgl^{-1} 2,4-5), cotyledonary leaf segments produce compact and brown callus. When cotyledonary leaf segments are cultured in MS + 2 mgl^{-1} BA + 0.5 mgl^{-1} NAA, clusters of seed-like somatic embryos are produced directly on the surface of the explants (Fig. 2, Table 2).

The callus tissues, induced from both the explants, are subcultured to fresh medium of same constituents (MS + 2.5 mgl^{-1} 2, 4-D) every 3-weeks. After 3-4 subcultures, the nature of callus becomes fast-growing, friable and beaded embryogenic, resembling zygotic embryos at various stages of early development up to the globular stage.

Embryogenic nature of culture is determined through observation in different stages of embryogenic development. Globular embryos (stage 1), characterized by a translucent head, are generally visible protruding from the surface of embryogenic cultures. Heart shaped (stage 2) somatic embryos are characterized by a smooth, opaque embryonal head with a little tapering base. Torpedo shaped (stage 3) embryos are also opaque and have little bifurcated cotyledonary initials starting to develop. Fully mature somatic embryos (stage 4) are characteristically white and have well-defined cotyledons with hypocotyls.

2.4 Culture maintenance

The embryogenic callus is maintained up to one year by subculturing every 3-week on the same medium and under the similar culture conditions as during its initiation (MS medium with 2.5 mgl^{-1} 2,4-D). Callus older than one year is not competent for embryogenesis. Somatic embryogenesis is a terminal event in their culture system and regenerative cultures can't be maintained (Mullins and Srinivasan, 1976). In contrast, Krul and Worley (1977) documented that cultures could be maintained for at least six months and subsequently, "indefinitely" (Krul, 1985). We tried various media to maintain embryogenic callus but further development was not possible.

Table 1. Composition of the medium used for the different stages of callus-derived somatic embryogenesis in Indian olive (*Elaeocarpus robustus*).

Compounds[1]	Embryogenic callus (mgl^{-1})	Embryo development (mgl^{-1})	Embryo germination (mgl^{-1})	Somatic seedlings maturation (mgl^{-1})
NH_4NO_3	1650	1650	1650	1650
KNO_3	1900	1900	1900	1900
$CaCl_2$ .2 H_2O	440	440	440	440
$MgSO_4$ .7 H_2O	370	370	370	370
KH_2PO_4	170	170	170	170
KI	0.83	0.83	0.83	0.83
H3BO3	6.2	6.2	6.2	6.2
MnSO4. 4 H_2O	22.3	22.3	22.3	22.3
ZnSO4 . 7 H_2O	8.6	8.6	8.6	8.6
Na_2MoO_4. 2 H_2O	0.25	0.25	0.25	0.25
CuSO4. 5 H_2O	0.025	0.025	0.025	0.025
CoCl2. 6 H_2O	0.025	0.025	0.025	0.025
FeSO4. 7 H_2O	27.8	27.8	27.8	27.8
Na_2. EDTA. 2	37.3	37.3	37.3	37.3
H_2O	100	100	100	100
Meso-inositol	0.5	0.5	0.5	0.5
Nicotinic acid	0.5	0.5	0.5	0.5
Pyridoxine HCl	0.1	0.1	0.1	0.1
Thiamine HCl	2	2	2	2
Glycine	30000	30000	30000	30000
Sucrose	2.5	-	-	-
2,4-D	-	2.5	-	-
BA	-	0.5	-	-
IBA	-	-	300	300
Glutamine	-	-	1.5	1.5
GA3	0.7%	0.7%	0.7%	0.7%
Bacto Difco-agar				

[1]All macro- and micro elements are the ingredients of MS medium

3. SOMATIC EMBRYO DEVELOPMENT

For the development of somatic embryos from embryogenic callus, 0.5 ± 0.05g callus are gently separated with forceps and transferred to 100 ml conical flasks containing 40 ml MS medium (MS salts with (per liter) 100 mg meso-inositol, 0.5 mg nicotinic acid, 0.5 mg pyridoxin-HCL, 0.1 mg thiamine-HCL, 2 mg glycine and 3% sucrose). Globular to torpedo shaped embryos develop (Fig. 4) from embryogenic callus when cultured in MS medium containing 2.5 mgl^{-1} BA + 0.5 mgl^{-1} IBA. Gradually somatic embryos showing bipolar structure develop. Repetitive somatic embryogenesis is frequent and form many small embryoids. Embryogenic callus can't be maintained in 2,4-D but it can be maintained for more than two years by subculturing every 3 weeks in MS medium supplemented with 2.5 mgl^{-1} BA + 0.5 mgl^{-1} IBA.

Table 2. Stages of Indian olive regeneration system through somatic embryogenesis and their specific medium additives, culture conditions, durations and associated responses

Stage	Medium additives[1] (mgl^{-1})	Light[2]	Duration[3] (wk)	Remarks
1. Callus induction from eoicotyl explant	2,4-D (2.5)	Light	3	Creamy white, semi friable callus formation
2. Embryogenic callus	2,4-D (2.5)	Light	9	Embryogenic callus with large aggregates
3. Embryo development	BA(2.5) + IBA (0.5)	Light	6	Somatic embryos develop
4. Embryo germination	GA_3 (1.5) + Glutamine (300)	Light	5	Somatic embryos germination
5. Direct embryogenesis from cotyledon	BA (2.0) + NAA (0.5)	Light	3	Direct globular-torpedo shaped embryo formation
6. Germination of direct embryo	i) IBA (1.0) ii) GA_3 (1.5) + Glutamine (300)	Light Light	2 6	Radicle elongation Embryo germinate to seedling
7. Secondary embryo from direct embryo	BA (2.0) + NAA (0.5)	Light	6	Embryo misshaping with brown color and secondary embryo formation
8. Somatic seedling maturation	GA_3 (1.5) + Glutamine (300)	Light	3	Somatic seedling mature for transplantation

[1]Full-strength MS salts,
[2]Incubation at 16-h photoperiod (50 µmol m^{-2} s^{-1})
[3]Subculture at 3-wk intervals throughout culture duration

4. SECONDARY SOMATIC EMBRYO

Somatic embryos directly form on the cotyledonary leaf explant by sub-culturing on the same medium (MS + 2 mgl^{-1} BA + 0.5 mgl^{-1} NAA) don't flourish, however the developed embryos are large and deformed, turn brown, and form secondary somatic embryos (Fig. 3, Table 2). The secondary embryos are fully matured and loosely attached to tissue at the root pole. So, the embryos directly develop on the explant are transferred to the embryo germination medium or sub-cultured for secondary embryo formation.

5. SOMATIC EMBRYO GERMINATION

Somatic embryos (globular-torpedo shaped) are separated individually and auxin pulse treatment is given on the MS medium containing 1 mgl^{-1} IBA (indole-3-butyric acid). After 2 weeks, somatic embryos elongate radicle (Fig. 5); transfer to germination medium containing agar gelled (0.7%) MS basal salts supplemented with 3% sucrose + 1.5 mgl^{-1} GA_3 + 300 mgl^{-1} glutamine (Table 1 & 2); incubation under fluorescent light in a 16/8 h light/dark cycle at 24 ± 1°C. Somatic embryos germinate and convert into complete normal somatic seedlings (Fig. 5) within 40 days. The secondary somatic embryos and the embryos developed from epicotyl-derived callus are cultured in the germination medium to form somatic seedlings. Normally 60% somatic embryos form somatic seedlings, which are maintained *in vitro* in germination medium (Table 1 & 2) until they are 5-8 cm in length and then they are hardened and acclimatized.

6. ACCLIMATIZATION AND FIELD TRANSFER

For acclimatization of the somatic seedlings, the culture vessels containing seedlings are taken out from the plant growth chamber and keep them at normal room temperature and under indirect day light for 10 days. The plantlets are removed carefully from the culture vessels, and washed gently under running tap water in order to remove agar attached with the roots. Immediately, they are dipped in 5% Bavistin (fungicide) solution for 5 min and transplanted in the cavity of polystyrene seedling trays (64 cavity each). The cavity is filled with autoclaved mixture of compost and vermiculite (7:3). At the time of transplantation, care is taken to prevent root damage. The trays containing the seedlings are covered with transparent polyethylene sheet for maintaining 80-85% relative humidity under a double layer of 20% shade net in the greenhouse and plantlets with supporting materials are moistened twice a day. The polyethylene sheets are gradually removed to expose plantlets to the outer environment and subsequently removed after 20 d. Plantlets are regularly watered every 2 d depending on the weather and sprayed liquid fertilizer (10% solution of 4N: 4P: 2K). Within 3 months, plantlets are acclimatized and transferred to the nursery. After two months, plantlets are transplanted into 10-litre polybags filled with compost and alluvial soil (2:3). They are watered and fertilized as needed and after six months the plants are transplanted in the open field, where the plants have been growing normally.

7. CONCLUSION AND FURURE PROSPECTS

Our results suggest that the problems related to somatic embryogenesis are similar to other woody species. However, with this protocol, sufficient somatic seedlings have been produced for the establishment of superior genotypes. Somatic embryos directly developed on the cotyledonary leaf segments are capable of germination by direct transfer on the germination medium. Once initiated from epicotyl segment the embryogenic cultures can be efficiently multiplied and maintained without loss of embryogenic potential by regular subculture of embryogenic callus on media supplemented with low levels plant growth regulators.

Although somatic embryos are obtained from epicotyle derived-callus, embryo development and germination rates are low, embryo-to-plant conversion being limited by poor shoot development. So far somatic embryogenesis in this species originates from germinating seedlings. The regenerated plants can, therefore, be cloned of a sexually recombined genotype, but not of a single donor tree.

Somatic embryos have not yet been obtained from mature material of Indian olive. In order to realize the potential of this technique for clonal multiplication and deployment, further studies are necessary: firstly, to induce somatic embryo production from explants of mature selected trees; secondly, to achieve synchronized embryo development, and thirdly, to optimize medium composition so as to satisfy the better physiological requirements of embryos, thereby enabling more embryos to mature and enhancing conversion rate.

8. REFERENCES

Anonymous, 1964. The wealth of India, Raw Materials Vol-1: E. Dictionary of Indian Raw Materials and Industrial products, pp. 431-433. Publications and Information Directorate, CSIR, New Delhi, India.

Caius, J.F. 1986. The Medicinal and Poisonous Plants of India. pp. 476-477.

Das, D.K. 1987. Edible Fruits of Bangladesh Forests. Bulletin on Plant Taxonomy Series. p. 13. Bangladesh Forest Research Institute, Chittagong, Bangladesh.

Drury, C.H. 1985. The Useful Plants of India. pp. 316-319. Naya Prokash, Calcutta., India.

Jain, S.M., P.K. Gupta and R.J. Newton (Eds). 1995. Somatic Embryogenesis in Woody Plants, Vol. 2- Angiosperms. Kluwer Academic Publishers. Dordrecht / Boston / London.

Krul, W.R. 1985. *In vitro* propagation of grape. United States Patent No. 4,532,733.

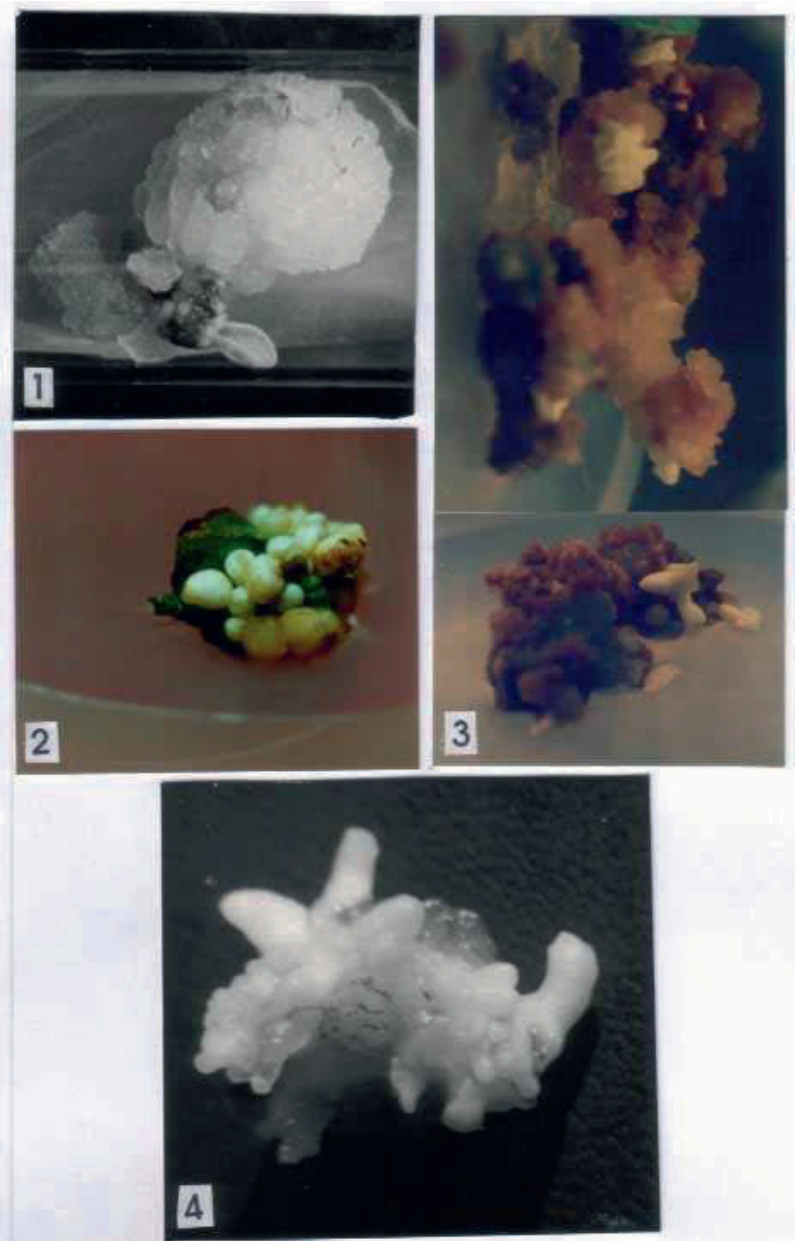
Krul, W.R. and J.F. Worley. 1977. Formation of adventitious embryos in callus cultures of "Seyval", a French hybrid grape. J. Amer. Soc. Hort. Sci. 102:360-363.

Kirtikar, K.R., Basu, B.D. 1981. Indian Medicinal Plants. Vol. I. pp. 406-407. Reprinted Second Edition, 1975. Bishen Singh Mahendra Pal Singh, Dehra Dun, India.

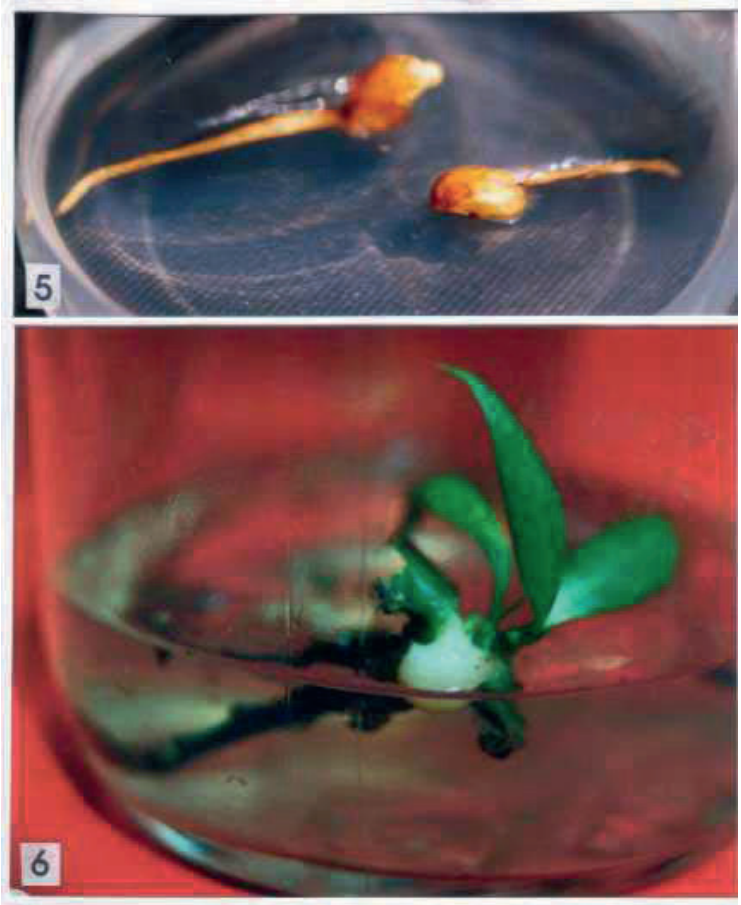
Mullins, M.G. and C. Srinivasan. 1976. Somatic embryos and plantlets from an ancient clone of grapevine (cv. Cabernet-Sauvignon) by apomixes *in vitro.* J. Exp. Bot. 27:1022-1030.

Murashige, T, Skoog, F. 1962. A revised medium for rapid growth and bioassys with tobacco tissue cultures. Physiol. Plant. 15: 473-497.

Roy, S.K., Islam, M.S., Hadiuzzaman, S. 1998. Micropropagation of *Elaeocarpus robustus.* Plant Cell Rep. 17: 810-813.



Figures 1-4. Somatic embryogenesis in Indian olive (*Elaeocarpus robustus*). 1. Callus induction in epicotyl segment cultured on MS medium with 3% sucrose and 2.5 mgl^{-1} 2,4-D. 2. Cluster of seed-like somatic embryos directly developed on the cotyledonary leaf segment cultured on MS medium + 3% sucrose + 2 mgl^{-1} BA + 0.5 mgl^{-1} NAA. 3. Formation of secondary somatic embryos from misshaped primary embryos developed on the cotyledonary leaf segment when subcultured in MS medium + 3% sucrose + 2 mgl^{-1} BA + 0.5 mgl^{-1} NAA. 4. Formation of different stages of somatic embryos from epicotyl induced callus cultured in MS medium with 2.5 mgl^{-1} BA + 0.5 mgl^{-1} IBA.



Figures 5-6. Germination of somatic embryos. 1. Formation and elongation of radicle from somatic embryos (as mentioned in Fig. 2) cultured in MS medium with 1 mgl^{-1} IBA. 6. Fully grminated somatic embryo cultured in MS medium with 1.5 mgl^{-1} GA_3 + 300 mgl^{-1} glutamine.

RESCUE OF ENDANGERED PALMS BY IN VITRO METHODS: THE CASE OF 'BOTTLE PALM'

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1. INTRODUCTION

The presence of only a single meristem and the low viability of seeds are the main reasons for recalcitrance of palms in culture. *In vitro* seed germination has been used to improve the germination rates of palms (Zaid and Tisserat 1984). In vitro propagation by somatic embryogenesis represents an alternative approach that has been reported in *Cocos nucifera* (Chan et al. 1998), *Elaeis guineensis* (Teixeira et al. 1993), *Phoenix dactylifera* (Veramendi and Navarro, 1996) and *P. canariensis* (Huong et al. 1999). As the conservation of palm seeds through storage at ambient temperature or in liquid nitrogen is difficult because of the high moisture content of the seeds, storage of somatic embryos might provide an alternative approach.

The 'bottle palm', *Hyophorbe lagenicaulis* (L. Bailey) H.E. Moore, is endemic to Mauritius, where it is confined to lowland palm savannah on Round Island. It grows to 6 m in height and has a bottle-shaped trunk. The leaves are red or orange tinted when young and green at maturity. By the 1970s, the species faced extinction in the wild. The wild population fell to 7 or 8 plants, because rabbits and goats were introduced to its habitat. After the successful removal of the herbivores from the island in 1986, Round Island has become a managed nature reserve. Currently, there are more than 250 palm trees that constitute the wild population of this species. However, germination of seeds in the wild takes 5-6 months and is sporadic. For this reason, *in vitro* germination of seeds and the induction of somatic embryos were investigated by Sarasan et al. (2002).

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The article describes *in vitro* methods that are being developed to propagate *H. lagenicaulis* and which might be used for other palms.

2. *IN VITRO* GERMINATION OF ZYGOTIC EMBRYOS

Hyophorbe lagenicaulis fruits are collected from the Botanic Gardens Conservation Department, Mauritius, and Royal Botanic Gardens, Kew, England, which are classified by characters of the fruit wall and endosperm into categories of increasing maturity; M1 (green fruit with soft endosperm), M2 (green fruit with semi-hard endosperm) and M3 (dark brown fruit with hard endosperm). Remove outer flesh and inner fibrous layers of the fruit and clean the seeds with scouring pad and leave them overnight in distilled water. Surface sterilise seeds by dipping first for 40 sec in 70% alcohol, and then immerse for 40 min in an aqueous solution of 0.5% (w/v) sodium dichloroisocyanurate (an effective bleach that causes little damage to plant material), containing a drop of Tween-20 on an orbital shaker (100 rpm). Wash sterilised seeds in sterile distilled water and excise embryos. The endosperm hardens with age and M2 & M3 embryos are removed with scalpel blade by chipping off the endosperm.

Transfer excised embryos to the culture medium containing MS salts and vitamins (Murashige and Skoog, 1962), sucrose (60 gl^{-1}), and activated charcoal (2 gl^{-1}). Maintain the cultures at 26±2°C and provide light with cool white fluorescent lamps (PPFD 25 µmol $m^{-2}s^{-1}$) for a 12h photoperiod.

Zygotic embryos enlarge and double in volume within three days of culture. Add activated charcoal in the culture medium to prevent browning of tissues, which otherwise slows growth. Avoid contamination of cultured zygotic embryos for preventing loss of cultures. This is particularly important for the conservation of endangered plants, as availability of the material from the wild is limited.

Signs of germination, including the appearance of haustorial, plumular and radicular nodules, occur in M1 embryos within 7-11 days and in both M2 and M3 embryos within 7 days. Embryos that do not germinate within first two weeks don't develop any further. Seedlings are classified according to whether the

haustorium is: a) absent, b) small or c) large. The proportion in each class varies amongst M1, M2 and M3 embryos (Fig.1), haustorium being present in significantly fewer M1 embryos (32.1%) than M2 and M3 embryos. Those somatic embryos lacking haustorium fail to germinate (Fig. 1).

3. INITIATION OF SOMATIC EMBRYOS FROM SEEDLINGS

3.1. Somatic embryo induction

Juvenile and mature-phase explants have previously been used for the induction of somatic embryos in palms (Teixeira et al. 1993, Veramendi and Navarro, 1996). The investigation described here differs from others in that a strategy of using sectioned germinating embryos was explored.

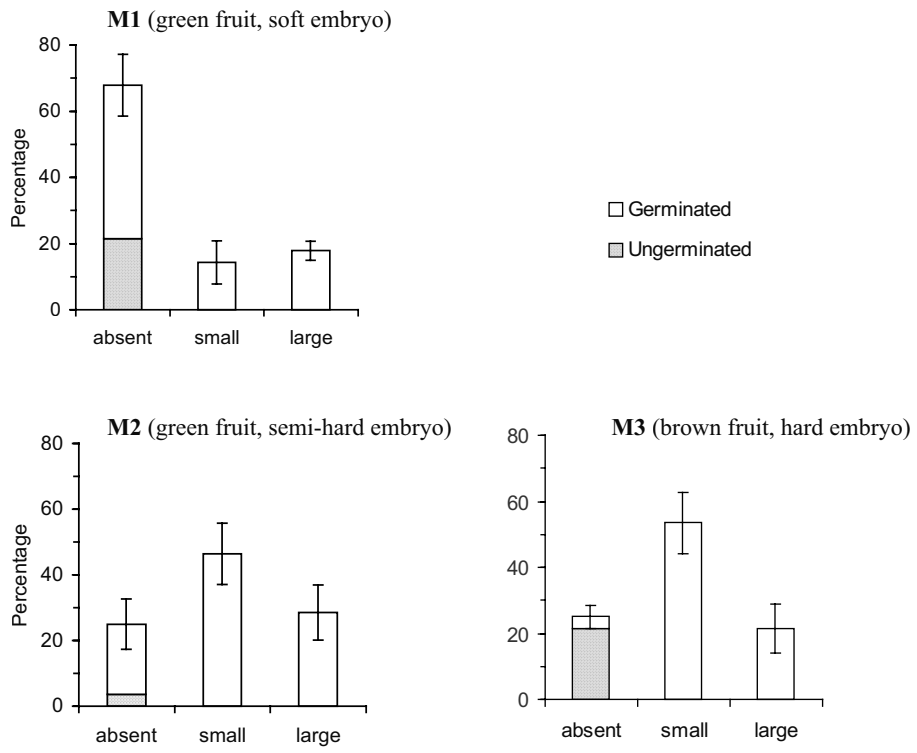

Figure 1. Percentages of embryos from M1, M2 and M3 seeds, in which the haustorium was absent, small or large. Embryos that germinated or remained ungerminated on MS medium containing 30 gl^{-1} sucrose and 3 gl^{-1} activated charcoal are distinguished. Error bars indicate standard deviations for 7 replicates (Sarasan et al., Plant Cell Rep., 2002, reproduced with permission)

Take 2-3-week-old *in vitro* seedlings with large haustoria, and cut sections longitudinally into two halves through the haustorium, plumule and root (Fig. 2A). In treatment 1 (T1) half seedlings (one per Petri dish) are not further divided, whereas in treatment 2 (T2) the haustorium, plumule and the root are separated.

The sectioned seedlings are then partially embedded in embryo induction medium. The culture medium for the induction of somatic embryo is composed of sucrose (30 gl^{-1}), 2,4-dichlorophenoxyacetic acid (2,4-D, 3 mgl^{-1}) and is solidified with agar. For further more tests, see table 1. The culture medium is poured in aliquots of 25 ml into single-vented Petri dishes (90 mm diameter), and properly sealed with parafilm. All cultures are incubated in darkness at 26 ±2°C.

Table 1. Growth regulators tested for induction and germination of somatic embryos

Medium	Growth regulators (mgl^{-1})																					
	2,4-D					BA				NAA				TDZ				GA3				
Induction (SI)	1	2	3	4	5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	0.1	0.5	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1	0.5	1	2	-	-	-		
Germination	-	-	-	-	-	0.1	0.5	1	2	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	0.5	-	-	-	0.1	0.5	1	2	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	0.5	-	-	-	-	-	-	-	-	-	-	-	0.1	0.5	1	2	

Globular staged somatic embryos form directly on the cut surface (Fig.2B) of longitudinal sections in somatic embryo induction medium (SI medium) with in 6 weeks of culture. Use 3-week-old seedlings with haustoria for cutting longitudinal sections. A large number of somatic embryos form (Fig. 3) on sectioned, undivided half-seedlings (T1 seedlings) than on sectioned seedlings separated into haustoria, plumules and roots (T2 seedlings). Student's t-tests show that their frequencies are significantly higher ($p<0.01$) on separated haustoria, plumules and radicles. Callus forms along with somatic embryos on T2 but not in T1 seedlings, and the development beyond the globular stage occur only on T1 seedlings. On T1 seedlings, globular and elongated embryos are initially fused in clusters, which separate as development proceed. At maturity, 28% embryos on the haustoria, 50% on the plumules and 60% on the radicles of T1 seedlings remain fused.

Browning of the haustorium leads to browning of somatic embryos, which can be prevented by transferring embryos to the fresh medium. When charcoal is added to the culture medium to prevent browning, embryos form callus. Browning accelerates when sucrose is replaced with maltose in the liquid medium. Lower concentrations of 2,4-D (0.1, 0.5, 1 and 2 mgl^{-1}) in the culture medium form few embryos and at a higher concentration (5 mgl^{-1}) more callus is formed and rapid browning of tissues. The substitution of 2,4-D with either BA or TDZ (0.1, 0.5, 1 and 2 mgl^{-1}) does not change the results. When somatic embryos are transferred individually to the fresh culture medium containing 2,4-D ($3mgl^{-1}$) and sucrose, secondary embryos form which appear similar to the primary somatic embryos. Secondary embryos are formed directly on the primary embryos after four weeks.

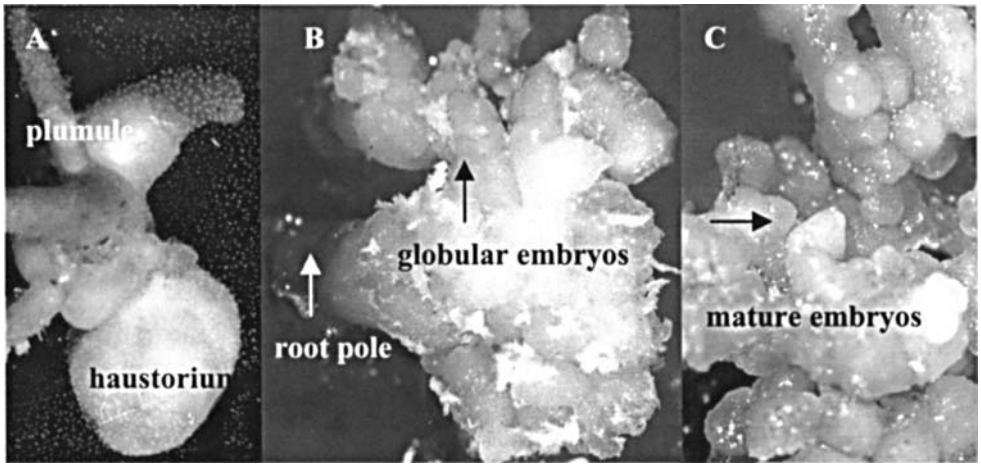

Figure 2. Germination of zygotic embryos and stages of somatic embryogenesis. A; a zygotic embryo 2 weeks after introduction to in vitro culture, B. somatic embryos forming at the root pole, C; mature embryos forming on plumule.

The haustorium of palms is involved in the hydrolysis of the reserves in the endosperm and the mobilisation of nutrients required for embryo germination. Studies in coconut have indicated the existence of vascular bundles in the haustorium that converged towards the embryonic axis (Verdeil *et al*. 1998). Longitudinal sectioning exposes the vasculature of the seedling and facilitates contact with the medium. Retention of communication between the haustorium, plumule and radicle is apparently important because, when they are separated (treatment T2), there is a marked reduction in the number of embryos formed. However, the genotype of the selected explants may have influenced the type of response. In a separate trial on seeds collected from the trees in Royal Botanic Gardens Kew the number of embryos produced is less than from seeds collected from the wild in Mauritius.

Browning is initiated in the haustorium both during the induction of somatic embryos and the germination of zygotic embryos and may be associated with the activity of enzymes produced for the digestion of stored food reserves. The inclusion of charcoal in culture media to prevent browning might reduce the availability of nutrients and hormones. In oil palm, the auxin requirement was raised 50-fold by inclusion of activated charcoal in the medium (Teixeira *et al*. 1993). In *Phoenix canariensis*, the presence of activated charcoal completely inhibited the induction of embryogenic callus, even when the concentration of auxin was raised (Huong *et al.* 1999). In the study on *H. lagenicaulis*, the inclusion of charcoal leads to callusing, loss of embryogenic potential and browning, but morbidity is avoided by subculturing somatic embryos at 2-week intervals.

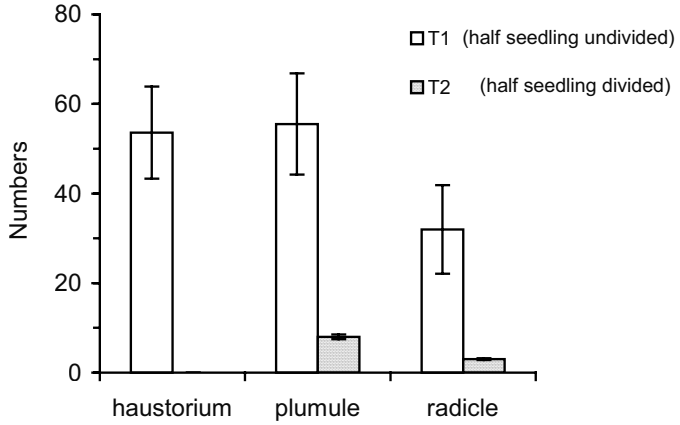

Figure 3. The number of somatic embryos that formed on the haustorium, plumule and radicle of longitudinally sectioned seedlings on MS medium containing 30gl^{-1} sucrose and 3 mgl^{-1} 2,4-D. . Error bars indicate standard deviations for 6 replicates (Sarasan et al., Plant Cell Rep., 2002, reproduced with permission)

3.2. Somatic embryo germination

Culture mature somatic embryos on agar-solidified germination media containing MS salts and vitamins, and growth regulators as indicated in Table 1. Cultures are maintained in Petri dishes for 6-10 weeks. At 2-week interval, transfer discoloured somatic embryos to the same culture medium.

After transfer to germination media, most embryos increase in size but none regenerates plantlets that could be transferred to soil. On medium containing MS at full strength and $0.1 mgl^{-1}$ BA, 7% embryos show plumular development. Hypertrophy of the embryonic axis, caused by callusing and browning, impair further development. The addition of GA_3 (1 mg l^{-1}) reduces browning and increases callusing.

The direct regeneration of primary and secondary embryos by adopting this protocol is less likely to generate somaclonal variants. than regeneration from callus tissue because the number of cell generations in the disorganised growth phase is minimised. The generation of genetic variants, which might include abnormal, ill-adapted plants, would be particularly unwelcome in endangered species of small population size. Effects on major morphological features and fertility would manifest only after several years as palms have long life cycle.

5. CONCLUSIONS

A higher rate of seed germination *in vitro* is a major advantage compared to the sporadic germination that occurs *in vivo*. Development of somatic embryos in palms is difficult and needs complex culture conditions. However, a protocol to produce somatic embryos in large scale is essential to conserve the germplasm of endangered palms as storage of palm zygotic embryos is erratic and unreliable in most cases. Somatic embryos are ideal material for cryopreservation and can be applied to other endangered palms.

Germination of somatic embryos was found to be problematic and, along with cryopreservation of somatic embryos, will be the subject of further investigations using seeds collected both from wild and other sources.

REFERENCES

Chan JL, Saenz L, Talavera C, Hornung R, Robert M, Oropeza C (1998) Regeneration of coconut (*Cocos nucifera* L.*)* from plumule explants through somatic embryogenesis. Plant Cell Rep 17:515-521

Huong LTL, Baiocco M, Huy BP, Mezzetti B, Santillocchi R, Rosati P (1999) Somatic embryogenesis in Canary Island date palm. Plant Cell Tissue Organ Cult 56:1-7

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Sarasan V, Ramsay MM, Roberts AV (2002) In vitro germination and induction of direct somatic embryogenesis in 'Bottle Palm' [*Hyophorbe lagenicaulis* (L. Bailey) H. E. Moore], a critically endangered Mauritian palm. Plant Cell Rep 20:1107-1111

Teixeira JB, Sondahl MR, Kirby EG (1993) Somatic embryogenesis from immature zygotic embryos of oil palm. Plant Cell Tissue Organ Cult 34:227-233

Veramendi J, Navarro L (1996) Influence of physical conditions of nutrient medium and sucrose on somatic embryogenesis of date palm. Plant Cell Tissue Organ Cult 45:159-164

Verdeil JL, Hocher V, Triques K, Laykurwa R, Rival, A, Durand-Gesselin T, Engelmann F, Sangare A, Hamon S (1998) State of research on coconut embryo culture and acclimatization techniques in the IDEFOR (Cote d'Ivoire) and ORSTOM/CIRAD laboratories (France). In: Batugaland PA and Engelmann F (eds) Coconut Embryo Culture, CGIAR, Rome Italy, pp 17-26

Zaid A, Tisserat B (1984) Survey of the morphogenetic potential of excised palm embryos *in vitro*. Crop Res. 24:1-9

SOMATIC EMBRYOGENESIS IN AMERICAN GRAPES (*Vitis* x *labruscana* L.H. Bailey)

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1. INTRODUCTION

Successful embryogenesis and subsequent maintenance and plant conversion in grapevines has been restricted to a few species including *Vitis vinifera, V. longii* and *V. rupestris,* or hybrids composed of at least one of these species (reviewed by Reisch and Pratt, 1996; Gray and Meredith, 1992). In the last decade *V. rotundifolia* (Robacker, 1993), *V. berlandieri* x *V. riparia* (Mauro et al., 1995), *V. latifolia* (Salunkhe et al., 1999), and *V. riparia* (Xue et al., 1999) were added to the list, but not *V. x labruscana* L.H. Bailey. Nakano et al. (1997) attempted to make embryogenic cultures of *V.* x *labruscana*, but were completely unsuccessful. Kikkert et al. (1997) reported 0.1 to 3% embryogenesis in *V.* x *labruscana* cultivars, but made no mention of maintenance or conversion of their cultures to plants.

Many grape geneticists agree that the most efficient way to genetically transform grapevines involves somatic embryogenesis (Perl and Eshdat, 1998). Recently, grapevine cells have been genetically transformed and plants regenerated using somatic embryogenic cultures as the original target tissue,

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but most of them are *V. vinifera* cultivars (Perl and Eshdat 1998). However, due to high genotype dependence, some grape species and cultivars remain recalcitrant to the process of embryogenesis and transformation. *V.* x *labruscana* cultivars have been among the most recalcitrant. The protocol detailed below describes the procedure for the induction and maintenance of embryogenic cultures for two *V. x labruscana* cultivars ('Fredonia' and 'Niagara'). We further report that these lines can be maintained for at least three years, and they could be readily converted to plants during that period as well as used successfully in genetic transformation experiments.

2. EMBRYOGENIC CULTURE

2.1. Explant type and disinfestation

The explant of choice to establish somatic embryogenic cultures from *V. x labruscana* cultivars is the ovary and its associated tissues (receptacle + tissues attached to the ovary) obtained from immature flowers. To avoid high contamination of field-grown material, dormant but fully vernalized cuttings of grapevines with large and fertile buds are gathered from parent vines during winter and rooted in a pot under greenhouse environment. When shoots start emerging they are sprayed daily with a solution containing 2.0 gl^{-1} Captan and 0.01% Tween 20 until the flower clusters reach the right maturity for embryogenesis experiments. Ten to 14 days from anthesis, flower clusters are collected and sealed in a plastic bag for a 72h chilling treatment at 4^{o}C. After chilling flower clusters are disinfested with a 1.3% sodium hypoclorite (NaOCl) solution containing 0.1% (v/v) Tween 20 for 10 minutes and rinsed 4 times with sterile distilled water (5 min/rinse). The ovary and its associated tissues are dissected from the immature flowers under aseptic conditions aided by a stereomicroscope and then explanted onto embryogenic *culture initiation medium* (CIM, Table 1).

2.2. Inoculation and initiation

Because fungal and bacterial contamination spread fast in the tissue culture environment, it is recommended that each explant is placed in a separate vessel. In our laboratory we use culture tubes (25 X 150 mm) containing 5.0 ml

of CIM; one explant per tube. To minimize medium dehydration, the culture tubes are sealed with Parafilm® after inoculation. These test tubes are then incubated in the dark at 21°C ± 1°C. Successful embryogenic cultures develop a proembryonic mass-like structure within 50 days after inoculation at a rate from 16% to 25% for 'Niagara' and 'Fredonia', respectively. These structures are slow growing, friable, white to dark, with a nodular texture, and differ from other type of tissues growing on explants in this medium (Fig. 1).

2.3. Long-Term Maintenance of Embryogenic Lines

Although CIM is efficiently used to initiate embryogenesis in *V.* x *labruscana*, the medium is not adequate to maintain long-term embryogenic cultures, which turn dark and

Table 1. The composition of Embryogenic Culture Initiation Medium (CIM)

Description	Concentration
Inorganic nutrients	Full strength Nitsch and Nitsch (NN, 1969) salts
Sucrose	30 gl^{-1}
Myo-inositol	100 mgl^{-1}
Casein hydrolysate	0.8 gl^{-1}
Indole-3-acetyl-L-asparticacid(IASP, dissolved in dimethyl sulfoxide)	17 μM (added after autoclaving[1])
2,4-dichlorophenoxyacetic acid (2,4-D)	9 μM
6-benzyladenine (BAP)	1 μM
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])
Gelling agent (Phytagel)	2.5 gl^{-1}

[1] The medium was autoclaved for 20 min at 121 °C.

eventually die. Therefore, to maintain long-term embryogenic lines of *V.* x *labruscana* cultivars, proembryonal masses obtained in CIM should be cultivated in a long- term maintenance medium (LTMM, Table 2).

In order to maintain embryogenic cultures for extended periods, proembryonic masses originated from CIM are divided into small portions, approximately 2.0 mm in diameter and inoculated onto *Long Term Maintenance Medium* (LTMM, Table 2). The cultures are maintained in 100 x 15 mm disposable Petri plates containing about 30 ml per plate LTMM under dark incubation at 21°C ± 1°C.

After a few days in LTMM, 'Fredonia' and 'Niagara' embryogenic cultures rejuvenate by developing globular embryogenic masses (Figure 2). In a recent analysis of 50-day-old 'Niagara' and 'Fredonia' embryogenic cultures, Motoike et al. (2001) observed a diameter increase of 6.0 and 7.0 mm, respectively, on the basal cross section of cultures, measured at the surface of the medium where the cells had been in contact. In our laboratory *V.* x *labruscana* embryogenic lines have been maintained up to 3 years under frequent subculturing in LTMM, with no obvious loss of embryogenic competence. Also, the proembryonic lines maintained in LTMM were more synchronized (Motoike et al., 2001). This control permitted us to transform cells and screen for transformants efficiently. Since each stage of embryo development is differentially sensitive to selection agents (Motoike et al., 2002), the synchronization made screening for transgenic embryos at different stages of development possible.

2.4. Embryo Development and Maturation

Vitis x *labruscana* sp. produce orthodox seeds, which means after they are completely formed, the seeds or embryos enter into a maturation phase. The maturation phase of orthodox seeds is characterized by a critical dehydration

Table 2. The composition of Long Term Maintenance Medium (LTMM).

Description	Concentration
Inorganic nutrients	Full strength Nitsch and Nitsch (NN, 1969) salts
Sucrose	30 gl^{-1}
Myo-inositol	100 mgl^{-1}
Casein hydrolysate	0.8 gl^{-1}
Indole-3-acetyl-L-asparticacid(IASP, dissolved in dimethyl sulfoxide)	4μM (added after autoclaving[1])
2,4-dichlorophenoxyacetic acid (2,4-D)	2μM
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])
Gelling agent (Phytagel)	2.5 gl^{-1}

[1] The medium was autoclaved for 20 min at 121°C.

and consequent arrest of embryo development (Litz and Gray, 1992). The maturation phase in these embryos is a transitory but indispensable stage

between embryo development and embryo germination phases. However, *V.* x *labruscana* somatic embryos do not normally enter into a maturation phase after the torpedo stage unless they are induced to in a specially formulated medium, an embryo development and maturation medium (EDMM, Table 3).

The embryo development and maturation phase is carried out in 100 x 15 mm disposable Petri plates filled with 30 ml EDMM. Five or more proembryonic masses (ca 2.0 mm in diameter) are plated in each plate and then incubated in a photoperiod of 16h days (cool white fluorescent light) at temperatures between 20 and 22^{o} C. The photosynthetically active radiation (PAR) at the level of the medium surface is 131µmol m^{-2} s^{-1}. Within 30 days several mature embryos arise from the proembryonic masses (Fig. 3).

Table 3. The composition of Embryo Development and Maturation Medium (EDMM)

Description	Concentration
Inorganic nutrients	Full strength Nitsch and Nitsch (NN, 1969) salts
Sucrose	30 gl^{-1}
Myo-inositol	100 mgl^{-1}
Casein hydrolysate	0.8 gl^{-1}
Indole-3-acetyl-L-asparticacid(IASP, dissolved in dimethyl sulfoxide)	17 µM (added after autoclaving[1])
β-naphthoxyacetic acid (NOA)	10 µM
1,2,3-thiadiazol-5-yl-N'-phenylurea (TDZ)	1 µM
Abscisic acid (ABA)	1 µM
Activated charcoal (Darco S 51)	2.5 gl^{-1}
Polyethylene glycol (PEG, Amresco-OH)	50 gl^{-1}
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])
Gelling agent (Phytagel)	2.5 gl^{-1}

[1] The medium was autoclaved for 20 min at 121^{o}C.

2.5. Embryo Germination and Conversion

Somatic embryo germination is characterized by cotyledon expansion and chlorophyll formation, followed by radicle and hypocotyl elongation (Merkle and Wiecko, 1990). The process of shoot meristem development and subsequent leaf initiation from the apical meristem is known as conversion (Nickle and Yeung, 1994). Poor germination and conversion is typical in many embryogenic culture systems (Litz and Gray, 1992). Faure

et al. (1996) attributed the poor germination and conversion rates of cultured embryos to precocious germination during the maturation stage.

In the present protocol, successful germination of *V.* x *labruscana* mature somatic embryos can be accomplished in two different media, 'Fredonia' germination medium (FGM, Table 4) and 'Niagara' germination medium (NGM, Table 5).

The germination process is carried out in 100 x 15 mm disposable Petri dishes. More than thirty somatic embryos can be germinated in a single plate. After inoculation the plates are incubated in the dark for seven days and then transferred to light in a culture room maintained under a photoperiod of 16h (cool white fluorescent light) at 20 to 22^{o} C and the PAR at the medium surface around 131µmol $m^{-2}s^{-1}$. In these conditions Motoike et al. (2001) obtained 93% and 56% germination for 'Fredonia' and 'Niagara' somatic embryos, respectively.

After germination *V.* x *labruscana* somatic embryos complete their conversion in a conversion medium (CM, Table 6). The conversion is performed in baby food jars (ca 9.0 x 5.0 cm). The germinated plantlets are allowed to grow in this medium for 30 days and then transferred to soil in a greenhouse (Fig. 4).

Table 4. The composition of 'Fredonia' Germination Medium (FGM)

Description	Concentration
Inorganic nutrients	Full strength Nitsch and Nitsch (NN, 1969) salts
Sucrose	30 gl^{-1}
Myo-inositol	100 mgl^{-1}
Vitamins	Staba (1969)
6-benzyladenine (BAP)	0.4µM
□-naphthalene-acetic acid (NAA)	0.5µM
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])
Gelling agent (Phytagel)	2.5 gl^{-1}

[1] The medium was autoclaved for 20 min at 121^{o}C.

Motoike et al. (2001) using this protocol found that the conversion rates of germinated *V* x *labruscana* somatic embryos were relatively low, 18% and 15%, respectively, for 'Fredonia' and 'Niagara'. However, these conversion rates were

sufficient to regenerate transformed plants in genetic transformation experiments (Motoike et al., 2002).

Table 5. The Composition of 'Niagara' Germination Medium (NGM)

Description	Concentration
Inorganic nutrients	Full strength Nitsch and Nitsch (NN, 1969) salts
Sucrose	30 gl^{-1}
Myo-inositol	100 mgl^{-1}
Vitamins	Staba (1969)
Indole-3-acetyl-L-aspartic acid (IASP, dissolved in dimethyl sulfoxide)	4.0μM (added after autoclaving[1])
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])
Gelling agent (Phytagel)	2.5 gl^{-1}

[1] The medium was autoclaved for 20 min at 121°C.

Table 6. The composition of Conversion Medium (CM).

Description	Concentration
Inorganic nutrients	75% C_2D salts (Chee and Pool, 1983)
Sucrose	22.5 gl^{-1}
Myo-inositol	100 mgl^{-1}
Vitamins	Staba (1969)
□-naphthalene-acetic acid (NAA)	1.0 μM
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])
Gelling agent (agar, Sigma-Aldrich Co.)	8 $g.l^{-1}$

[1] The medium was autoclaved for 20 min at 121°C.

3. APPLICATIONS

Somatic embryogenic cultures can be used for several purposes, which include cryopreservation, in vitro selection of somaclonal variants, bioreactors, encapsulation for the production of artificial seeds and genetic transformation. Among several applications the *V.* x *labruscana* somatic embryogenic culture were successfully used in genetic transformation experiments. The protocol detailed below describes an efficient method for *Agrobacterium tumefaciens*-mediated transformation of *Vitis* x *labruscana* c.v. 'Fredonia' based on a published procedure (Motoike et al., 2002) where 'Fredonia' proembryonic cultures were cocultivated with *A. tumefaciens*

strain LBA 4404 carrying constructor *Agl-gus* BS35 built in pBI 101 (Figure 2). *Agl-gus* BS35 contains both a kanamycin resistance gene (*NPTII*) under the control of nopaline synthase (*NOS*) promoter, and the β-glucuronidase reporter gene (*GUS*), fused to a flower-specific and developing fruit-specific promoter, *Agl5*, which allows for the expression of *GUS* in flowers and developing fruits including embryos of transformed grapevines.

3.1. Preparation of *Agrobacterium tumefaciens* Cells

Agrobacterium cells are grown overnight, under vigorous shaking at 21°C ± 1°C in culture tubes containing 5 ml liquid Luria-Bertani Medium (LB, Sambrook et al., 1989) with 50 mgl^{-1} kanamycin and 20 mgl^{-1} rifampicin. After 12 hours growth, the *Agrobacterium* culture is centrifuged at 5000 x *g* for 10 minutes, and the pellet is rinsed and resuspended in the same volumes of liquid Nitsch and Nitsch (NN, 1969) medium enriched with 100 μM acetosyringone (Scorza et al., 1995). The *Agrobacterium* cells are then activated for the transformation process by shaking them vigorously for six hours at the environmental conditions described previously (Scorza et al., 1995).

3.2. Co-cultivation of Targeted Embryogenic Cells with *Agrobacterium*

Embryogenic cells from *V.* x *labruscana* cv 'Fredonia' are inoculated by

Table 7. The composition of the Cocultivation Medium (CCM).

Description	Concentration
Inorganic nutrients	Full strength Nitsch and Nitsch (NN, 1969) salts
Sucrose	30 gl^{-1}
Myo-inositol	100 mgl^{-1}
Casein hydrolysate	0.8 gl^{-1}
Indole-3-acetyl-L-asparticacid(IASP, dissolved in dimethyl sulfoxide)	4μM (added after autoclaving[1])
2,4-dichlorophenoxyacetic acid (2,4-D)	2μM
Polyvinylpolypyrrolidone (PVPP)	1%
Acetosyringone	100 μM (added after autoclaving[1])
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])
Gelling agent (Phytagel)	2.5 gl^{-1}

[1] The medium was autoclaved for 20 min at 121 °C.

immersing them in a test tube containing the activated *Agrobacterium* cell solution for 10 minutes. About 500 mg of fresh embryogenic culture cells are used for transformation. After inoculation the excess *Agrobacterium* is removed by blotting the embryogenic cells on a stack of sterile paper towels. The embryogenic cells are then co-cultivated with *Agrobacterium* in the dark at 21°C ± 1°C in a Petri dish containing 30 ml of co-cultivation medium (CCM, Table 7), for 48 hours.

3.3. Incubation of Co-cultivated Embryogenic Cells

After co-cultivation, the embryogenic cells are washed in liquid NN medium containing 500 mgl^{-1} carbenicillin to inhibit further *Agrobacterium* growth, and then transferred to incubation medium (IM) in Petri dishes (150 x 15 mm). The IM has two layers, a solid phase (Table 8) and a liquid phase (Table 9). The solid phase is made of 25 ml solid IM, and it is overlaid with 5 ml of liquid IM. Embryogenic cells are then incubated for seven days in the dark at 21°C ± 1°C, and then transferred to selection media.

Table 8. Composition of the Solid Phase Incubation Medium (solid IM).

Description	Concentration
Inorganic nutrients	Full strength Nitsch and Nitsch (NN, 1969) salts
Sucrose	30 gl^{-1}
Myo-inositol	100 mgl^{-1}
Casein hydrolysate	0.8 gl^{-1}
Indole-3-acetyl-L-asparticacid(IASP, dissolved in dimethyl sulfoxide)	4 μM (added after autoclaving[1])
2,4-dichlorophenoxyacetic acid (2,4-D)	2 μM
Polyvinylpolypyrrolidone (PVPP)	1%
Carbenicillin	500 mgl^{-1}
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])
Gelling agent (Phytagel)	2.5 gl^{-1}

[1] The medium was autoclaved for 20 min at 121°C.

3.4. Selection of Transformants

The selection of transformed embryogenic cells is made in three steps using LTMM with 500 $mg.l^{-1}$ carbenicillin and step-wise kanamycin concentrations.

The first selection step contains levels of kanamycin that impose a 50% reduction in growth of non-transformed embryogenic cells (54 mgl^{-1}). The second selection step contains levels of kanamycin that impose 75% reduction in growth of non-transformed embryogenic cells (107 mgl^{-1}). The third and final selection step contains the highest level of kanamycin (200 mgl^{-1}), which we assume based on previous studies is high enough to completely stop growth of non-transformed embryogenic cells.

Table 9. The composition of Liquid Phase Incubation Medium (liquid IM)

Description	Concentration
Inorganic nutrients	Full strength Nitsch and Nitsch (NN, 1969) salts
Sucrose	30 gl^{-1}
Myo-inositol	100 mgl^{-1}
Casein hydrolysate	0.8 gl^{-1}
Indole-3-acetyl-L-asparticacid(IASP, dissolved in dimethyl sulfoxide)	4 μM (added after autoclaving[1])
2,4-dichlorophenoxyacetic acid (2,4-D)	2 μM
Dithiothreitol (DTT)	2.5 $mgml^{-1}$
Carbenicillin	500 mgl^{-1}
pH	5.6 (adjusted before the addition of gelling agent and autoclaving[1])

[1] The medium was autoclaved for 20 min at 121°C.

To perform selection, small embryogenic clumps (1.5 to 2.0 mm in diameter) from IM are transferred to Petri dishes containing the first step selection medium and incubated for 30 days in the dark at 21°C ± 1°C. At the end of that time, actively growing cells are transferred to the second step selection medium and then into the final selection medium. At the end of final selection stage, actively growing cells are transferred to EDMM with 500 mgl^{-1} carbenicillin and 200 mgl^{-1} kanamycin. The embryogenic cells are grown in Petri dishes and incubated in a culture room maintained under a 16h photoperiod (cool white fluorescent light) between 20 to 22° C. The PAR at the level of the medium surface is previously described. The selection process takes 4 weeks to complete.

3.5. Germination and Conversion of Transformed Embryos

At the end of the development and maturation stage in EDMM, mature embryos are harvested from individual colonies and transferred to FGM and then to CM. The germination and conversion of these embryos, as well as the subsequent establishment of plants in a secure greenhouse, are done according to the procedures discussed above. In addition, both the germination and conversion steps are accomplished by using 200 mgl^{-1} kanamycin.

4. FURTHER PROTOCOL MODIFICATIONS

In this chapter an efficient protocol for somatic embryogenesis in *V.* x *labruscana* was presented. The protocol details the method for induction, long term maintenance, maturation and germination and conversion of somatic embryos. However the conversion rates of germinated *V.* x *labruscana* somatic embryos appear to be relatively low with 18% and 15%, respectively, for 'Fredonia' and 'Niagara', requiring a further protocol improvement.

5. REFERENCES

Chee R. and R. M. Pool. 1983. In vitro vegetative propagation of *Vitis*: application of previously defined culture conditions to a selection of genotypes. Vitis 22: 363-374.

Faure, O., J. Aarrouf, and A. Nougarede. 1996. Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L.): embryonic organogenesis. Ann. Bot. 78: 23-28.

Gray, D. J. and C. P. Meredith. 1992. Grape, p. 229-262. In: Hammerschlag, F. A. and R. E. Litz (eds.). Biotechnology of perennial fruit crops. CAB International, Cambridge.

Kikkert, J. R., G. S. Ali, M. J. Striem, M. Martens, P. G. Wallace, L. Molino, and B. I. Reisch. 1997. Genetic engineering of grapevine (*Vitis* sp) for enhancement of disease resistance. Acta Hortic. 447: 273-279.

Litz, R. E. and D. J. Gray. 1992. Organogenesis and somatic embryogenesis, p. 3-34. In: Hammerschlag, F. A. and R. E. Litz. (eds.). Biotechnology of perennial fruit crops. CAB International, Cambridge.

Mauro M. C., S. Toutain, B. Walter, L. Pinck, L. Otten, P. Coutos-Thevenot, A. Deloire, and P. Barbier. 1995. High efficiency regeneration of grapevine plants transformed with the GFLV coat protein gene. Plant Sci. 112: 97-106.

Merkle, S. A. and A. T. Wiecko. 1990 Somatic embryogenesis in three magnolia species. J. Am. Soc. Hortic. Sci. 115: 858-860.

Motoike, S.Y., R.M. Skirvin, M.A. Norton and A.G. Otterbacher. 2001. Somatic embryogenesis and long term maintenance of embryogenic lines from fox grapes. Plant Cell, Tissue, and Organ Culture 66:121-131.

Motoike, S.Y., R.M. Skirvin, M.A. Norton, and A.G. Otterbacher. 2002. Development of methods to genetically transform American grape (Vitis x labruscana L. H. Bailey). Journal of Horticultural Science & Biotechnology 77(6): 691-696.

Nakano M., T. Sakakibara, Y. Watanabe, and M. Mii. 1997. Establishment of embryogenic cultures in several cultivars of *Vitis vinifera* and V. *x Labruscana*. Vitis 36: 141-145.

Nickle, T. C. and E. C. Yeung. 1994. Further evidence of a role for abscisic acid in the conversion of somatic embryos of *Daucus carota.* In Vitro Plant Cell. Dev. Biol. 30P: 96-103.

Nitsch, J. P., and C. Nitsch. 1969. Haploid plants from pollen grains. Science 163: 85-87.

Perl, A. and Y. Eshdat. 1998. DNA transfer and gene expression in transgenic grapes. Biotechnol. Genet. Eng. Rev. 15: 365-368.

Reisch, B. I. and C. Pratt. 1996. Grapes, p. 297-369. In: Janick, J. and J. N. Moore (eds.). Fruit breeding, Vol. II. John Wiley and Sons, New York.

Robacker, C. 1993. Somatic embryogenesis and plant regeneration from Muscadine grape leaf explants. HortScience 28: 53-55.

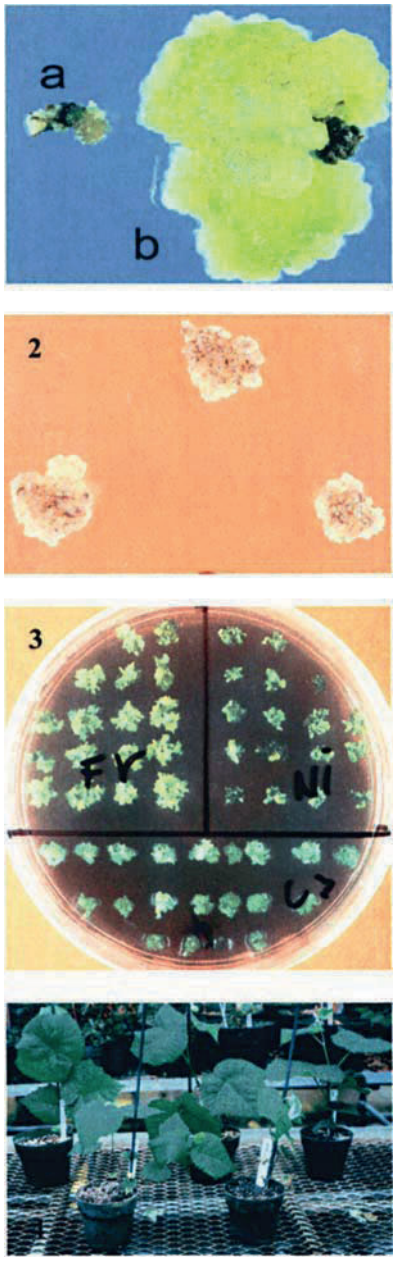
Salunkhe, C. K., P. S. Rao, and M. Mhatre. 1999. Plantlet regeneration via somatic embryogenesis in anther callus of *Vitis latifolia* L. Plant Cell Rep. 18: 670-673.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Plainview, NY.

Scorza, R., J. M. Cordits, D. W. Ramming, and R. L. Emershad. 1995. Transformation of grape (*Vitis vinifera* L.) zygotic-derived somatic embryos and regeneration of transgenic plants. Plant Cell Rep. 14: 589-592.

Staba, J. E. 1969. Plant tissue culture as a technique for the phytochemist. Recent Adv. Phytochem. 2: 80.

Xue, B., K. S. Ling, C. L. Reid, S. Krastanova, M. Sekiya, E. A. Momol, S. Sule, J. Mozsar, D. Gonsalves, and T. J. Burr. 1999. Transformation of five grape rootstocks with plant virus genes and a *virE2* gene from *Agrobacterium tumefaciens*. In Vitro Plant Cell. Dev. Biol. 35: 226-231.



Different stages of *V.* x labruscana somatic embryogenesis:

Figure 1 – (top) induction phase, a proembryogenic mass, b non-embryogenic mass;
Figure 2 – long term maintenance proembryogenic mass;
Figure 3 – embryo development and maturation phase;
Figure 4 – converted plants from somatic embryos.

PISTACHIO (PISTACIA VERA L.)

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1. INTRODUCTION

The pistachio tree (*Pistacia vera* L.), a deciduous, dioecious, and wind-pollinated tree species, is a member of the Anacardiaceae, a family that also includes cashew, mango, poison ivy, poison oak, pepper tree, and sumac. The genus *Pistacia* includes at least 11 species (Zohary, 1952); other authors recognise as many as 15 (Whitehouse, 1957). *P. vera* L. is the only economic importance for bearing edible fruits and serving as rootstocks. *P. vera* L. is an Irano-Turanian species, the main range of which covers the middle Asian republics of Uzbekistan, Tadzhikistan, Kirgiziya and southern most parts of Turkmenia and Kazakhstan. In the north, *P. vera* extend to about latitude 43°N. to the Karatau, Kirgizskiy and Talasskiy Alatau mountain ranges, while in the south and south-western Afghanistan, in the Paropamisus mountain. in the Herat province it reaches a latitude of 35°N. It grows most abundantly and on the most extensive areas in Tadzhikistan, where it occupies about 115.000 ha; in the whole of central Asia natural thickets cover about 300.00 ha (Browicz 1988).

World production of pistachio continues on an upward trend. Total world pistachio production in 2002 amounted to 593,5 Millions of Libres (ML) (California Pistachio Commission WEB sites). Iran is the highest pistachio producer (253,5 ML) and other producers are: United States of America (160,3 ML), Syria (88,0), Turkey (77,2), Greece (14,3 ML) and Italy (0,2 ML).

Pistachio fruits are consumed indifferent forms such as a luxury table nut, in pastry, confectionery and ice cream industries. An increasing world population and rise in demand for pistachio nuts has increased the need to produce more pistachio trees through clonal planting. In current and traditional horticultural practice, the pistachio nut is propagated by budding or grafting on rootstocks-seedlings and rooted cuttings on rootstocks-seedlings. The pistachio nut does not reproduce true-to-type when propagated by seed. Grafting and budding methods must be employed since difficulties in rooting of cuttings and incompatibility between

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rootstock and scion, frequently necessitates inter-grafting. Therefore, traditional breeding and selection of pistachio is expensive and time consuming.

Cultivar improvement and commercial propagation has rather been difficult because of high heterozygosity and the shortage of regenerated superior plant material. However, plant biotechnology offers rapid cloning of elite germplasm. Somatic embryogenesis, i.e., development of somatic embryoids from cultured somatic cells/tissues, is an important aspect first reported by Onay et al. (1995) in pistachio. However, this method has not been used for commercial propagation because only juvenile explants respond and possible field performance of the regenerated plantlets has not been adequately tested. Somatic embryogenesis is being viewed as one of the important techniques of plant tissue culture for mass cloning of elite plants through production of somatic seeds (Onay et al. 1996). A comparative study for inducing embryogenic tissue from kernels of mature and immature fruits of pistachio after 14 weeks of culture, embryogenic tissue formed on 78% of the explants when immature fruits were used, but only on 25% of mature fruits (Onay, 2000a). An excellent review on somatic embryogenesis of pistachio has been published by Onay and Jeffree (2000). Onay (2000b) hypothesised that somatic embryos arise from single cells or small cell aggregates by an initial asymmetric division at the epidermic layer of embryogenic mass, and seem to develop from a few small meristematic cells within the embryogenic mass of cells. In pistachio, somatic embryogenesis has succeeded and plants have recovered from: 1) immature fruits (Onay et al. 1995), 2) mature zygotic embryos (Onay 2000a), and 3) juvenile leaf explants (Onay and Jeffree, 2000).

In this chapter, detailed protocol inducing somatic embryogenesis is given for the establishment of embryogenic mass, and development of somatic embryos i.e. maturation, germination, plantlet development and plantlet acclimatisation using explants from immature fruits explants of pistachio.

2. MATERIALS

1. Absolute ethanol, H_2O_2, NaOCl (10-14% available chlorine), sterile water
2. Flow hood, Petri dishes (9 cm and 3 cm diameters), erlenmeyers, pipettes, forceps, and scalpels
3. Decoated kernels obtained from immature fruits (*Figure 1*) collected 12 weeks after anthesis
4. Media (see Tables 1 and 2).

Table 1: Pistachio basic culture media (BCM)

Constituent	**mg/L**	**Constituent**	**mg/L**
Basal Salts			
Ammonium nitrate	1650.0		
Boric acid	6.20		
Calcium chloride anhydrous	332.20		
Cobalt chloride hexahydrate	0.0250	Organic Additives	
Cupric sulphate pentahydrate	0.0250		
Disodium EDTA dehydrate	37.260	Sucrose	varies
Ferrous sulphate heptahydrate	27.80	Casein hydrolysate	varies
Magnesium sulphate anhydrous	180.70	l-ascorbic acid	varies
Manganese sulphate monohydrate	16.90	Myo-Inositol	100.0
Potassium iodide	0.830	Thiamine hydrochloride	10.0
Potassium nitrate	1900.0	Nicotinic acid (Free acid)	1.0
Potassium phosphate monobasic	170.0	Pyridoxine hydrochloride	1.0
Sodium molybdate dehydrate	0.250	Agar	varies
Zinc sulphate heptahydrate	8.60	pH	5.7

Table 2: Formulations of pistachio media[(1)]

	BCM-1	**BCM-2**	**BCM-3**	**BCM-4**
	Stage I Initiation	Stage II Maintenance	Stage III Maturation	Stage IV Germination
Sucrose	30.000	20.000	40.000	40.000
N6-Benzyadenine	1.0	None or 0.5	1.0-4.0	-
Abscisic acid	-	-	0.5-4.0	0.5-4.0
Casein hydrolysate	500	200	200	200
l-ascorbic acid	50	50	50	50
Agar	-	-	7000	7000

[(1)]All units are in mg/L (or ppm)
The pH of all media are adjusted to 5.7

Basic media composition is listed in Table 1. Required modifications for each culture stage are listed in Table 2.

Mix together all ingredients, with the exception of ABA, and bring to volume prior to autoclaving (20 minutes at 121ºC, 15psi). Filter ABA to sterilise and add to sterile media aseptically. Add agar to make solid BCM-3 and 4 plates. Pour 30 ml/dish in 9 cm Petri dishes, or 10 ml/dish in 3 cm Petri dishes.

3. METHOD

The regeneration method can be divided into six main steps: initiation of embryogenic cultures from explants, maintenance and multiplication of embryogenic cultures, establishing embryogenic cell cluster-suspension, maturation

of somatic embryos, germination of somatic embryos and regeneration of plantlets from the somatic embryos.

3.1. Initiation of Embryogenic Cultures

Use decoated kernels obtained from immature fruits for initiation of embryogenic cultures. Collect immature fruits in the middle of July (about 8 weeks after fertilisation) until the second week of August (12 weeks after anthesis). The optimal explant stage for initiation is when the immature fruits were harvested on the second week of August.

Steps

1. Isolate the kernels from the immature fruits.
2. Treat the kernels with 10% NaOCl (v/v) for 10 min.
3. Wash the kernels 3-5 times with sterile distilled water in the laminar-flow hood.
4. Transfer the sterile kernels in a Petri dish.
5. Remove the seed coat with scalpel and forceps.
6. Place 4-5 decoated kernels (half kernels or as intact) onto 50 ml BCM-1 induction medium in 250 ml erlenmeyer flasks.
7. Seal the flasks with a double layer of aluminium foil.
8. Place on an orbital shaker at 98 rpm at a light intensity of 25μmol m^{-2} sec^{-1} photon flux density and a temperature of 25ºC.
9. Subculture the developed tissues to the BCM-2.

Figure 1. Immature Pistacia vera L. fruits.

Description of embryogenic response

Among several PGRs and combinations tested, only the BA treatments in liquid MS medium gave a granular, compact and pale green tissue. This is called an embryogenic tissue (ET) developed directly from cultured immature kernels without involving any intermediate callus stage (*Figure 2*). The first stages of the development of embryogenic tissue visible on the external part of the explants were never observed before 4 weeks in culture. The explants cultured at the highest concentration of BA (more then 4 mg/L) and other PGRs did not induce the ET and formed degenerate black tissue.

3.2. Maintenance and Multiplication of Embryogenic Tissue

The ET were subcultured regularly for more than a year by 10-12 days intervals onto BCM-2.

Steps

1. Separate the ET from the adjacent tissue and transfer onto maintenance medium (BCM-2) without or with reduced concentration of BA.
2. Maintain embryogenic tissue by subcultures every 10-12 days onto fresh BCM-2.
3. Place on an orbital shaker at 98 rpm at a light intensity of 25µmol m^{-2} sec^{-1} photon flux density and a temperature of 25ºC.

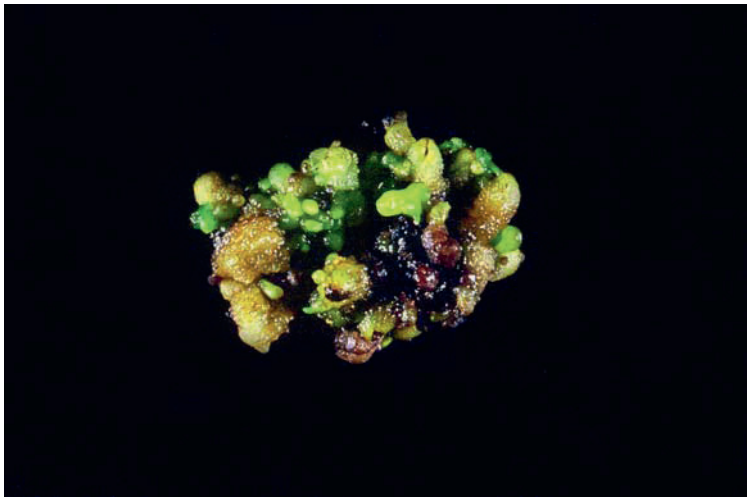

Figure 2. Intact embryogenic tissue (kernel) after 4 weeks in liquid MS medium containing 1 mg/l BA.

3.3. Establishing Embryogenic Cell Masses (ECM)

At this stage, attempt to establish a fine cell suspension from cluster of ET resulted in failure. Embryogenic cultures multiply by cleavage polyembryony as clavate structures on the primary explants. After two subcultures (10-12 days intervals) in the BCM-2, rapidly growing clusters of ECM was obtained (*Figure 3*).

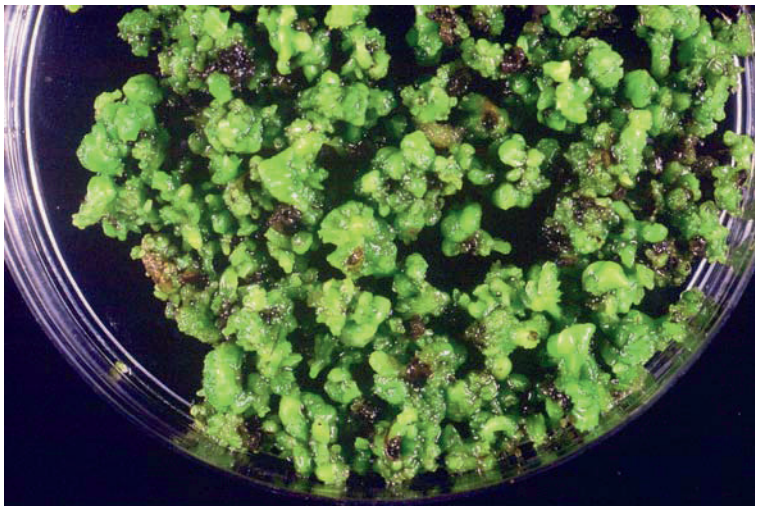

Figure 3. EMS initiated from intact embryogenic tissue after 2 subcultures (with 10-12 days interval) in liquid MS medium.

Steps

1. Transfer 0.5 g (wet weight) EMS into a 250 ml flask containing 50 ml BCM-2 liquid medium.
2. Seal the flasks with a double layer of aluminium foil and place the flask on a rotary shaker at 98 rpm at a light intensity of 25 µmol. m^{-2} sec^{-2} photon flux density and a temperature of 25ºC.
3. Discard the spent medium and measure the fresh weight (FW) and dry matter of EMS.
4. Subculture the regenerated EMS into fresh liquid BCM-2 every 10 or 12 days. Transfer 0.5 g of the regenerated EMS into a 250 ml flask containing 50 ml of fresh BCM-2 liquid medium. Maintain ECM liquid cultures by regular 10-12 days subculture.

3.4. Maturation of Somatic Embryos

Mannitol and PEG are not metabolised by pistachio ECM to any great extent, and they don't lead to somatic embryogenesis. Better synchrony in

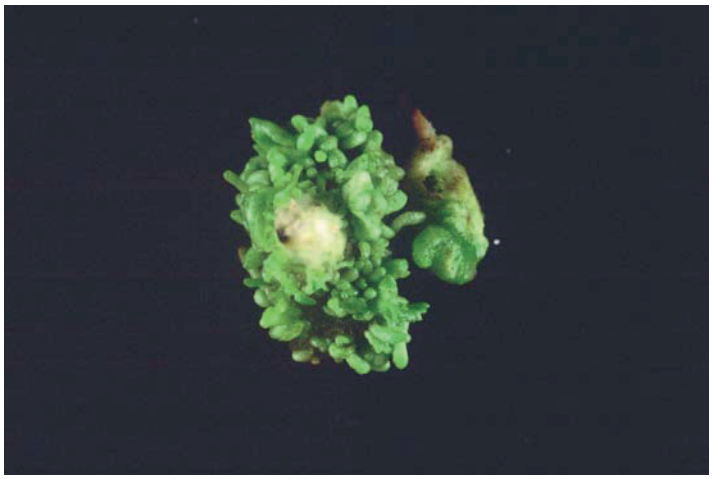

Figure 4. Somatic embryo maturation in the presence of ABA in BCM-3 culture medium.

embryo (*Figure 4*) maturation is observed in ABA and BA supplemented cultures grown (BCM-3) in the presence of sucrose, which attributes its role as an osmoticum.

Steps

1. Transfer embryogenic cell masses into BCM-3 with or without 0.5-4 mg/l ABA or 0.5 mg/L BA. After 3 or 4 weeks of ABA or BA treatments, transfer developing somatic embryo clusters into BCM-4 medium.
2. Incubate the culture plates in continuous light at 25ºC.
3. After 4-6 weeks EMS cultures develop cotyledonary somatic embryos (*Figure 5*).

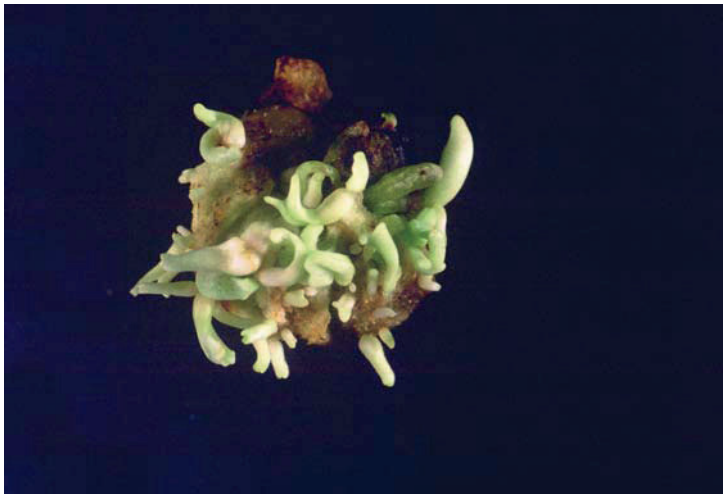

Figure 5. Pistachio cotyledonary somatic embryos at the end of development on BCM-4.

3.5. Germination and Conversion to Plantlets

Neither germination nor plantlet development medium contained BA or ABA. To determine the effects of BA, racemic (±) ABA and sucrose treatments during maturation on the subsequent germination and plantlet regeneration, clusters of mature somatic embryos were transferred from BCM-3 to BCM-4 (Onay et al. 2000).

Steps

1. Select mature cotyledonary embryos from the development medium using forceps and scalpels. Transfer the selected embryos to solidified BCM-4 germination medium.
2. Incubate the cultures in continuous light at 25ºC.
3. After 3-5 weeks, embryos germinate and develop somatic seedlings (*Figure 6*).
4. Acclimatise the plantlets in pots containing a sterile mixture of peat and perlite (1:1, v/v) in a growth room at 90 ± 5% RH for 3-4 weeks before transfer to normal glasshouse conditions (20ºC night temperature, 65-80% RH, 18 h day length with day light) supplemented by mercury fluorescent lamps.
5. Feed the explants with a solution of N:P:K (2:1:1) was provided weekly to plants undergoing acclimatisation.
6. Transplant somatic seedlings into pots containing a mixture of peat and perlite (1:1, v/v) or peat and grit (1:1; v/v).
7. Grow the somatic seedlings in pots for 3 months in the greenhouse (*Figure 7*) and then transplant into the field.

Figure 6. Pistachio somatic seedlings after germination on BCM-4.

Figure 7. Pistachio somatic seedlings are in the greenhouse.

3.6. Encapsulation of Somatic Embryos and ECMes

The viability of the encapsulated EMS and somatic embryos should be investigated immediately following encapsulation, and after storage for 60 days at 4°C (Onay 1996; Onay et al. 1996).

Steps

1. Isolate mature somatic embryos, blott dry on sterile filter paper for 1 min, and dip for a few minutes into a mixture of 3% (w/v) sodium alginate in MS medium supplemented with 4% (w/v) sucrose, with or without 1.0 mg/L BA.
2. Pick up single embryo with forceps, together with 0.05-0.1 ml of alginate solution, approximately 4-7 mm in length and drop into a 0.6% (w/v) solution of $CaCl_2.2H_2O$.
3. Keep the solidified drops (beads), each containing a single embryo in the $CaCl_2$ solution for 2 hours on a shaker (180 rpm) in continuous light (10 µmol m^{-2} s^{-1}). After the incubation period, recover the beads by decanting off the $CaCl_2$ solution and then wash three times with liquid MS medium.
4. Transfer the resulting encapsulated embryos (*Figure 8*) in 9.0 cm diameter Petri dishes containing 20 ml of BCM-1 medium solidified with agar 0.7% (w/v) and supplemented with 4% sucrose.
5. Store the encapsulated embryos in the dark at 4°C for two months in an aseptic condition on two filter papers soaked in liquid BCM supplemented with 4% (w/v) sucrose.
6. After storage, the beads were cultured in 9.0 cm Petri dishes on 20 ml of BCM solidified with agar (0.7% w/v) supplemented without or with 1.0 mg/L BA and 4% (w/v) sucrose.
7. Seal the Petri dishes with Parafilm and incubate in the light (25 µmol m^{-2} s^{-1}) at 25°C.

Well developed plantlets are usually obtained within 5-7 weeks of culture, and when they potted into a sterile mixture of perlite-grit (0.3-1 mm standard grades-quartzag) (1:1, v/v) compost. No phenotypic or genotypic variation was observed among the plantlets.

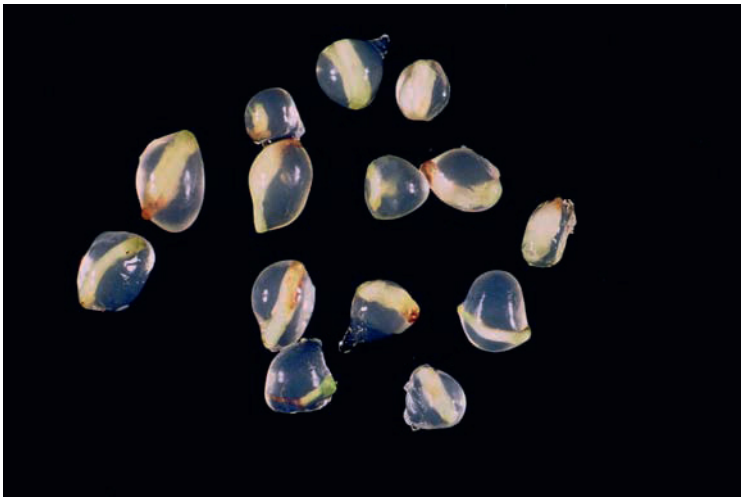

Figure 8. Individual pistachio somatic embryos encapsulated in Ca-alginate beads ready for culture.

4. STEPS FOR FURTHER MODIFICATIONS

So far, research conducted on somatic embryogenesis of *P. vera* L. is insignificant when compared with other woody species. The major limitations using somatic embryogenesis in clonal propagation of pistachio are low-embryogenic culture-initiation frequencies and high labor costs. However, somatic embryogenesis could be developed as a commercial technique to reduce the cost of plant production.

Cloning juvenile pistachio material, in conjunction with breeding programs, may be an effective means of producing improved planting stock. The protocol described here can be used only in the laboratory on a small scale and significant progress is needed to expand this technology to large scale production at commercial levels. To optimise the scale up plant micropropagation from the somatic embryos, the in vitro methods should be improved in five aspects: i) It is necessary to determine the effects of genotypes on the initiation potential of the embryogenic cultures. Bioreactor technology [e.g. temporary immersion system] could be adopted to proliferate suspension cultures yielding large numbers of stage one embryos in a uniform physiological condition suited to maturation. The potential for long-term storage of somatic embryos and ECMes of *P. vera* L. in liquid nitrogen could also be tested; ii) Cold storage, desiccation, physical and chemical components of the media must be tested to promote embryo germination and to improve the efficiency of conversion into plantlets; iii) The suitable conditions for acclimatisation of the plantlets after transfer to soil have to be determined; iv) Genetic fidelity of somatic seedlings is essential, and for that molecular marker analysis is needed [e.g AFLPs, DNA fingerprinting etc.]; v)

Initiation of somatic embryogenesis from somatic tissue of mature trees is also a very important goal, as it may lead to rejuvenation. However, somatic embryogenesis of mature trees is generally more difficult than their juvenile counterparts. Nevertheless mature trees are often preferred for cloning because they impart superior characteristics to the next generation and that are liable to be largely lost in sexual recombination.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

Browicz, K. (1988). Chronology of the euxinian and hyrcanian element in woody flora of Asia, plant systematic and evolution, by Springer-Verlag, pp. 305-314.

Onay A (1996) *In vitro* organogenesis and embryogenesis of Pistachio, *P. vera*, L. PhD Thesis, University of Edinburgh.

Onay A & Jeffree CE (2000) Somatic Embryogenesis in Pistachio. In: Somatic embryogenesis in woody plants / edited by S.Mohan Jain, Pramod K. Gupta, Ronald J. Newton (Forestry Sciences). Chapter 10, Section B, Kluwer Academic Publishers, The Netherlands, vol; 6: 361-390.

Onay A, Jeffree CE, Theobald C & Yeoman MM (2000). Analysis of the effects of maturation treatments on the probabilities of somatic embryo germination and embling development in Pistachio, *Pistacia vera L.* using a logistic regression method. Plant Cell, Tissue and Organ Culture. Volume 60 No. 2: 121-129.

Onay A, Jeffree CE & Yeoman MM (1995). Somatic embryogenesis in cultured immature kernels of Pistachio, *Pistacia vera* L. Plant Cell Reports. 15: 192-195.

Onay A, Jeffree CE & Yeoman MM (1996). Plant regeneration from encapsulated embryoids and an embryogenic mass of Pistachio. Plant Cell Reports, 15: 723-726.

Onay A (2000a). Somatic embryogenesis in cultured kernels of Pistachio, *Pistacia vera* L cv. Siirt. In: Proceedings of the Balkan Peninsula:into the next Millenium Volume II(Ed. N. Gözükırmızı).pp: 109-115, Istanbul University, Turkey.

Onay A (2000b). Histology of somatic embryo initiation and development in pistachio (*Pistacia vera* L.). Turkish Journal of Botany. 24: 91-95.

Whitehouse, W.E. (1957). The pistachio nut-A new crop for the Western United States. *Economic Botany*, pp. 281-321.

Zohary, M. (1952). A monografphical study of the genus *Pistacia*. *Palestanian Journal of Botany* (Jerusalem) 5. 187- 228.

GRAPE (*VITIS VINIFERA L.)*

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1. INTRODUCTION

Grapevine (*Vitis* spp.) ranks second after mango amongst the most important fruit crops cultivated worldwide. Somatic embryogenesis has been reported from anthers or embryogenic cell suspension cultures in *Vitis rupestris*, *V. latifolia*, *V. vinifera* and *V. longifolia* (Hirabayashi et al 1976; Bouquet et al 1982; Gray and Mortenson 1987; Jayashanker et al 1999; Salunkhe et al 1999), from ovules in *Vitis longii* and *vinifera* cv. Cabernet-Sauvignon (Gray and Mortensen 1987; Mullins and Srinivasan 1976) and from ovaries in *Vitis longii* ' Microsperma' (Gray and Mortensen 1987). Regeneration via organogenesis and somatic embryogenesis from leaves and petioles has been reported in *Vitis rupestris* (Martinelli et al 1993) and *V. rotundifolia* (Robacker 1993). However, somatic embryogenesis with low frequency was induced using leaf disc of *V. vinifera* and *V. aestivalis* (Matsuta 1990) and tendrils of *V. vinifera* (Salunkhe et al 1997). The limitation of above methods is that anthers, ovules and zygotic embryos are available for experimentation for only a brief period. Developing a methodology for getting somatic embryogenesis with a high frequency from leaf discs, which is readily available material throughout the year, would be particularly useful for genetic improvement of *Vitis vinifera.* In this chapter we report a protocol for somatic embryogenesis and plant regeneration through leaf disc culture method.

2. MATERIALS

1. 2 % (V/V) detergent (ALA- trade name of a detergent), 2 % (W/V) Bavistin (2-methoxy-carbamoyl benzimidazole- a systemic fungicide), 0.1 % $HgCl_2$, 0.8 % sodium hypochlorite
2. Laminar air flow hood, petri plates, pipettes, forceps and scalpel
3. 40 mm^2 leaf disc pieces
4. Stereo-microscope
5. Media (see Tables 1, 2 & 3)

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Table 1: Grape Murashige and Skoog basal culture medium (MS)

Constituents	Mg/L	Constituents	Mg/L
Macronutrients		*Iron Source* (Fe. EDTA)	
NH_4NO_3	1650	$FeSO_4$. 7 H_2O	27.8
KNO_3	1900	Na_2. EDTA	37.3
$CaCl_2$	440		
$MgSO_4$	370	*Vitamins*	
KH_2PO_4	170		
		Nicotinic acid	0.5
		Pyridoxine hydrochloride	0.5
Micronutrients		Thiamine hydrochloride	0.5
KI	0.83	Glycine	2.0
H_3BO_3	6.2	Myo-inositol	100.0
$MnSO_4$. $4H_2O$	15.6	Sucrose	30 x 10^3
$ZnSO_4$. $7H_2O$	8.6		
$NaMoO_4.2H_2O$	0.25	pH	5.7
$CuSO_4.5H_2O$	0.025	Difcobacto-agar	8000.0
$CoCl2.6H_2O$	0.025		

Table 2: Grape Nitsch and Nitsch basal medium (NN)

Constituent	mg/L	Constituent	mg/L
Macronutrient		*Iron Source*	
$MgSO_4$. $7H_2O$	185	$FeSO_4$. $7H_2O$	27.8
KH_2PO_4	68	Na_2. EDTA	37.3
KNO_3	950	*Vitamin Source*	
NH_4NO_3	720	Thiamine hydrochloride	0.5
		Pyridoxine hydrochloride	0.5
Micronutrient		Nicotinic acid	5.0
$MnSO_4$. $4H_2O$	25	Glycine	2.0
$ZnSO_4$. $7H_2O$	10	myo-inocitol	100.0
Na_2MoO_4. $2H_2O$	0.25	Sucrose	30x10^3
$CuSO_4$. $5H_2O$	0.025	pH	5.7
$CoCl_2.6H_2O$	0.025	Difco bacto-agar	8000.0

Table 3: Formulations of grape media

	MS-1, 2	**MS-1, 2or3**	**NN-1or2**	**NN-b,3**	**HNS**
MS or NN media	Stage I	Stage II	Stage III	Stage IV	Stage V
with hormones	ICM	PSM	SSE	GSE	TPS
(mgl^{-1})					
MS basal media with Hormones					
BA	1	1			
2,4-D	0.1	0.1			
IAA	1	1			
IBA		0.1			
NN basal media with hormones				b or b $_{1/2}$	
BA				1	
IAA			1		
IBA			0.1 0.1		

HNS		
Ingredients	*Conc. gm/l*	*Final conc. (mM)*
Macronutrients		
Ca $(NO_3)_2$. $4H_2O$	0.94	4.0
$MgSO_4$. $7H_2O$	0.52	2.0
KNO_3	0.66	6.0
$NH_4H_2PO_4$	0.12	1.0
Micronutrients		
		Final conc.(μM)
H_3BO_3	28	45
$MnSO_4.H_2O$	34	20
$CuSO_4.5H_2O$	1.0	0.4
$ZnSO_4.7H_2O$	2.2	0.7
$(NH4)_6\ Mo_7O_{24}.4H_2O$	1.0	0.2
H_2SO_4 (Conc.)	5 ml	

A 0.1 ml vol. of the micronutrient solution is mixed with 1 liter of the macronutrients and pH adjusted to 6.7

Abbreviations: ICM: Induction of callus and its maintenance; PSE: Primary somatic embryogenesis; SSE: Secondary somatic embryogenesis; GSE: Germination of somatic embryos; TPS: Transfer of plantlets to soil; BA: Benzyladenine; IAA: Indole 3-acetic acid; IBA: Indole 3-butyric acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; MS-1: Murashige & Skoog,1962 basal with BA 1mgl^{-1} and 2,4-D 0.1mgl^{-1}; MS-2: MS basal with IAA 1mgl^{-1}; MS-3: MS basal with IBA 0.1mgl^{-1}; NN b : Nitsch & Nitsch 1969, basal; NN b ½ : Half-strength of macro, micro and iron source salts of NN basal; NN-1: NN basal with IBA 0.1 mgl^{-1} ; NN-2: NN basal with IAA 1mgl^{-1} ; NN-3: NN basal with BA 1 mgl^{-1} and IBA 0.1 mgl^{-1} ; HNS : Hoagland and Arnon,1950 nutrient solution ; Tissue culture Difco bacto-agar is 8 gl^{-1} and pH of all media are adjusted to 5.7

3 METHOD

The protocol described below (see also Das et al 2002) was applicable to all four Indian grapevine (*Vitis vinifera L.*) cultivars viz Pusa seedless, Beauty seedless, Perlett and Nashik (obtained from Indian Agricultural Research Institute, New Delhi, India). The regeneration method can be divided into five main steps: induction of callus and its maintenance, somatic embryogenesis (primary), secondary somatic embryogenesis, germination and conversion of plantlets from the somatic embryos, and transfer of plantlets into soil.

3.1. Induction of callus and its maintenance

For callus induction from leaf discs following steps are involved:

1. Take upper three leaves from the shoot tip and submerge for 5 min in a 2% (V/V) detergent (ALA-trade name of a detergent) and then rinse in tap water for 10 min.
2. Leaves are treated with 2 % (W/V) Bavistin (2-methoxy-carbamoyl- a systemic fungicide) for 10 min and then rinse with sterile distilled water.
3. Leaves are treated with 0.1 % $HgCl_2$ for 5 min and rinse twice with sterile distilled water.
4. Leaves are treated with 0.8 % sodium hypochlorite and rinse thrice with distilled water.
5. Following proper sterilization, leaves are excised into 40 mm^2 pieces and about 8-10 pieces are cultured with their lower surface touching on MS-1 medium per petri dish (90 mm), incubated at 25 ± 2^{0}C in dark for two weeks. During this period whole leaf disc proliferates and form callus.
6. This callus could be maintained for a long time (more than two years) if cultured on MS-2, incubated at 25 ± 2^{0}C and light intensity (60 µE m^{-2} s^{-1}) in a 16 h light and 8 h dark cycle.

3.2. Somatic embryogenesis (Primary)

From callus to somatic embryogenesis (primary) involves following steps:

1. After callusing the cultures are kept under low light intensity (15 µE m^{-2} s^{-1} cool white) in a 16 h light and 8 h dark cycle for 2-4 weeks before transferring to high light intensity (60 µE m^{-2} s^{-1}) in a 16 h light and 8 h dark cycle for 8 weeks.
2. Cultures should be transferred every four weeks to fresh MS-1 medium.
3. Cultures transferred from darkness to low light intensity will form pro-embryos (Fig 1A), which will differentiate into somatic embryos (Fig 1B) if these are shifted to high light intensity.
4. When cultures are transferred directly from darkness to high light intensity before first culturing them under low light conditions, pro-embryos will not differentiate into embryos and the whole tissue will turn pink.
5. Embryo elongation and maturation can be achieved on MS-1 semi solid medium or MS-2 liquid medium, however, the cultures should be kept under diffused light at 25 ± 2^0C.

3.3. Secondary somatic embryogenesis

From primary somatic embryos to secondary somatic embryos formation following steps are involved:

1. Primary somatic embryos either picked from solid cultures or taken from liquid cultures be placed horizontally on NN-1 or 2 semi solid medium and gelled with 0.8 % Difco bacto-agar.
2. These cultures are incubated at 25 ± 2^0C, in low light intensity (15 µE m^{-2} s^{-1}) in a 16 h light and 8 h dark cycle and sub-cultured on fresh medium every 4-week interval.
3. After 8-16 weeks a large number of secondary somatic embryos will be formed and if these embryos continue to remain on the same NN-1 fresh medium four weeks, then tertiary somatic embryos (repetitive or recurrent type of somatic embryogenesis) are induced.
4. A similar phenomenon of formation of tertiary embryos is seen if secondary somatic embryos are cultured on NN b, incubated at 25 ± 2^0C under high light intensity (60µE m^{-2} s^{-1}).

3.4. Germination of somatic embryos

In grapevine, somatic embryo germination has been a limiting step, probably due to embryo dormancy. We have developed a reliable method for the germination of somatic embryo. Three modes of germination were observed:

1. Culture primary somatic embryos (Fig 1C) either on NN b or NN-3 and subjected to 4^0C for 2 weeks in the dark, followed by standard culture conditions. The embryos will start germinating and show good rooting (Fig 1D & E). Consequently they germinate into plantlets when transferred to NN b medium supplemented with casein hydrolysate (250 mgl^{-1}) for elongation (Fig 1F).
2. Without low temperature treatment, primary somatic embryos form callus and differentiate into a large number of shoot buds. These shoot buds can be excised, rooted and plantlets formed on NN-1 medium containing casein hydrolysate (250 mgl^{-1}).
3. Secondary and tertiary somatic embryos can be germinated, rooted and form plantlets on NN b or NN $b_{1/2}$ medium supplemented with casein hydrolysate (250 mgl^{-1}) without low temperature treatment.

3.5. Transfer of Plantlets to Soil

Only fully-grown plantlets (~ 10 cm) should be taken out from culture tubes and washed in tap water to remove agar attached to roots. These plantlets should be transferred to pots filled with sterilized vermiculite. The transferred plants (Fig 1G) should be watered regularly with HNS, should be kept at 25 ± 2^0C and high light intensity (90µE m^{-2} s^{-1}) and completely covered with polythene bags. After one week, polythene bags can be punctured with a needle and plants allowed to remain for two more weeks under these conditions. Acclimatized and hardened plantlets (no somaclonal variation) can be transferred to soil for growth and flowering (Fig 1H).

4. CONCLUSION

This regeneration protocol via repetitive or recurrent type of somatic embryogenesis is a good system for mass-scale production of true to the type plants since genetic constitution could be maintained and no somaclonal variation among *in vitro* grown plants is observed. Although grapes are vegetatively propagated through cuttings yet cut parts are highly prone to infection. To avoid these contamination and transportation cost, artificial seeds could be developed by encapsulation of somatic embryos, which could be stored for a long period. Germplasm also could be stored through cryopreservation. Conventional breeding

is largely limited due to long pre-bearing age, high polyploidy and heterozygosity. Molecular techniques i.e. genetic engineering could be applied for development of good agronomic traits for genetic improvement of grape through genetic transformation due to the availability of a very efficient regeneration and transformation protocol through leaf material which is available round the year.

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5. REFERENCES

Bouquet A., Piganeau A. M. Influence du genotype sur la production de cals, d' embryoids et de plantes entieres par culture d' anthers in vitro dans Ie genre Vitis. Compt Rend Acad Sci Paris 1982; 295: 569-574

Das D.K., Reddy M.K., Upadhyaya K. C., Sopory S.K. An efficient leaf-disc culture method for the regeneration via somatic embryogenesis and transformation of grape (*Vitis vinifera L.*). Plant Cell Rep 2002; 20: 999-1005

Gray D. J., Mortensen J.A. Initiation and maintenance of long term somatic embryogenesis from anthers and ovaries of *Vitis longii* ' Microsperma'. Plant Cell Tiss and Org Cult 1987; 9: 73-80

Hoagland D.R., Arnon D.I. The water culture method of growing plants without soil, Calif Agric Exp Stn 1950; Bull No. 347, Berkeley, Calif., USA.

Jayashankar S., Gray D. J., Litz R.E. High-efficiency somatic embryogenesis and plant regeneration from suspension cultures of grapevine. Plant Cell Rep 1999; 18: 533-537

Martinelli L., Bragagna P., Poletti V., Scienza A. Somatic embryogenesis from leaf and petiole derived callus of *Vitis rupestris*. Plant Cell Rep 1993; 12: 207-210

Matsuta N. Effect of auxin on somatic embryogenesis from leaf callus in grape (Vitis spp.) Japan J. Breed. 42: 879-883

Mullins M.G., Srinivasan C. Somatic embryos and plantlets from an ancient clone of the grapevine (cv. Cabernet- Sauvignon) by apomixis *in vitro*. J. Exp Bot 1976; 27: 1022-1030

Murashige T., Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962; 15: 473-497.

Nitsch J.P., Nitsch C. Haploid plants from pollen grains. Science 1969; 163: 85-87.

Robacker C. Somatic embryogenesis and plant regeneration from muscadine grape leaf explants. Hort Sci 1993; 28: 53-55

Salunkhe C.K., Rao P.S., Mhatre M. Induction of somatic embryogenesis and plantlets in tendrils of *Vitis vinifera L.* Plant Cell Rep 1997; 17: 65-67.

Salunkhe C.K., Rao P.S., Mhatre M. Plantlet regeneration via somatic embryogenesis in anther callus of *Vitis latifolia L.* Plant Cell Rep 1999; 18: 670-673

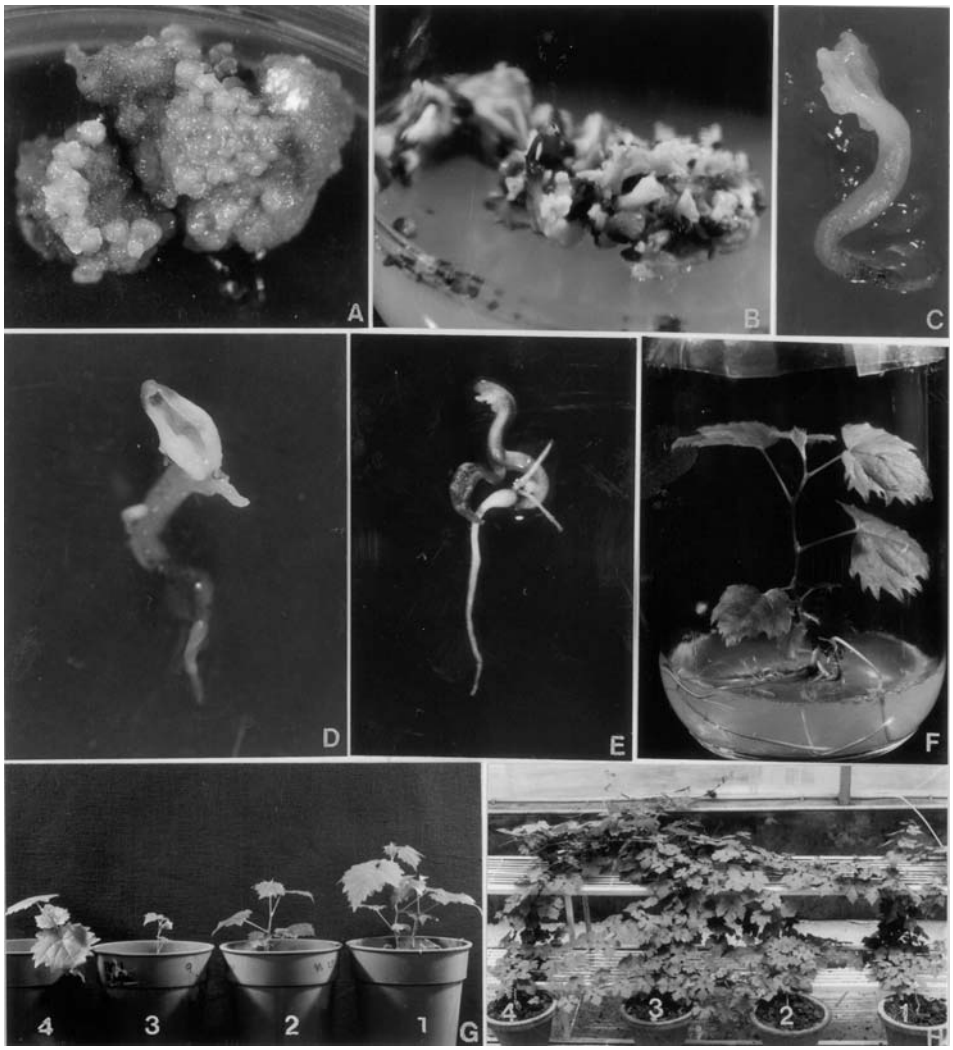

Fig 1. Somatic embryogenesis and plantlet formation in four Indian cultivars of grape (*Vitis vinifera L.*)

A) Callus (Proembryo stage). **B)** Somatic embryos differentiated from embryogenic callus. **C)** An enlarged single embryo. **D & E)** Somatic seedling with elongated root. **F)** Full grown *in vitro* somatic seedling. **G)** Plantlets in vermiculite:1. Pusa seedless, 2. Beauty seedless, 3. Perlett, and 4. Nashik. **H)** Plantlets on field soil in greenhouse: 1. Pusa seedless, 2. Beauty seedless, 3. Perlett, and 4. Nashik

DATE PALM *Phoenix dactylifera* L.

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1. INTRODUCTION

Date palm (*Phoenix dactylifera* L.), a diploid with 2n = 36, is a member of the monocot family Arecacea classified as a dioecious tall evergreen. Date palm female trees bear fruits at 3 to 5 years and are fully mature at 12 years. The fruit is a nutritious source of sugar, minerals, and vitamins. Date palm population, estimated 90 million trees worldwide, is concentrated between latitudes 10° and 30° north of the Equator, mainly in arid regions of the Middle East and North Africa, where it is thought to have been cultivated for over several thousands of years (Zohary and Hopf, 1993). According to FAOSTAT database (apps.fao.org), world dates production has risen from 2535 thousand tons in 1980 to 5037 thousand tons in 2000. Major producers of date palm are Egypt, Iran, Saudi Arabia, Pakistan, Iraq, Algeria, United Arab Emirates, Oman, Sudan, Libya, Tunisia, Morocco, Mauritania, and United States.

Date palm can be propagated by seeds basically for breeding or ornamental purposes but cannot be used to propagate desired cultivars because cross-pollination in the date palm results in new heterogeneous varieties of unknown characteristics usually with inferior date quality. Alternatively, propagation by offshoots is more widely used since true-to-type trees are produced. However, only about 20 offshoots are produced during the first 10 to 15 years of the tree life. For large-scale propagation of date plam plants by tissue culture, several review articles have appeared in the literature (Tisserat, 1984a; Omar et al., 1992; Benbadis, 1992).

Various explants have been used to initiate date palm *in vitro* cultures including immature zygotic embryos (Reynolds and Murashige, 1979), mature zygotic embryos (Reuveni, 1979; Zaid and Tisserat, 1984), leaf segments (Tisserat, 1979; Fki et al., 2003), inflorescence tissues (Bhaskaran and Smith, 1992; Fki et al.,

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 309–319.

2003), and *in vitro* plantlets leaf segments (Sudhersan et al., 1993). However, most frequently used explants are apical shoot tips and lateral buds which proved most responsive and practical for micropropagation purpose (Tisserat, 1984b). It has been demonstrated that plant regeneration in date palm is possible through organogenesis and somatic embryogenesis depending upon genotype and hormonal supplements. This chapter, however, is intended to focus on aspects of somatic embryogenesis. Strategies aimed at improving *in vitro* conditions for date palm somatic embryogenesis and understanding physiological and morphological *in vitro* responses have been the focus of recent studies (Omar and Novak, 1990; Sudhersan et al., 1993; El Hadrami and Baaziz, 1995a; Zaid and Hughes, 1995; Veramendi and Navarro, 1996; Veramendi and Navarro, 1997; Al-Khayri, 2001, 2002, 2003; Al-Khayri and Al-Bahrany, 2001, 2003a, 2003b; Fki et al., 2003). Progress has been made in distinguishing embryogenic and non-embryogenic date palm calli, based on peroxidase activity (Baaziz et al., 1994; El Hadrami et al., 1995b) and IAA-oxidase activity (El Bellaj et al., 2000). A method has been developed for determining chromosome number or ploidy level of date palm regenerants based on flow cytometric analysis (Fki et al., 2003).

The protocol described here is based on callus induction from apical shoot tip explants and has proved applicable to numerous commercially important date palm cultivars (Al-Khayri, 2001; Al-Khayri and Al-Bahrany, 2001, 2003a). Procedures including explant preparation, callus proliferation, somatic embryo development, plant formation, and acclimatization are described.

2. PROTOCOL OF SOMATIC EMBRYOGENESIS IN DATE PALM

2.1 Culture Medium

1. The basal medium used for date palm tissue culture is based on MS salts (Murashige and Skoog, 1962) with modifications. Prepare stock solutions as described in Table 1, combine the appropriate volume of each stock in water, and add other additives as specified. This basal medium is used throughout the protocol, except for the rooting stage where half-strength MS salts is recommended.
2. Note that this protocol consists of six stages differing in hormones and activated charcoal content as described in Table 2. Add these additives accordingly and adjust the medium to final volume.
3. Adjust medium to pH 5.7 with 1 *N* KOH, and dispense in 150 x 25-mm culture tubes (15 ml medium per tube) for initiation stage or in 125-ml culture flasks or GA-7 Magenta vessels (50 ml per vessel) for subsequent sages. Autoclave for 15 min at 121°C and $1x10^5$ Pa (1.1 kg cm^{-2}).

Table 1. Constituents of date palm *in vitro* culture medium including salt components and organic additives, preparation of stock solutions, and final concentration of culture medium. Note that hormonal supplements and activated charcoal are specified elsewhere according to culture stage.

Constituents	Chemical formula	Stock conc. (g/L)	Medium conc. (mg/L)
Major nutrients[1]			
10X stock, use 100 ml per L medium			
Ammonium nitrate	NH_4NO_3	16.5	1650
Potassium nitrate	KNO_3	19.0	1900
Calcium chloride-2H2O	$CaCl_2 \cdot 2H_2O$	4.4	440
Magnesium sulfate-7H2O	$MgSO_4 \cdot 7H_2O$	3.7	370
Potassium orthophosphate	KH_2PO_4	1.7	170
Sodium phosphate	$NaH_2PO_4 \cdot 2H_2O$	1.7	170
Minor nutrients[1]			
100X stock, use 10 ml per L medium			
Potassium iodide	KI	0.083	0.83
Boric acid	H_3BO_3	0.62	6.2
Manganese sulfate-4H_2O	$MnSO_4 \cdot 4H_2O$	2.23	22.3
Zinc sulfate-7H_2O	$ZnSO_4 \cdot 7H_2O$	0.86	8.6
Sodium molybdate-2H_2O	$Na_2MoO_4 \cdot 2H_2O$	0.025	0.25
Cupric sulfate-5H_2O	$CuSO_4 \cdot 5H_2O$	0.0025	0.025
Cobalt chloride-6H_2O	$CoCl_2 \cdot 6H_2O$	0.0025	0.025
Iron –EDTA[1]			
100X stock, use 10 ml per L medium			
Iron sulfate-7H_2O	$FeSO_4 \cdot 7H_2O$	2.78	27.8
Ethylenediamine tetraaceticacid disodium salt dihydrate	$Na_2EDTA \cdot 2H_2O$	3.73	37.3
Vitamins			
100X stock, use 10 ml per L medium			
Myo-Inositol		12.5	125
Nicotinic acid		0.1	1
Pyridoxine hydrochloride		0.1	1
Thiamine hydrochloride		0.1	1
Glycine		0.2	2
Calcium pantothenate		0.1	1
Biotin		0.1	1
Other additives			
Glutamine			200
Ascorbic acid			100
Citric acid			100
Sucrose			40000
Agar			7000
Hormones and charcaol			
Hormones	according to stage as specified in Table 2		
Activated charcoal	according to stage as specified in Table 2		
pH			5.7

[1]These constituents (major and minor nutrients and Fe-EDTA) are used at half strength in preparation of medium used for rooting stage.

Table 2. Stages of date palm regeneration system, specific hormones and activated charcoal additives, culture conditions, durations, and responses associated with each stage.

Stage	Medium additives[1] (mg/L)	Light[2]	Duration[3] (wk)	Remarks
1. Culture initiation	2,4-D (100), 2iP (3) Act. charcoal (1500)	Dark	9	Explants swell, small tissue/callus protrusions
2. Callus induction	NAA (10), 2iP (30) Act. charcoal (1500)	Light	3	Explants turn green, visible callus formation
3. Embryogenic callus proliferation	NAA (10), 2iP (6) Act. charcoal (1500)	Light	9	Marked explants expansion, embryogenic callus growth
4. Callus multiplication	NAA (10), 2iP (1.5)	Dark	9	Callus multiplication and long-term maintenance
5. Embryogenesis	None	Light	12	Somatic embryos develop, mature, and germinate
6. Rooting	NAA (0.2)	Light	12	Roots form, plantlets ready for acclimatization in soil

[1] Full-strength MS salt except for rooting where half-strength MS salt is used.
[2] Incubation at 16-h photoperiods (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) or complete darkness at 23ºC ± 2ºC.
[3] Transfer at 3-wk intervals throughout culture duration.

2.2 Explant Preparation

1. Separate healthy 3-year-old offshoots from mother plants preferably during cool seasons. Note that offshoots separated in summer usually perform poorly *in vitro*. Using a hatchet and a serrated knife, trim the leaves and remove outer leaves acropetally to expose the shoot tip region (Fig. 1 *A*, *B*). Careful manipulation is necessary to avoid fracturing of shoot tips which are characterized by brittleness. Expose the white tissue of the shoot tip region leaving a 10-cm high, 4-cm in diameter, cylindrical-shaped shoot tip tissue surrounded with white fleshy tissue of young leaves sheaths (Fig. 1 *C*). Cut around the base in a 45° angle, about 2 cm away from the circumference of the cylindrical-shaped tip region (Fig. 1 *D*). To reduce browning, place excised shoot tips in a chilled antioxidant solution of ascorbic acid and citric acid at 150 mg l^{-1} each.
2. Surface disinfected shoot tips in 70% ethanol for 1 min, followed by shaking or stirring for 15 min in 1.6% w/v sodium hypochlorite (30% v/v commercial bleach) containing two drops of Tween 20 per 100 ml solution. Rinse tissue with sterile distilled water three times and keep in a chilled sterile antioxidant solution all through explant manipulation process. Note that young offshoots, one year old or less appear to exhibit less browning *in vitro*; however, they supply only several explants per offshoot. Older offshoots occasionally provide axillary buds which can be used as explant sources treated in a similar manner to shoot tip explants.

3. Note that the shoot tip region consists of apical meristematic bud surrounded by a number of leaf primordia encased by fleshy white tissue of leaf sheaths. Only the meristematic regions and leaf primordia are suitable for embryogenic callus formation. Under aseptic conditions, using scalpel and forceps trim the ends and further remove the outer tissue to expose leaf primordia and shoot terminal, leaving about 5 cm in length and 1 cm in diameter tip tissue. Separate leaf primordia, surrounding the terminal shoot tip, at the point of attachment (Fig. 1 *E*) and cut each primordia into two transverse sections. Section the terminal tip into 4 to 8 longitudinal sections, approximately 0.5 to 1 cm^3. A single offshoot may yield 10 to 20 explants including terminal tip sections and leaf primordia segments.

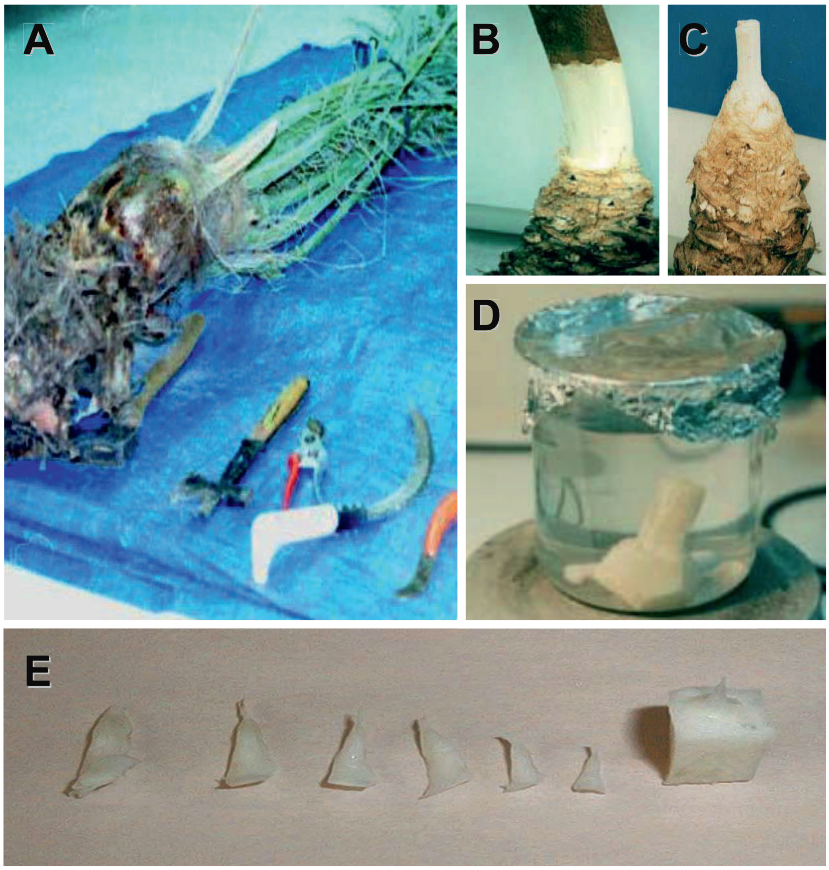

Figure 1. Date palm explant preparation. *A*) Offshoot and tools for shoot tip separation, *B*) Surrounding leaves and leaf sheaths removed gradually, *C*) Shoot tip region, *D*) Shoot tip region isolated and disinfected, *E*) Isolated leaf primorida and shoot tip explants (photo *E* is courtesy of Ms. Fadila Abed, INRAA).

2.3 Callus Culture

1. For culture initiation, prepare explants as described and culture on initiation medium as specified in Table 2.
2. Position whole leaf primordia explants vertically with the cut end inserted into the medium (Fig. 2 *A*). Because of potential latent internal contamination, a persistent problem in date palm tissue culture, it is advisable to culture explants in individual vessels throughout the procedure.
3. Incubate cultures as described in Table 2. Note that Table 2 also describes duration and conditions for all culture stages. Despite the addition of activated charcoal to the medium, browning often persists in date palm *in vitro* cultures. Transferring to a fresh medium at 3-wk intervals is recommended throughout the system to reduce phenolics accumulation.
4. Move entire explants to callus induction medium as described in Table 2. Note that light incubation is employed in this stage which stimulates greening of explants. Callus formation becomes clearly visible (Fig. 2 *B*).
5. Transfer entire explants to callus proliferation medium as described in Table 2. More callus growth occurs that can be easily separated from the original explants. Proportions of explants tend to turn brown to black during this stage; however, if maintained these explants would often produce whitish spots of embryogenic callus growth on their surfaces.
6. Separate callus growth from explants and transfer to callus multiplication medium (Table 2). Because of the lengthy incubation durations required to produce embryogenic callus in date palm, this stage provides an excellent opportunity to maintain callus for future experiments or embryo production. Callus maintained over two years has preserved embryogenic potential. Obviously, complications associated with lengthy maintenance, such as spontaneous mutation and possible lose of embryogenic capacity can be minimized by avoiding extended callus maintenance durations.

2.4 Cell Suspension Culture

1. Inoculate 1 g of callus into 150-ml culture flask containing 50 ml liquid medium. For cell culture establishment use callus multiplication medium (Table 2) but without agar. For somatic embryos development, use hormone-free liquid medium.
2. Incubate cultures at 23ºC ± 2ºC on an orbital shaker set to 100 rpm and 16-h photoperiods (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) provided by cool-fluorescent lamps.
3. For the first 2 wk, replace the culture medium every 3 to 5 days to reduce browning that often occurs initially.
4. Maintain cultures by decanting half of the liquid medium and adding an equal

volume of medium. After 3 months, each callus multiplication culture can be divided to establish two new suspension cultures.

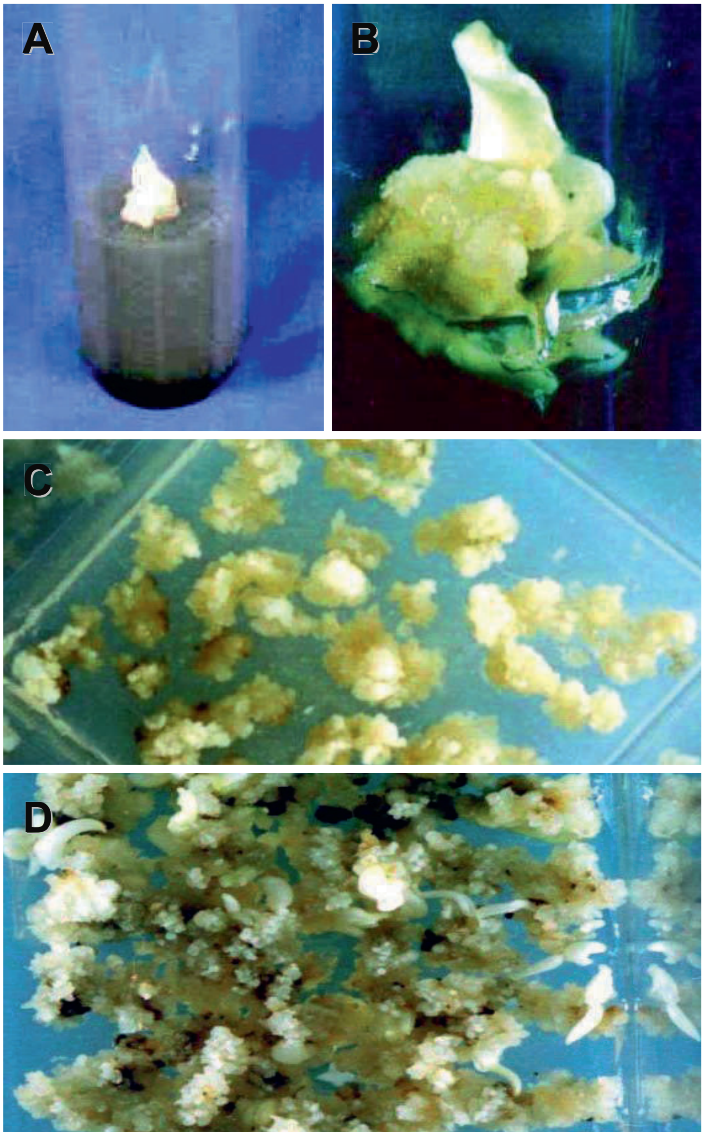

Figure 2. Date palm callus induction and somatic embryogenesis. *A*) Newly cultured explant, *B*) Callus formation at the cut edge of the explant, *C*) Embryogenic callus multiplication, *D*) Somatic embryos development on hormone-free medium.

2.5 Embryogenesis and Plant Establishment

1. Transfer callus from callus multiplication cultures (Fig. 2 *C*) to a hormone-free medium (Table 2) to encourage the development, maturation, and germination of somatic embryos. After a few weeks, globular embryos begin to appear which subsequently elongate forming bipolar-shaped embryos (Fig. 2 *D*). These embryos form green shoot growth but often root development is delayed and can be stimulated with auxin supplement.
2. To expedite complete plant formation, transfer mature or germinating embryos to rooting medium (Table 2). This stimulates root induction and shoot elongation forming complete plantlets with about 5-cm-long shoot (Fig. 3 *A*). Maintaining these plantlets until they reach 8 to 10 cm long increases their survival rate in soil.

2.6 Acclimatization and Field Transfer

1. Remove plantlets from culture tubes and gently rinse under a slow stream of water to remove residual agar medium from the root region (Fig. 3 *B*).
2. Position the plantlets upright in a beaker containing enough water to submerge the roots and cover with a transparent plastic bag for 3 d.
3. Treat plantlets with fungicide, 500 mg l^{-1} Benlate, and plant in 5-cm plastic pots containing potting mix consists of 1 soil: 1 peat moss: 1 vermiculite (Fig. 3 *C*). Water the transplants with 100 mg l^{-1} N-P-K fertilizer (20-20-20) and subsequently as needed. Mist the plantlets with water during the process of soil transfer to prevent desiccation.
4. Keep potted plantlets in clear plastic enclosures under culture room conditions for 3 wk during which gradually reduce humidity by adjusting air flow in the plastic enclosures (Fig. 3 *D*). Then, the transplants can be transferred to a shaded area of a greenhouse. Alternatively, the plantlets can be maintained under a shaded plastic tent in a greenhouse immediately after transplanting to soil. Introduce the plantlets to ambient conditions by gradually uncovering the plastic tent over a period of 3 wk. Any time during acclimatization, if plantlets show signs of water stress, immediately mist with water, close the acclimatization enclosures, and after a few days try again to gradual exposure the ex vitro plantlets to ambient relative humidity.
5. After 3 to 6 months, and subsequently as often as necessary, transfer the plantlets to larger pots and maintain in a greenhouse. Note that date palm growth vigor varies among cultivars. The plants can be transferred to a shade house and nurtured for 12 to 24 months (Fig. 3 *E*). The plants can then be planted in the field (Fig. 3 *F*). Normally, fruits are produced after 3 years of field growth.

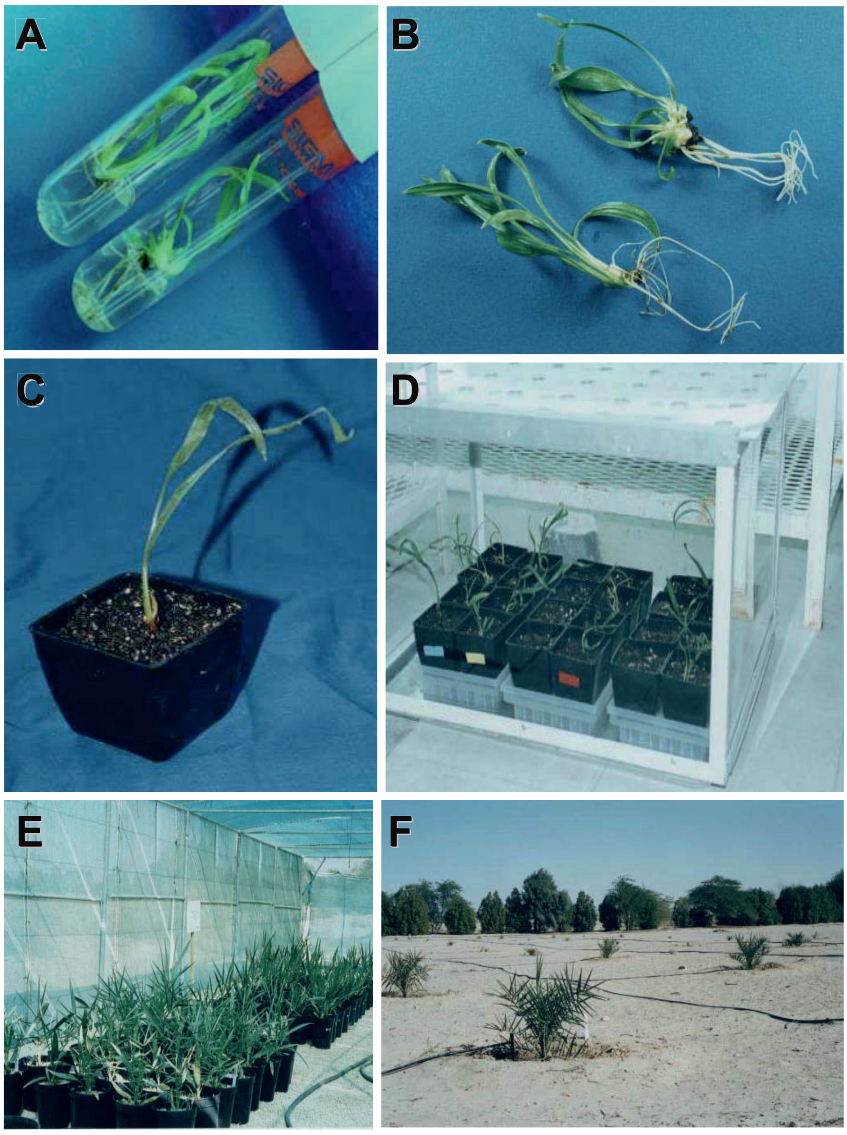

Figure 3. Date palm *in vitro* plant establishment. *A*) *In vitro* plantlets on rooting medium, *B*) Complete plantlets ready for hardening, *C*) Ex vitro plantlet in potted soil, *D*) Plantlets during acclimatization, *E*) Ex vitro plants maintained in a shade house, *F*) Tissue culture-derived date palms growing in the field.

3. RESEARCH PROSPECTS

Considering the importance of date palm, it must be said that date palm lags behind other species in the broadness of research scopes and extent of in-depth studies. In the area of micropropagation, research in commercial production, scale up, to produce propogules with low-cost efficiency is an important issue. The current price of in-vitro-derived date palm has not met the consumer expectations. Automation and bioreactor technologies, which may contribute to efficiency in commercial micropropagation, have not been yet exploited in date palm. Optimization of medium components, expediting existing micropropagation protocols, utilizing somaclonal variation, achieving, and commercial application of synthetic seed technology are some topics that merit further investigations. Research aimed at developing transformation system suitable for date palm is lacking despite of its great importance; particularly, in light of the current threat to date palm production imposed by the red palm weevil (*Rhynchophorus ferrugineus* Oliver) and bayoud disease caused by *Fusarium oxysporum* f. sp. *Albedini.* Through introgression of appropriate genes, eg. *Bt* and *chitinase* genes, research in date palm genetic engineering may produce enhanced resistance to these pests as it has been demonstrated in other plant species.

4. REFERENCES

Al-Khayri, J.M. 2001. Optimization of biotin and thiamine requirements for somatic embryogenesis of date palm (*Phoenix dactylifera* L.). *In vitro* Cell. Dev. Biol. Plant 37: 453-456.

Al-Khayri, J.M. 2002. Growth, proline accumulation, and ion content in NaCl-stressed callus cultures of date palm (*Phoenix dactylifera* L.). *In vitro* Cell. Dev. Biol. Plant 38: 79-82.

Al-Khayri, J.M. 2003. *In vitro* germination of somatic embryos in date palm: Effect of auxin concentration and strength of MS salts. Cur. Sci. 84: 101-104.

Al-Khayri, J.M., Al-Bahrany, A.M. 2001. Silver nitrate and 2-isopentyladenine promote somatic embryogenesis in date palm (*Phoenix dactylifera* L.). Sci. Horti. 89: 291-298.

Al-Khayri, J.M., Al-Bahrany, A.M. 2003a. Genotype-dependent *in vitro* response of date palm (*Phoenix dactylifera* L.) cultivars to silver nitrate. Sci. Horti. xx:xxx-xxx *in press*.

Al-Khayri, J.M., Al-Bahrany, A.M. 2003b. Growth, water content, and proline accumulation in drought-stressed callus of date palm. Biol. Plant. xx: xxx-xxx *in press.*

Baaziz, M., Aissam, F., Brakez, Z., Bendiab, K., El Hadrami, I., Cheikh, R. 1994. Electrophoretic patterns of acid soluble proteins and active isoforms of peroxidase and polyphenoloxidase typifying calli and somatic embryos of two reputed date palm cultivars in Morocco. Euphytica 76: 159-168.

Benbadis, A.K. 1992. Coconut and date palm. In: Hammerschlag, F.A., Litz, R.E. (ed.), pp. 383-400, Biotechnology of Perennial Fruit Crops. CAB International, Wallingford.

Bhaskaran, S.H., Smith, R.H. 1992. Somatic embryogenesis from shoot tip and immature inflorescence of *Phoenix dactylifera* L. cv. Barhee. Plant Cell Rep. 12: 22-25.

El Bellaj, M., El Jaafari, S., El Hadrami, I. 2000. IAA-oxidase: regulator and potential marker of somatic embryogenesis in date palm *(Phoenix dactylifera* L*.).* Cahiers Agri. 9: 193-195.

El Hadrami, I., Baaziz, M. 1995a. Somatic embryogenesis and plant regeneration from shoot-tip

explants in *Phoenix dactylifera* L. Biol. Plant. 37: 205-211.
El Hadrami, I., Baaziz, M. 1995b. Somatic embryogenesis and analysis of peroxidases in *Phoenix dactylifera* L. Biol. Plant. 37: 197-203.
Fki, L., Masmoudi, R., Drira, N., Rival, A. 2003. An optimized protocol for plant regeneration from embryogenic suspension cultures of date palm, *Phoenix dactylifera* L., cv. Deglet Nour. Plant Cell Rep. 21:517-524.
Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Omar, M.S., Novak, F.J. 1990. *In vitro* plant regeneration and ethylmethanesulphonate (EMS) uptake in somatic embryos of date palm (*Phoenix dactylifera* L.). Plant Cell Tiss. Org. Cult. 20: 185-190.
Omar, M.S., Hameed, M.K., Al-Rawi, M.S. 1992. Micropropagation of date palm (*Phoenix dactylifera* L.). In: Bajaj, Y.P.S. (ed.), Biotechnology in Agriculture and Forestry, Protoplast and Genetic Engineering, Vol. 18, pp. 471-492Springer-Verlag, Berlin.
Reuveni, O. 1979. Embryogenesis and plantlets growth of date palm (*Phoenix dactylifera* L.) derived from callus tissues. Plant Physiol. 63: 138.
Reynolds, J.F., Murashige, T. 1979. Asexual embryogenesis in callus cultures of palms. *In vitro* 15: 383-387.
Sudhersan, C., Abo El-Nil, Al-Baiz, A. 1993. Occurrence of direct somatic embryogenesis on the sword leaf of *in vitro* plantlets of *Phoenix dactylifera* L., cultivar Barhee. Cur. Sci. 65: 887-888.
Tisserat, B. 1979. Propagation of date palm (*Phoenix dactylifera* L.) *in vitro*. J. Exp. Bot. 30: 1275-1283.
Tisserat, B. 1984a. Date palm. In: Sharp, W.R., Evans, D.A., Ammirato, P.V., Yamada, Y. (ed.), Handbook of Plant Cell Culture, Vol. 2, pp. 505-545. Macmillan Publishing Company, NY.
Tisserat, B. 1984b. Propagation of date palms by shoot tip cultures. HortScience 19: 230-231.
Veramendi, J., Navarro, L. 1996. Influence of physical conditions of nutrient medium and sucrose on somatic embryogenesis of date palm. Plant Cell Tiss. Org. Cult. 45: 159-164.
Veramendi, J., Navarro, L. 1997. Influence of explant sources of adult date palm (*Phoenix dactylifera* L.) on embryogenic callus formation. J. Hort. Sci. 72: 665-671.
Zaid, A., Hughes, H. 1995. Water loss and polyethylene glycol-mediated acclimatization of *in vitro*-grown seedlings of 5 cultivars of date palm (*Phoenix dactylifera* L.) plantlets. Plant Cell Rep. 14:385-388.
Zaid, A., Tisserat, B. 1984. Survey of the morphogenetic potential of excised palm embryos *in vitro*. Crop Res. 24: 1-9.
Zohary, D., Hopf, M. 1993. Date palm *Phoenix dactylifera*. Domestication of Plants in the Old World, 2nd ed. Clarendon, Oxford.

SOMATIC EMBRYOGENESIS PROTOCOL: *CITRUS*

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1. INTRODUCTION

Citriculture is widely carried out in both tropical and subtropical regions and is one of the most economically important fruit crops. Although a high genetic variability is present in the genus *Citrus* and its wild relatives, improvement by conventional breeding is difficult because of the presence of nucellar embryos, a high degree of heterozygosity, and long juvenile periods, in addition to problems in sterility. Recent advances in *in vitro* tissue culture and genetic transformation offer new opportunities for the citrus scion and rootstock improvement. This chapter will describe *in vitro* tissue culture and genetic transformation protocols that can be used to obtain useful material for citrus physiological and genetic studies.

2. SOMATIC EMBRYOGENESIS

Since polyembryony is a trait widespread in *Citrus* and nucellar embryos are produced *in vivo*, many studies on *in vitro* somatic embryo regeneration have involved the culture of isolated nucelli, nucellar embryos, or whole fertilized or unfertilized ovules. As regenerated somatic embryos usually originate from nucellar tissues, they have a genetic makeup that is identical to that of the plant source, excluding mutational events. A direct correlation between the degree of *in vivo* polyembryony and the ability to regenerate somatic embryos *in vitro* has been reported (Mitra and Chaturvedi, 1972). Embryogenic calli in *Citrus* usually originate from nucellar tissues and are only rarely generated from somatic tissue that is neither nucellar nor ovular in origin (anthers, juice vesicles, stigmas and styles). Protocols for the high frequency induction of somatic embryos in *Citrus* using undeveloped ovule or stigma and style culture are described below.

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2.1. Source of explants

The selection of elite citrus plants is essential for the development of efficient systems of somatic embryogenesis. For these purposes, explants should be collected from selected elite specimens that are visibly free from any symptoms of disease, stress, or spontaneous mutations (i.e. variegated fruits and leaves, variation in color, size and shape of fruits, and various other plant abnormalities). The important points to bear in mind when deciding upon the choice of explant are that i) callus formation appears to depend on the status of the tissue, ii) callus initiation occurs more readily in tissues that are still juvenile, and iii) that explants must contain living cells. When floral tissues and fruits are old, chances of callus and embryo formation from undeveloped ovules, stigma, or style explants decrease. Stigma and styles derived from immature flowers and undeveloped ovules from unripe fruits have higher embryogenic potentials.

2.2. Composition of growth medium

The composition of the media used for *in vitro* regeneration of citrus somatic embryos is based on the inorganic salts recommended by Murashige and Skoog (1962) and on the organic compounds suggested by Murashige and Tucker (1969) (Table 1). Sucrose (50 g l^{-1}) is usually used as the carbon source. Usually growth regulators, when needed, are added directly to the medium before autoclaving. The pH of the medium is adjusted to 5.7$\pm$0.1 with 0.5 M potassium hydroxide or hydrochloric acid. Normally, 8 g l^{-1} agar is used (unless stated otherwise) to solidify media for citrus tissue culture. After the addition of agar, the medium is sterilized by autoclaving at 121°C for 20 min. Several components are thermolabile (i.e. antibiotics and various growth regulators) and should not be autoclaved. These substances should be sterilized by passage through a 0.22μm filter unit and added aseptically to the culture medium that has been autoclaved and allowed to cool to approximately 40°C. The medium is then dispensed under sterile conditions in plant tissue culture vessels (i.e. in Petri dishes, test tubes, jars). To avoid dehydration, vessels should be sealed with laboratory sealing film. After sterilization, the medium can be stored at room temperature for several weeks before use.

Table 1. Composition of MT medium for citrus tissue culture (Murashige and Tucker 1969)

Component	Concentration (mg l^{-1})
Inorganic salts	Same as MS medium (1962)
Glycine	2
Myo-inositol	100
Nicotinamide	5
Pyridoxine-HCl	10
Thiamine-HCl	10

Component	Concentration (mg l^{-1})
Sucrose	50,000
Malt extract	500

2.3. Somatic embryogenesis from undeveloped ovules

Starrantino and Russo (1980) first reported somatic embryogenesis from undeveloped ovule culture. The percentage of embryogenic explants can be obtained within range from 0 and 70% depending on the genotype. Usually, this regeneration procedure does not work with monoembryonic genotypes (Moore 1986).

2.3.1. Sterilization of explants

Open pollinated fruits at different ages (from 1 to 15 months after anthesis) are harvested from trees growing in the field. The fruits can be stored for long periods at 4°C. Ripe fruits can be stored about 1-5 months, while unripe fruits can be conserved for up to 4-7 months. Before sterilization each fruit is washed and the skin is peeled off. The fruits are surface sterilized by immersion for 5 min in ethanol (70% v/v) followed by 30 min in 2% (w/v) sodium hypochlorite.

2.3.2. Establishment of explants in culture

Without rinsing, fruits are cut open using forceps and scalpel under sterile conditions (Fig. 1A). Single segments are cut lengthways and by tearing the segment walls from each other, the segments are then opened (Fig. 1B). The undeveloped ovules are usually located along the part of the segment wall adjacent to the fruit axis (Fig. 1C). With the aid of a stereo microscope, the undeveloped ovules (0.3-1.5 mm in width and 1-3 mm in length) are carefully excised from segments and transferred to a Petri dish containing semisolid MT medium (to avoid loss of water). Ovule integuments (testa and tegmen) are removed (Fig. 1D) with the aid of a stereo microscope. The removal of the ovule integuments is not absolutely necessary, although it increases the percentage of responsive explants, ensuring a better contact between the explant and the medium. The excised ovules without integuments are placed (up to 25 explants) into a Petri dish (100 x 20 mm) containing 25 ml of MT semisolid medium supplemented with 0-6 mg l^{-1} 6-benzylaminopurine (BA) or alternatively supplemented with 0.01 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg l^{-1} BA (Grosser et al., 2000). The explants are transferred to a growth chamber at 25-27 °C under a 16 h day length with a photosynthetic photon flux density of 50 µmol m^{-2} s^{-1} and left from 1 to 8 months depending on the cultivar. Explants and calli are subcultured at 30-40-day intervals.

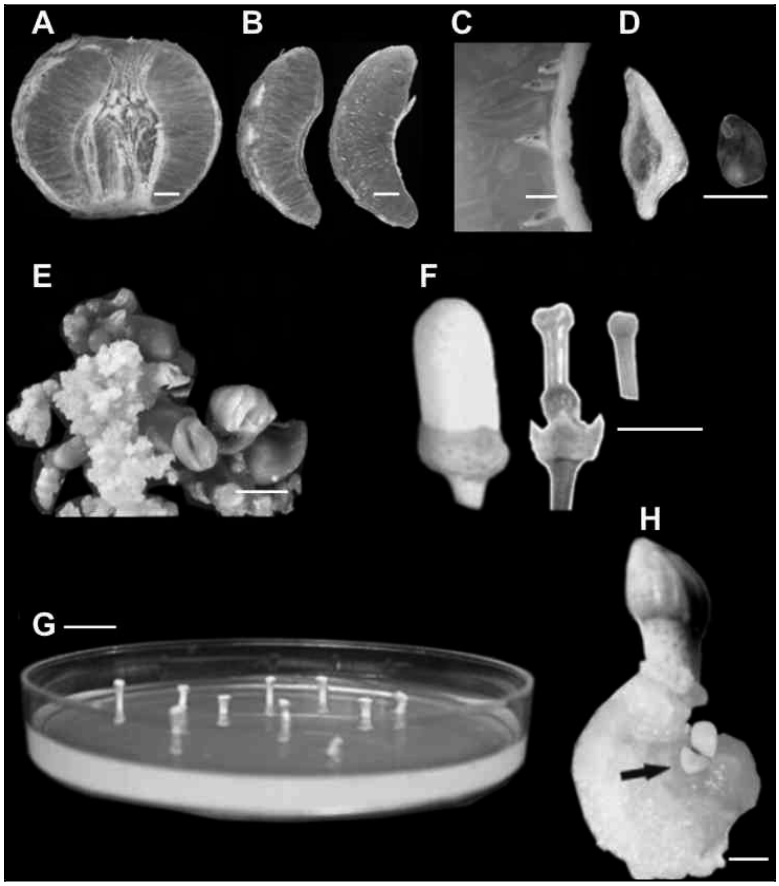

Figure 1. Somatic embryogenesis in Citrus from undeveloped ovule or stigma and style culture. **A:** Fruits are cut open under sterile conditions (bar = 1 cm). **B:** Single segments are cut lengthways (bar = 1 cm). **C:** The undeveloped ovules are located along the fruit axis (bar = 1.5 mm). **D:** Ovules with (left) and without (right) integuments (bar = 1 mm). **E:** Somatic embryos at different stages of development at the surface of undeveloped ovule derived callus (bar = 1 mm). **F:** Stigma and style explants excised from the flowers (bar = 1 cm). **G:** Stigma and style are placed vertically with the cut surface in contact with the medium (bar = 1 cm). **H:** Somatic embryo (arrow) developed at the surface of stigma / style derived callus (bar = 1 mm)

2.3.3. Embryo differentiation

In some cases, somatic embryos may develop directly from the cultured explant without an intermediate callus phase after 15-30 days of culture. Usually, somatic embryos develop in a non-synchronous manner after 50-120 days at the surface of undeveloped ovule derived calli (Fig. 1E). If calli show a low embryogenic competence to induce or enhance somatic embryogenesis, it is possible to incubate the citrus cells on MT medium supplemented with 2% glycerol (as the sole carbon source). The embryogenic cell lines regenerated from undeveloped ovule cultures

can be maintained on MT semisolid medium supplemented with 0-3 mg l^{-1} BA (subcultured at 30-day intervals) for several years.

2.3.4. Embryo germination and plant development

Regenerated somatic embryos (1-2 mm in diameter) are isolated from the callus and transferred to test tubes (155 x 23 mm) containing 20 ml of hormone free MT semisolid medium. One embryo is incubated in each test tube and transferred to a growth chamber at 25-27 °C under a 16 h day length. Within 2-3 months on MT medium, plantlets reach 5-7 cm in length. Alternatively, embryo germination can be attempted in MT semisolid medium supplemented with 1 mg l^{-1} gibberellic acid (GA_3) and 0.02 mg l^{-1} □-naphthaleneacetic acid (NAA) (Grosser and Gmitter 1990).

2.3.5. Acclimatization of regenerated plants

Acclimatization of plantlets is one of the most critical steps of the regeneration protocols. Plantlets are removed carefully from the test tubes and washed in sterile distilled water, removing as much of the medium as is possible without injuring the plant tissues (residues of medium are rich in nutrients that can promote pathogen growth). Plantlets are pricked into plastic pots containing sterilized soil. After transplanting, plants are incubated in a basal heating bench at 22/27 °C (ambient/soil temperature), a 16 h day length with a photosynthetic photon flux density of 100 μmol m^{-2} s^{-1}. Usually, the plantlets of *Citrus* regenerated *in vitro* have a cuticle that is poorly developed and an insufficient root system as well. Transpiration loss must be reduced as much as possible by maintaining high humidity levels (95±5% relative humidity) in the atmosphere for about 30 days. Nevertheless, high relative humidity levels in some cases can promote diseases and pests. They can be prevented by treatment with insecticides (Methomyl, methyl-O-(methylcarbamoyl) thiolacetohydroxamate) and fungicides (Fosetyl Al, phosphonic acid monoethyl ester aluminum salt) at 20-30-day intervals (De Pasquale et al. 1999). Once the root system has been established the regenerated plants need to be hardened-off before they are transferred to the greenhouse. A gradual reduction in relative humidity can be achieved by partially opening the lid of the bench. About 30 days later, the lid can be completely removed and most plants can survive under greenhouse conditions.

2.4. Somatic embryogenesis from stigma and style culture

Somatic embryogenesis from stigma and style cultures was first reported by Carimi et al. (1994). The percentage of embryogenic explants range between 0% and 58% depending on the genotype. Usually, lemons, tangerines, and navel sweet oranges show a higher embryogenic potential, whereas mandarins and grapefruit show a lower percentage of embryo regeneration. The culture of stigma and style has been successfully employed in the regeneration of many cultivars of the

following *Citrus* species: *C. aurantifolia, C. aurantium, C. deliciosa, C. limetta, C. limon, C. madurensis, C. medica, C. meyerii, C. myrtifolia, C. paradisi, C. sinensis* and *C. tardiva*. A different regeneration potential is also observed in different genotypes within the same species.

2.4.1. Sterilization of explants

Flowers are collected before opening and are either used immediately or alternatively, can be stored at 4°C for about 7-10 days. Whole flowers are surface-sterilized by immersion for 2 min in ethanol (70% v/v in water) and for 15 min in 2% (w/v in water) sodium hypochlorite. They are then rinsed once in sterile distilled water for 5 min. If the petals of the flowers are not tightly closed, the sterilization solutions can penetrate within the flower and injure the explant surface cells causing the cultures to turn brown and necrotic in a few days.

2.4.2. Establishment of explants in culture

With the aid of forceps and scalpel, flowers are cut open under sterile conditions. Only flowers in which the sterilization solution did not penetrate the petals should be used in order to increase the percentage of responsive explants. The stigma and style are excised from the flowers (Fig. 1F) with a sterile sharp blade and placed vertically onto plastic Petri dishes with the cut surface in contact with the medium (Fig. 1G). Problems of dehydration can dramatically reduce the number of responsive explants; this can be circumvented by treating reduced numbers of explants simultaneously. Up to 20 explants can be placed into a Petri dish (100 x 20 mm) containing 25 ml of MT semisolid medium supplemented with 0-3 mg l^{-1} BA. It is important to make sure that the basal portion of the style is firmly pressed into the medium without burying the explant. Cultures are transferred to growth chamber at 25-27 °C under a 16 h day length with a photosynthetic photon flux density of 50 µmol m^{-2} s^{-1}, and left for between 2 and 8 months depending on the cultivar. Explants and calli are subcultured at 30-40-day intervals. Contamination of explants can be a major problem and, if undetected, bacterial and fungal contamination can severely compromise cultures. Frequent inspection of cultures, particularly within the first month after culture initiation, should allow the recovery of healthy explants if contamination is detected promptly. Moreover, it is important to carefully inspect the cultures for signs of contamination before any subculture is attempted. Alternatively, placing fewer explants in smaller Petri dishes (7 explants / Petri dish 55 x 15 mm) reduces the risk of contamination.

2.4.3. Embryo differentiation

Usually callus formation occurs from the cut surface of the style base at 2-5 weeks after culture initiation and somatic green embryos develop at the surface of callus (Fig. 1H). Normally, somatic embryos appear 2-3 months after culture initiation (i.e. lemon, lime), but in some cases somatic embryos can appear after 7 months of

culture (mandarin, grapefruit, sour orange). The embryogenic cell lines regenerated from stigma and style culture can be maintained on BA (0-3 mg l^{-1}) supplemented MT semisolid medium for several years and retain their embryogenic capacity.

2.4.4. Embryo germination, plant development and acclimatization of regenerated plants

The procedures for embryo germination, plant development and acclimatization of regenerated plants are the same as those described for somatic embryogenesis from undeveloped ovules.

2.5. Cell suspension cultures

A procedure for the induction of embryogenic suspension cell cultures based on the culture of unfertilized sweet orange ovules (*C. sinensis* var. Brasiliensis Tanaka) has been described by Sakai et al. (1991). The method for the initiation of liquid cultures can also be applied to embryogenic calli of several *Citrus* species regenerated from different types of explants (i.e. undeveloped ovules or stigmas and styles). For embryogenic nucellar callus induction, unfertilized ovules are excised from flower buds nearly flowering, placed on MT semisolid medium supplemented with 10 mg l^{-1} BA and maintained in a growth chamber at 25°C under a 16 h day length. Explants and calli are subcultured at every 30-day interval on a maintenance medium that has the same composition as the callus initiation medium. Alternative procedures for the initiation of embryogenic calli are the same as described in the section “Somatic embryogenesis from undeveloped ovules” or “Somatic embryogenesis from stigma and style culture”.

In order to initiate a liquid culture, about 1-2 g of embryogenic calli are placed in 250 ml Erlenmeyer flasks containing 50 ml of MT liquid medium supplemented with or without 3-10 mg l^{-1} BA. Flasks are capped with aluminum foil and maintained in a growth chamber on a horizontal rotary shaker (90-110 rpm) at 25$\pm$1°C under a 16 h day length cycle. Cells are subcultured in fresh medium at 10-15 day intervals. To establish uniform liquid cultures, the culture conditions are kept as constant as possible at least for 2-4 months, until the growth coefficient becomes stable. In some cases it is possible to obtain habituated cultures and grow the cells in liquid medium without growth regulators. In order to reduce the dimension of the cell units, cell suspensions are passed periodically through a nylon sieve having a pore size of 250-500 μm. In order to induce somatic embryogenesis, about 1 g of cells is inoculated in a 250 ml Erlenmeyer flask containing 50 ml of hormone free MT liquid medium supplemented with 2% glycerol (without sucrose). The suspension cultures are maintained on a horizontal rotary shaker (90-110 rpm) at 25-27 °C. Usually, somatic embryos differentiate within 4-6 weeks after glycerol treatment (Ben-Hayyim and Neumann 1983).

3. PROTOPLASTS ISOLATION

Protoplast technology and its applications in *Citrus* are highly advanced compared with other trees. Vardi et al. (1975) reported the first example of successful citrus protoplast isolation and culture, followed by callus formation and embryo differentiation. Subsequently, several *Citrus* species have been regenerated from protoplasts via somatic embryogenesis. Moreover, citrus protoplasts have been used for mutagenic treatment (X-ray radiation and ethylmethanesulphonate), genetic transformation, and to regenerate somatic hybrid and cybrid plants. Ohgawara et al (1985) obtained for the first time somatic hybrids of citrus involving *Citrus* (*C. sinensis* and *Poncirus trifoliata*). Recently, Grosser et al. (2000) reviewed use of protoplast fusion for citrus improvement. A typical procedure based on the method described by Grosser and Gmitter (1990) for citrus protoplast isolation and fusion with polyethylene glycol (PEG) is described below.

3.1. Source material

Citrus protoplasts can be isolated from different sources including embryogenic cells (cultured on either solid or liquid media), non-embryogenic callus, and leaves. Embryogenic cell cultures (on solid or liquid media) may provide protoplasts with the best potential for proliferation and embryo regeneration. The procedures for the initiation of embryogenic cell suspension cultures are the same described above in the section "Cell suspension cultures". Leaves are the other routine source for protoplast isolation in *Citrus*. The reason is that leaf protoplasts are generally easy to isolate and large amounts of protoplasts are produced; however they do not develop into embryos. *In vitro* cultured nucellar seedlings are becoming more commonly used as a source of leaf material. The procedures for the growth *in vitro* of nucellar seedlings are the same described in the section "Genetic transformation".

3.2. Enzyme treatment

Suspension cell cultures: Generally, 6-10-day-old embryogenic cell suspension cultures (characterized by a high growth coefficient) are harvested with a sterile disposable wide-mouth pipette and about 0.5-1.0 g of cells are transferred (2-4 ml of suspension) to a Petri dish (55 x 15 mm). The suspension medium is removed with a Pasteur pipette and replaced with 3 ml of BH3 (see Table 2). About 1-2 ml enzyme solution (see Table 3) is added drop wise with a Pasteur pipette to facilitate cell separation; the dish is then closed with laboratory sealing film and maintained for 6-12 h in the dark on a horizontal rotary shaker (50 rpm) at 28°C.

Leaves: Prior to enzyme treatment, small leaves (about 15-25 mm in length) are collected under sterile conditions from *in vitro* grown nucellar seedlings (see the section "Epicotyl explant preparation from nucellar seedling") and cut into fine

strips with a sharp scalpel. About 5 leaves (about 30-60 mg fresh weight) are incubated in a 100 ml Erlenmeyer flask containing a mixture of BH3 medium and

Table 2. Composition of BH3 medium for citrus protoplast culture (Kao and Michayluk 1975; Grosser and Gmitter 1990)

Component	Concentration mg l^{-1}	Component (organic addenda)	Concentration mg l^{-1}
KH_2PO_4	170	Fructose	250
$MgSO_4 \cdot 7H_2O$	370	Ribose	250
$CaCl_2 \cdot 2H_2O$	440	Xylose	250
Na_2 EDTA	37.3	Mannose	250
$FeSO_4 \cdot 7H_2O$	27.8	Rhamnose	250
$MnSO_4 \cdot H_2O$	22.3	Cellobiose	250
$ZnSO_4 \cdot 7H_2O$	8.6	Galactose	250
H_3BO_3	6.2	Glucose	250
KCl	1,500	Sodium pyruvate	20
KI	0.83	Citric acid	40
$Na_2MoO_4 \cdot 2H_2O$	0.25	Malic acid	40
$CuSO_4 \cdot 5H_2O$	0.025	Fumaric acid	40
$CoCl_2 \cdot 6H_2O$	0.025	Vitamin B_{12}	0.02
Glutamine	3,100	Calcium pantothenate	1
Thiamine - HCl	10	Ascorbic acid	2
Pyridoxine - HCl	10	Choline chloride	1
Myo-inositol	100	p-aminobenzoic acid	0.02
Nicotinamide	1	Folic acid	0.4
Malt extract	500	Riboflavin	0.2
Casein hydrolysate	250	Biotin	0.01
Mannitol	81,970	Vitamin A (retinol)	0.01
Sucrose	51,350	Vitamin D_3	0.01
Coconut water	20 ml l^{-1}	(cholecalciferol)	

The pH of the solution is adjusted to 5.7±0.1. The solution is sterilized by passage through a 0.22µm filter unit and stored at 4°C

enzyme solution (10 ml total volume). A ratio (v:v) of 8:2 (BH3 medium : enzyme solution) is used for tender leaves, while a ratio of 6:4 is used for more mature leaves. Vacuum infiltration of the enzyme solution into the tissue considerably improves the efficacy of this treatment (15 min at 50 kPa). Leaves are maintained for about 12 h on a horizontal rotary shaker (50 rpm) at 28 °C in the dark. Usually, a combination of 51.35 g l^{-1} sucrose (0.15 M) and 81.9 g l^{-1} mannitol (0.45 M) in BH3 medium is sufficient (osmoticum 0.6 M), but in some cases it is necessary to increase the osmoticum concentration (up to 0.7 M).

Table 3. Composition of the enzyme solution for the isolation of citrus protoplasts (Grosser and Gmitter 1990)

Component	Concentration (mg l^{-1})
Mannitol	127,000
$CaCl_2$	1.33
MES*	1.17
NaH_2PO_4	0.17
Cellulase Onozuka RS	10,000
Macerozyme	10,000
Pectolyase Y-23	2,000

*MES = 2-(*N*-Morpholino)ethanesulfonic acid. The pH of the solution is adjusted to 5.6±0.1. The solution is sterilized by passage through a 0.22µm filter unit and stored at 4°C

3.3. Protoplast harvest and purification

Citrus protoplasts are easily separated from incomplete digested cells or tissues by sieving the enzyme-protoplast mixture through a nylon sieve with a pore size of 45 µm. Further purification of the enzyme-protoplast mixture (if the debris concentration is too high) can be achieved with a 25% sucrose / 13% mannitol gradient. After filtration, the enzyme-protoplast mixture is transferred to sterile screw-capped tubes (15 ml) and centrifuged for 7 min at 100-300 x*g*. The liquid phase is removed and the protoplasts are gently resuspended in 5 ml of 25% sucrose containing CPW nutrients (Table 4). About 2 ml, 13% mannitol containing CPW nutrients carefully loaded (avoid mixing of the two phases) over the sucrose cushion. The gradient is further centrifuged for 6 min at 100 x*g* to separate the debris (pellet) and the protoplast fraction (ring at the interface between the two phases). With the aid of a Pasteur pipette, the protoplasts are carefully collected

Table 4. Composition of CPW solution for the purification of citrus protoplasts (Frearson et al. 1973; Grosser and Gmitter 1990)

Component	Concentration (mg l^{-1})
KH_2PO_4	27.2
KNO_3	101
$CaCl_2$	150
$MgSO_4$	250
$Fe_2(SO_4)_3 \cdot 6H_2O$	2.5
KI	0.16
$CuSO_4$	0.025

The pH of the solution is adjusted to 5.8±0.1. The solution is sterilized by passage through a 0.22µm filter unit and stored at 4°C

and transferred to a new tube and centrifuged for 4 min at 100 x*g*. The supernatant is removed and the pellet is diluted with about 10 volumes of BH3 medium (Table 2). Alternatively, the protoplast density is calculated with hemocytometer and then adjusted to about 2-4 x 10^5 ml^{-1}.

3.4. Protoplast fusion using PEG

Usually somatic hybrids in *Citrus* are generated by the fusion of protoplasts isolated from embryogenic cell lines of one parent with protoplasts isolated from non-embryogenic cells or tissues (i.e. leaf-derived protoplasts) the second parent. Equal volumes of purified protoplasts of the two parental sources are mixed together and a small volume of resuspended protoplasts (2-3 drops) is then transferred to a Petri dish (55 x 15 mm). After gently adding two drops of PEG solution (see Table 5), protoplasts are incubated for 10 min. Next, two drops of freshly prepared elution solution (90% A Solution and 10% B Solution [v:v], see Table 5) are added and incubated for 15 min. This is followed by the addition of 12 drops of BH3 medium around the margin of fusing protoplasts, which is then left for 5 min. The PEG and elution solutions are carefully pipetted off (avoiding the elimination of protoplasts), replaced with 15 drops of BH3 medium for washing, and left for 10 min. The washing step must be repeated 2-3 times.

Table 5. Composition of citrus protoplast fusion solutions (Grosser and Gmitter 1990)

Component	PEG Solution *	A Solution	B Solution
Polyethylene glycol (MW 8,000)	40%	-	-
Glucose	54.1 g l^{-1}	72.1 g l^{-1}	-
$CaCl_2$	7.32 mg l^{-1}	7.32 mg l^{-1}	-
Dimethylsulfoxide	-	10%	-
Glycine	-	-	22.5 g l^{-1}
PH	6	6	10.5**

* Old batches of PEG solution can reduce protoplast viability. ** The pH of the solution B is adjusted to 10.5 with KOH in pellets. The solutions are sterilized by passage through a 0.22 μm filter unit, store in the dark at 4°C

3.5. Protoplast culture and somatic embryo regeneration

After the final wash, protoplasts are cultured directly in the center of the fusion Petri dish in about 6-20 drops of protoplast culture medium (the final protoplast concentration should be nearly 0.8-1.0 x 10^5 protoplasts / ml). In order to maintain high humidity, 15-20 drops of protoplast culture medium are added around the perimeter of the Petri dish. Protoplasts are incubated for about 5 weeks in the dark at 28 °C. BH3 medium is considered the optimum protoplast culture medium for protoplasts of recalcitrant genotypes. However, protoplasts can also be cultured in

MT liquid medium (see Table 1) supplemented with 205.4 g l^{-1} sucrose or a mixture of MT and BH3 (1:1 v/v). See Vardi and Galun (1988) for more detailed information on osmoticum source, plating density, and plating efficiency. Usually, the first cell division is observed after 10-14 days of incubation in protoplast culture medium (Grosser and Gmitter 1990). The plating efficiency of citrus protoplasts, defined as the percentage of protoplasts giving origin to microcolonies, usually ranges from 0% to 35%. To reduce the osmoticum, after 3-4 additional weeks cultures are supplemented with about 11 drops of a mixture (2:3 v/v) of BH3 and MT medium (supplemented with 125 g l^{-1} sucrose). Cultures are maintained in low light at 28 °C. Two weeks later the osmoticum should be further reduced by adding about 1 ml of a mixture (1:1 v/v) of BH3 and MT medium (supplemented with 50 g l^{-1} sucrose). Next, MT liquid medium (500 µl) (supplemented with 50 g l^{-1} sucrose) is added at 2-week intervals. For further growth, vigorously growing microcolonies (1-2 mm in diameter) regenerated from protoplasts are poured into Petri dishes (100 x 15 mm) containing semisolid (7 g l^{-1} agar) MT medium (supplemented with 50 g l^{-1} sucrose). Cultures are maintained moist by adding liquid medium until cells adapt to solid medium growth conditions. Usually somatic embryos develop spontaneously about 10 weeks after protoplast isolation. In some cases, when calli show a low embryogenic competence to induce the formation of somatic embryos, it is possible to incubate the callus colonies derived from protoplast into Petri dishes (100 x 15 mm) containing semisolid (7 g l^{-1} agar) MT supplemented with 2% glycerol (without sucrose).

3.6. Embryo germination, plant development and acclimatization of regenerated plants

The procedures for embryo germination, plant development, and acclimatization of regenerated plants are the same as those described for somatic embryogenesis from undeveloped ovules.

4. RADIOSENSITIVE CURVE FOR INDUCED MUTATIONS

Citrus are highly heterozygous and progenies from cross breeding may result in several traits that are different from parents. However, induced mutations may change only one or a few specific traits of an elite genotype. Tissue culture has the potential to improve the effectiveness of mutation induction for citrus improvement. The determination of the appropriate radiation dose is one of the first steps in mutagenic treatment, and it is based on radiosensitivity. It involves the determination of the dose that causes a 50% reduction of vegetative growth of the irradiated material (LD_{50}) (Predieri 2001). Some examples of radiosensitivity of citrus tissues are reported in Table 6.

A typical procedure based on the method described by Vardi et al. (1975) for exposure of citrus protoplast to X-ray radiation is described below. Protoplasts are isolated from citrus embryogenic cell lines as described in the section "Protoplasts isolation". Protoplasts are resuspended in 0.6 M BH3 liquid medium and 2 days after isolation from the callus are exposed to X-rays at a dose of 2-4 krad. About 4h after treatment, the BH3 medium is removed. The protoplasts are gently resuspended in fresh BH3 medium and the protoplast density is adjusted to about 1-4 x 10^5 ml^{-1}. The procedures for protoplast culture and somatic embryo regeneration are the same as those described in the section "Protoplast culture and somatic embryo regeneration". Usually somatic embryos develop about 4 moths after protoplast treatment. The procedures for embryo germination, plant development, and acclimatization of regenerated plants are the same as those described for somatic embryogenesis from undeveloped ovules. Vardi et al. (1975) reported protoplast survival after X-ray irradiation (Fig. 2).

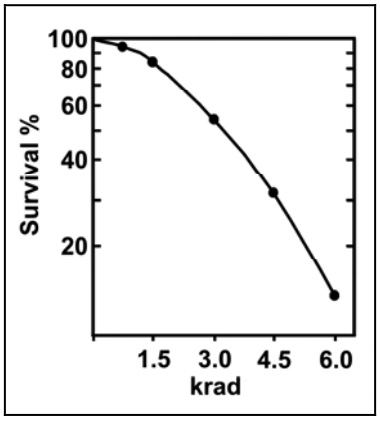

Figure 2. Survival of sweet orange protoplasts after X-ray radiation (Vardi et al. 1975). Survival is expressed as the percentage of colonies formed after 4 weeks relative to non-irradiated protoplasts

Table 6. Some examples of radiosensitivity of different sweet orange tissues (Vardi et al. 1975; Broertjes and Van Harten 1978; Ammirato et al. 1984)

Type of tissue	Mutagen	LD_{50} (krad)
Decapitated young seedlings	Gamma-rays	3
Protoplasts	X-rays	3.4
Bud wood	Gamma-rays	6
Seeds	Gamma-rays	10
Embryogenic callus	Gamma-rays	14

5. CRYOPRESERVATION

Cryopreservation of cells and meristems is fundamental for the long-term storage of citrus germplasm, reducing costs and ensuring maximal stability of the genetic and sanitary characteristics of stored germplasm. To date, cryopreservation methods of *Citrus* have been successfully applied to several different types of explants (seeds, ovules, embryos, embryonic axes, shoot tips and embryogenic cells). Sakai et al. (1991) described a simple and reliable procedure for cryopreservation of citrus nucellar cells. The authors reported that the average rate of cell survival after thawing is about 90% and that nucellar calli cryopreserved by this method maintain an embryogenic potential identical to that of non-frozen controls. Although they utilized callus derived from the culture of unfertilized ovules of navel orange (*C. sinensis* Osb. var. Brasiliensis Tanaka), the protocol can be readily applied to embryogenic calli of other *Citrus* species regenerated from different types of explants. Other methods are based on the use of expensive equipment such as programmable freezing units that allow the recovery up to 100% of cultures (Perez et al. 1997). A typical procedure based on the method describer by Sakai et al. (1991) for citrus embryogenic cell cryopreservation is described below. This method can be performed in any laboratory, as no sophisticated facilities are needed.

5.1. Initiation of embryogenic callus and cell suspension cultures

The procedures for the initiation of cell suspension cultures are the same described above in the section “Cell suspension cultures”.

5.2. Cryoprotection

About 0.4 ml packed cell volume from 6-day-old cell suspension cultures is washed with 2 ml of MT liquid medium supplemented with 136.9 g l^{-1} sucrose at 25°C. The cell suspension is gently mixed, centrifuged, and the supernatant fraction is removed. The cells are cryoprotected with 2 ml of MT liquid medium supplemented with 184.2 g l^{-1} glycerol and 136.9 g l^{-1} sucrose for 10 min at 25 °C. Cells are resuspended in the cryoprotective solution and a 0.4 ml aliquot of cell suspension is placed in 1.8 ml sterile cryotube.

5.3. Freezing

The cell suspension aliquots are cooled by placing the cryotubes horizontally in a freezer at -30°C. Freezing of cell suspension starts at about –15°C within about 8 min. After about 30 min, the cell suspension reaches –30°C and the cryotubes are then plunged directly into liquid nitrogen.

5.4. Thawing and regrowth of cells

The cryotubes are dropped into sterile water at 40°C until the thawing is complete. The cell suspension is then transferred (without washing out the cryoprotectant) into 2 ml of diluent containing 410.8 g l^{-1} sucrose in MT medium and maintained for 10 min at 25°C. For regrowth, samples of 0.5 ml of diluted cell suspension are transferred to a double layer of sterilized filter paper (50 mm in diameter). The cells and the layers of filter paper are placed on 25 ml of MT semisolid medium supplemented with 51.14 g l^{-1} sucrose and 3-5 mg l^{-1} BA in a Petri dish (100 x 20 mm). After 4-5 h incubation, the cells and the upper layer of filter paper are then transferred to fresh medium. The cells are incubated in a growth chamber at 25°C. Usually, regrowth of frozen cells starts 3-5 days after plating, and 12-17 days after plating enter the exponential growth phase.

5.5. Regeneration of somatic embryos

Cells grown on filter paper are transferred to a MT semisolid medium (9 g l^{-1} agar), supplemented with 50 g l^{-1} galactose and 50 ml of coconut water without sucrose. Within 2 months, cotyledonary embryos are regenerated. Somatic embryos develop into plants within 3 months (Sakai et al. 1991).

6. GENETIC TRANSFORMATION

In general, *Citrus* are recalcitrant to genetic transformation and the differences in transformation efficiency between genotypes even within a single *Citrus* species can be very quite different. Several citrus transformation protocols have been reported, based on the introduction of naked DNA into protoplasts or particle bombardment of embryogenic cells. However, the vast majority of the protocols in the literature are based on *Agrobacterium tumefaciens* mediated transformation of tissues (segments from *in vitro* germinated seedlings and internodal segments collected from young plants cultivated in the greenhouse) or undifferentiated cells (embryogenic nucellar calli). Usually, the non-oncogenic derivatives of A281 wild-type strain are utilized for transformation of *Citrus*.

In *Citrus*, explants derived from juvenile tissues (such as young seedlings or nucellar embryos) are usually used as starting material for genetic transformation of polyembryonic *Citrus* because of their high regenerative potential. However, plants regenerated from juvenile material present undesirable characteristics such as thorniness and excessive vegetative vigor. In addition, the long juvenile phase in *Citrus* induces a delay in flowering and prolongs the time required to analyze the characteristics of fruit. It has been shown that grafting mature buds (collected from adult plants) onto juvenile rootstocks can be used to obtain relatively juvenile material for genetic transformation. This technique allowed a transformation frequency of 6% and yielded transgenic sweet orange plants that flowered after 14

months in the greenhouse (Cervera et al. 1998). A typical procedure for citrus genetic transformation based on the method reported by Yu et al. (2002) is described below. We transformed epicotyl explants of sweet orange and Carrizo citrange with *Agrobacterium* strain EHA101 harboring binary vector pGA482GG and obtained a transformation frequency of 12 and 80%, respectively.

6.1. Epicotyl explant preparation from nucellar seedling

Seeds freshly removed from fruits are peeled and embryos are surface disinfected by immersion for 10 min in 0.5% (w/v in water) sodium hypochlorite solution containing 0.1% (v/v) Tween-20, and then rinsed three times in sterile distilled water for 5 min. Sterilized embryos are transferred to test tubes (155 x 23 mm) containing 25 ml of ½ (inorganic salts and vitamins) MT semisolid medium supplemented with 0.1 mg l^{-1} NAA, 500 mg l^{-1} charcoal, and 30 g l^{-1} sucrose. One embryo is incubated in each test tube. Embryos are transferred to growth chamber at 27 °C under a 16 h day length. About 20 days later epicotyl stems are harvested and cut transversely into 10 mm segments. Segments are then cut longitudinally into two halves to increase the wound area. Before inoculation with *Agrobacterium*, explants are pretreated by immersion for 3 h in liquid MT medium supplemented with 3 mg l^{-1} BA, 0.5 mg l^{-1} 2,4-D, 0.1 mg l^{-1} NAA, 39.3 mg l^{-1} acetosyringone, and 30 g l^{-1} sucrose (co-cultivation medium).

6.2. Rejuvenation of mature tissues through grafting on juvenile seedlings

Cervera et al. (1998) described an alternative procedure for explant preparation based on the recovery of relatively juvenile material through grafting mature scions on juvenile rootstocks.Buds collected from mature plants are grafted on seedlings of *C. volkameriana* Ten & Pasq. Growing in the greenhouse at 18-27°C. Newly elongated mature shoots are allowed to develop into one or two flushes. Stem pieces 20 cm long are stripped of their leaves and thorns, surface-disinfected by immersion for 10 min in 2% (w/v in water) sodium hypochlorite solution containing 0.1% (v/v) Tween-20, and then rinsed three times in sterile distilled water for 5 min. Internodal stem segments in a semi-hardened stage are cut (10 mm in length) transversely from the stem pieces and used for transformation.

6.3. Explant inoculation, co-cultivation and regeneration

Bacteria cultures with cell density less than 0.7 at OD_{600} are centrifuged at 1000 x*g* for 7 min and then resuspended in liquid co-culture medium at an OD_{600} of 0.15. For inoculation with *Agrobacterium*, explants are placed in 50 ml Erlenmeyer flasks containing 10 ml of this suspension and incubated at 25 °C for 10 min in an orbital shaker at 50 rpm. After inoculation, explants are removed and blot dried on sterile filter paper and placed horizontally with the longitudinally cut surface in contact with the co-cultivation semisolid medium and maintained in a growth

chamber at 25±1°C in the dark for 3 days. Following co-cultivation, explants are transferred to semisolid MT medium supplemented with 3 mg l^{-1} BA, 0.5 mg l^{-1} NAA, 30 g l^{-1} sucrose, 100 mg l^{-1} kanamycin (for selection of transformed cells), 250 mg l^{-1} cefotaxime and 250 mg l^{-1} vancomycin (in order to control bacterial growth) (regeneration medium). Explants are maintained in a growth chamber at 25±1°C in the dark for 2 weeks, followed by transfer to a 16 h photoperiod. After 5 weeks, the explants producing shoots are transferred to semisolid MT medium supplemented with 1 mg l^{-1} BA, 0.1 mg l^{-1} NAA, and 30 g l^{-1} sucrose. Explants are maintained on this medium until shoots reach 2 mm in length for shoot tip grafting or at least 10 mm for two-step rooting culture. Adventitious buds usually are regenerated 4-6 weeks after inoculation on the longitudinal cut surface.

6.4. Recovery of transformed plants by shoot tip grafting or by two-step rooting culture

The recovery of transformed shoots by shoot tip grafting is based on the procedure described by Peña et al. (1995). As rootstocks, *in vitro* grown seedlings of Troyer citrange (*C. sinensis* L. Osb. x *Poncirus trifoliata* L. Raf.) are used. Citrange seeds are peeled by removing both seed coats, surface-disinfected as described above, and pre-germinated on MT semisolid medium at 27°C for 1-2 weeks. After germination, seeds are sown individually on paper bridges (80 x 160 mm paper sheet folded twice longitudinally and once transversely to a final size of 20 x 80 mm) and transferred to test tubes (155 x 23 mm) containing 25 ml of hormone free MT liquid medium (1 seedling / test tube). They are then transferred to a growth chamber at 27°C in the dark for two weeks. Before grafting, citrange seedlings are decapitated leaving 1-1.5 cm of the epicotyl. The roots are shortened to 4-6 cm and the cotyledons with the axillary buds are removed. The transformed shoots about 2 mm in height are harvested from the stem segments and shoot tip grafted by placing them on the top cut surface of the decapitated citrange epicotyls, in contact with the vascular ring. Grafted plants are incubated in a liquid medium based on the inorganic salts as suggested by Murashige and Skoog (1962) and the organic compounds as suggested by Peña et al. (1995) (Table 7). The grafted plants are transferred to growth chamber at 25°C under a 16 h day length with a photosynthetic photon flux density of 45µmol m^{-2} s^{-1} for 3-5 weeks. Subsequently, epicotyls of the shoot tip grafted plants are grafted on vigorous seedlings growing in the greenhouse at 18-27 °C. The recovery of transformed shoots by a two-step rooting culture is based on the procedure described by Yu et al. (2002). This procedure can only be applied if the transformed shoots reach 10-15 mm in length.

Transformed shoots are cultured for 2 weeks on one-fourth strength MS inorganic salts and vitamins semisolid medium (Table 8), supplemented with 5 mg l^{-1} indole-3-butyric acid (IBA) and 0.5 mg l^{-1} NAA. After rooting, shoots are cultured for 3-4 weeks on one-fourth strength MS inorganic salts and vitamins semisolid medium (Table 8), supplemented with 20 mg l^{-1} kanamycin. The procedures for the plant

development and the acclimatization of transformed plants are the same as those described for somatic embryogenesis.

Table 7. Composition of liquid medium for grafted citrus plants (Peña et al. 1995)

Component	Concentration (mg l^{-1})
Inorganic salts	Same as MS medium (1962)
Myo-inositol	100
Nicotinamide	1
Pyridoxine-HCl	1
Thiamine-HCl	0.2
Sucrose	75,000

The pH of the medium is adjusted to 5.7±0.1 with 0.5 M potassium hydroxide and the medium is sterilized by autoclaving at 121°C for 20 min.

Table 8. Composition of medium for recovery of transformed citrus shoots (Yu et al. 2002)

Component	Concentration (mg l^{-1})	Component	Concentration (mg l^{-1})
KH_2PO_4	42.5	KI	0.21
KNO_3	475	$Na_2MoO_4 \cdot 2H_2O$	0.0625
NH_4NO_3	412.5	$CuSO_4 \cdot 5H_2O$	0.00625
$MgSO_4 \cdot 7H_2O$	92.5	$CoCl_2 \cdot 6H_2O$	0.00625
$CaCl_2 \cdot 2H_2O$	110	Glycine	0.5
Na_2 EDTA	9.31	Thiamine - HCl	0.025
$FeSO_4 \cdot 7H_2O$	6.96	Pyridoxine - HCl	0.125
$MnSO_4 \cdot 4H_2O$	5.58	Myo-inositol	25.0
$ZnSO_4 \cdot 7H_2O$	2.15	Nicotinamide	0.125
H_3BO_3	1.55	Sucrose	20,000

The pH of the medium is adjusted to 5.7±0.1 with 0.5 M potassium hydroxide and the medium is sterilized by autoclaving at 121°C for 20 min.

7. MOLECULAR MARKERS FOR GENETIC FIDELITY

Several types of molecular markers have been used in citrus genetic investigations, such as restriction fragment length polymorphisms (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites or simple sequence repeats (SSR), inter-simple sequence repeat (ISSR), to name a few. ISSRs have proven valuable for genetic investigations in *Citrus* (Fang and Roose 1997). They are generated by polymerase chain reaction (PCR) primers, which have a core sequence (di-, tri-, tetra- or pentanucleotide motifs) repeated in tandem (complementary to a single SSR) with a degenerate

anchor (extended into the flanking sequence by 2 to 4 nucleotide residues). The resultant PCR amplifies the sequence between two binding sites (SSR) in an opposite orientation within a suitable distance, yielding a multilocus marker system useful for genetic analysis. A typical reaction may yield 10-80 bands per lane depending on the species, primers, and PCR amplification conditions. ISSR-PCR are widely used as molecular markers because they have several advantages: no prior information (i.e. sequencing) to design the oligonucleotide primers is required, development costs are low, and protocols can easily be transferred to different *Citrus* species. The equipment required for ISSR analysis is relatively minimal and many companies make their own equipment. The major requirements are reported in Table 9.

Table 9. Major equipment required for ISSR analysis

Centrifuge for DNA extraction
Spectrophotometer for DNA concentration measurement
Thermal cycler for performing the polymerase chain reaction
Laminar flow hood to prepare PCR reaction mix in sterile conditions and avoid DNA contamination problems
Equipment for running and analysing agarose gels (i.e. power supply and electrophoresis units for nondenaturing polyacrylamide or agarose gels)
UV trans-illuminator for visualisation of PCR products stained with ethidium bromide
Refrigerator and freezer for reagent and sample storage
Other equipment necessary for DNA marker work (i.e. magnetic stirrer and pipettor)

7.1. DNA extraction

DNA extraction is based on the procedure described by Ruiz et al. (2000). A microfuge tube (1.5-2 ml) cap is used to collect about 8 leaf punches for each sample. Tubes are placed on ice, liquid nitrogen is added, and the tissues are homogenized by grinding with a stainless steel rod (rounded to fit the bottom of the tube). Extraction buffer (0.5 ml) (100 mM Tris, 50 mM ethylenediaminetetraacetic acid [EDTA], 500 mM NaCl, pH 8.0, and just before use 10 mM β-mercaptoethanol) is then added and the pulverised tissue is resuspend. After the addition of 33µl of 20% sodium dodecyl sulfate followed by vigorous agitation for a few seconds, tubes are incubated 10 min at 65°C. Next, 160µl 5 M potassium acetate are added, extract is gently mixed, and then placed on ice for 20 min. Samples are centrifuged in a microcentrifuge at maximum speed (14,000 x*g*) for 10 min at 4°C. The supernatant is filtered through Miracloth (Calbiochem) and transferred into a new tube. Isopropanol (1 vol) is added and the tube is gently inverted several times and incubated for 10 min at room temperature. The DNA is then precipitated by centrifugation at maximum speed (14,000 x*g*) for 5 min at 4°C. The supernatant is removed and the pellet is washed with 70% ethanol. The pellet is resuspended in 100 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM sodium EDTA pH 8.0). The tube is centrifuged at maximum

speed (14,000 x*g*) for 5 min at 4°C. The aqueous phase is transferred to a new Eppendorf tube and the DNA is precipitated again by the addition of 11µl 3 M sodium acetate and 72µl isopropanol. After mixing, the tube is centrifuged at maximum speed for 3 min at 4 °C. The pellet is washed with 70% ethanol and dried under vacuum. The DNA is resuspended in 30µl TE and stored at 4°C. Alternatively, the DNA can be isolated from different citrus tissues as described by Doyle and Doyle (1987).

It should be noted that quality and purity of the starting DNA template is crucial to the success of PCR (a high number of well-resolved bands) and that RNA contamination in the template interferes with the PCR reaction. Therefore, when necessary, DNA must be purified from RNA with RNase treatment.

7.2. Primer design

Fang and Roose (1997) reported the sequence of the primers that can be used to amplify citrus DNA for ISSR markers (Table 10).

The primer sequence is fundamental in determining the conditions of the PCR. The annealing temperature is dependent upon the primer melting temperature (T_m).

Table 10. ISSR primers used with citrus DNA (Fang and Roose 1997)

Primer sequence	Number of bands*	Primer sequence	Number of bands*
BDB(TCC)$_5$	28	(GA)$_8$YT	60
HVH(TCC)$_5$	46	(GA)$_8$YC	97
(TCC)$_5$RY	42	(GA)$_8$YG	65
HVH(TG)$_7$T	45	(AG)$_8$YT	97
VHVG(TG)$_7$	53	(AG)$_8$YC	73
DBDA(CA)$_7$	45	(AG)$_8$YG	63
HVH(CA)$_7$T	66	(AC)$_8$YT	40
BDB(CA)$_7$C	45	(AC)$_8$YA	55
DBD(AC)$_7$	32	(AC)$_8$YG	51
(CA)$_8$RG	58	(GT)$_8$YC	54
(CA)$_8$RY	54	(GT)$_8$YG	60

* Number of bands scored in navel sweet orange. B = non-A; D = non-C; H = non-G; R = purine; V = non-T; Y = pyrimidine

Fang et al. (1997) reported an annealing temperature of 52°C. However, it is preferably to empirically determine the most suitable annealing conditions by performing the reaction at several temperatures, starting approximately 5°C below the T_m. Several formulas exist to determine the theoretical T_m. The formula reported below can be used to estimate the melting temperature for oligonucleotides:

$T_m = 81.5 + 16.6 \log_{10} [M] + 0.41(\%G+C) - 675/n$

Where [M] is the molar concentration of monovalent cations, (%G+C) is the percentage of G and C in the oligonucleotide and n = number of bases in the oligonucleotide.

For primers with a high T_m, the annealing temperature should be increased in order to enhance the amount of specific products

7.3. PCR amplification of ISSR

PCR reactions are performed in sterile 0.250 or 0.500 ml Eppendorf tubes. The PCR reaction mix (a final volume of 25µl) contains 20 mM Tris-HCl (pH 8.4), 250µM dNTP, 3 mM $MgCl_2$, 50 mM KCl, 0.6 µM of each primer, 1 U of Platinum *Taq* polymerase (Life Technologies) and 25 ng of template DNA. The Eppendorf tubes are transferred to a thermal cycler (e.g. MJ Research thermocycler, Genenco, equipped with a Hot Bonnet) and the reaction is subjected to the following cycle program: initial denaturation step for 3 min at 94°C, followed by 27-33 cycles at 94°C for 30 s (denaturation), 53°C for 45 s (annealing) and 72°C for 120 s (extension), followed by a final extension step at 72°C for 6 min (Scarano et al. 2002). Fang and Roose (1997) observed that the inclusion of 2% formamide in the PCR reaction mix increased the number of fragments amplified, possibly by influencing primer-template annealing and melting temperatures. Formamide generally improves the results of PCR by reducing background and smearing on gels. Negative controls with water instead of template DNA should be performed to monitor for contamination. PCR-amplified DNA fragments are separated on a 1.5% agarose gel containing 1X TAE (40 mM Tris-acetate, 1 mM EDTA). About 5-10µl of reaction products (with an adequate amount of loading buffer) are loaded and the gel is run for 2-4 h at 90 V. The gel is stained with ethidium bromide (0.5µg/ml aqueous solution) for 30 min, destained in water for 10 min, and visualized under UV light. If the fingerprint pattern consists in a large number of fragments, they can be resolved by electrophoresis in DNA sequencing-type polyacrylamide gels. In this case, 5µl of reaction products can be visualized on silver-stained 6% polyacrylamide gel as described by Fang et al. (1997). Only those bands that show consistent amplification should be considered; smeared and weak bands should be excluded. Polymorphic ISSR markers are scored for the presence or absence of bands.

8. CONCLUSIONS

The genus *Citrus* is one of the most important fruit crop in the world. Besides, it is one of the most studied woody plants *in vitro*. Research continues to explore the potential of citrus cell and tissue culture as an assistant to plant breeding programs. *In vitro* techniques include somatic embryo regeneration, the generation of new nuclear and cytoplasmic

hybrids via protoplast fusion, embryo rescue, haploid regeneration via anther culture, sanitation of plants from virus and other pathogens, cryogenic storage of cells and meristems for germplasm conservation, as well as ploidy manipulation induced by cell and tissue culture. Moreover, the development of efficient procedures for the regeneration *in vitro* of somatic embryos of many *Citrus* species would be required as an intermediary for recombinant DNA technology approach to genetic improvement of scion varieties and rootstocks. The exploitation of in vitro embryo regeneration techniques may lead to the upgrading of large commercial production of citrus rootstocks. Research in the future must concentrate on improving the efficiency of somatic embryo regeneration protocol of monoembryonic genotypes, in particular clementine.

9. REFERENCES

Ammirato PV, Evans DE, Sharp WR, Yamada Y (1984) Handbook of plant cell culture, crop species. Vol 3. Macmillan, New York

Ben-Hayyim G, Neumann H (1983) Stimulatory effect of glycerol on growth and somatic embryogenesis in *Citrus* callus culture. Z Pflanzenphysiol 110:331-337

Broertjes C, Van Harten AM (1978) Developments in crop science (2). Application of mutation breeding methods in the improvement of vegetatively propagated crops: an interpretative literature review. Elsevier, Amsterdam

Carimi F, De Pasquale F, Crescimanno FG (1994) Somatic embryogenesis from styles of lemon (*Citrus limon*). Plant Cell Tissue Org Cult 37:209-211

Cervera M, Juárez J, Navarro A, Pina JA, Durán-Vila N, Navarro L, Peña L (1998) Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. Transgenic Res 7:51-59

De Pasquale F, Giuffrida S, Carimi F (1999) Minigrafting of shoots, roots, inverted roots and somatic embryos for rescue of *in vitro* regenerants of *Citrus*. J Am Soc Hortic Sci 124:152-157

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure from small quantities of fresh leaf tissue. *Phytochemical Bulletin* 19:11-15

Frearson EM, Power JB, Cocking EC (1973) The isolation and regeneration of *Petunia* leaf protoplasts. Dev Biol 33:130-137

Fang DQ, Roose ML, Krueger RR, Federici CT (1997) Fingerprinting trifoliate orange germplasm accessions with isozymes, RFLPs, and inter-simple sequence repeat markers. Theor Appl Genet 95:211-219

Fang DQ, Roose ML (1997) Identification of closely related citrus cultivars with inter-simple sequence repeat markers. Theor Appl Genet 95:408-417

Grosser JW, Gmitter FG (1990) Protoplast fusion and Citrus improvement. Plant Breeding Rev 8:339-374

Grosser JW, Ollitrault P, Olivares-Fuster O (2000) Somatic hybridization in *Citrus*: an effective tool to facilitate variety improvement. In Vitro Cell Dev Biol Plant 36:434-449

Kao KN, Michayluk MR (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126:105-110

Mitra GC, Chaturvedi HC (1972) Embryoids and complete plants from unpollinated ovaries and from ovules of *in vivo*-grown emasculated flower buds of *Citrus* spp. Torrey Bot Club 99:184-189

Moore GA (1986) *In vitro* propagation of *Citrus* rootstocks. HortScience 21:300-301

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473-497

Murashige T, Tucker DPH (1969) Growth factors requirements of citrus tissue culture. 1st Int *Citrus* Symp, 3:1155-1161

Ohgawara T, Kobayashi S, Ohgawara E, Uchimiya H, Ishii S (1985) Somatic hybrid plants obtained by protoplasts fusion between *Citrus sinensis* and *Poncirus trifoliata*. Theor Appl Genet 71:1-4

Peña L, Cervera M, Juárez J, Navarro A, Pina JA, Durán-Vila N (1995) *Agrobacterium*-mediated transformation of sweet orange regeneration of transgenic plants. Plant Cell Rep 14:616-619

Perez RM, Navarro L, Duran-Vila (1997) Cryopreservation and storage of embryogenic callus cultures of several *Citrus* species and cultivars. Plant Cell Rep 17:44-49

Predieri S (2001) Mutation induction and tissue culture in improving fruits. Plant Cell Tissue Org Cult 64:185-210

Ruiz C, Paz Breto M, Asíns MJ (2000) A quick methodology to identify sexual seedlings in citrus breeding programs using SSR markers. Euphytica 112:89-94

Sakai A, Kobayashi S, Oiyama I (1991) Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb.) by a simple freezing method. Plant Science 74:243-248

Scarano MT, Abbate L, Ferrante S, Lucretti S, Tusa N (2002) ISSR-PCR technique: a useful method for characterizing new allotetraploid somatic hybrids of mandarin. Plant Cell Rep 20:1162-1166

Starrantino A, Russo F (1980) Seedlings from undeveloped ovules of ripe fruits of polyembryonic citrus cultivars. HortScience 15:296-297

Vardi A, Spiegel-Roy P, Galun E (1975) Citrus cell culture: isolation of protoplasts, plating densities, effect of mutagens and regeneration of embryos. Plant Sci Lett 4:231-236

Vardi A, Galun E (1988) Recent advances in protoplast culture of horticultural crops: *Citrus*. Scientia Horticulturae 37:217-230

Yu C, Huang S, Chen C, Deng Z, Ling P, Gmitter FG (2002) Factors affecting *Agrobacterium*-mediated transformation and regeneration of sweet orange and citrange. Plant Cell Tissue Org Cult 71:147-155

OLIVE (*OLEA EUROPAEA* L.)

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1. INTRODUCTION

Olive (*Olea europaea* L.) is one of the oldest, most widespread and important crops of the Mediterranean basin. Many different olive genotypes are cultivated and a high degree of morphological and biological variation exists (Rugini and Lavee 1992). Olive cultivation from Mediterranean basin is presently expanding into areas of Australia, South and North America (Argentina, Chile, United States) and South Africa (Rugini and Fedeli 1990). The Mediterranean basin is the traditional area of olive cultivation and has 95% of the olive orchards of the world.

The olive species is originated most likely in Asia and then spread westwards along the Mediterranean Sea coasts (Blazquez 1996). The olive belongs to the *Oleaceae* family, which comprises of 29 genera and the genera *Olea* is one of them with 35 species (Heywood 1978). The domesticated olives belong to the genus *Olea*, species *europaea*, subspecies *sativa* and the number of the cultivated species is estimated more than 2500 cultivars. However a new classification is understudy as reported by Rugini and Baldoni [in press in Litz (ed), Hardwick T. Book Publisher]. All species of the genus *Olea* have the basic chromosome number 2n = 46 (x = 23). In the same area, subspecies *sylvestris*.is widespread in the wild.

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 345–360.

Olive oil is the first product and olives are also consumed as table olives in the Mediterranean countries. The consumption of olive oil has recently increased due to its nutritional value. The wood has a negligible importance even though its appreciation especially for artistic and building construction works.

The olive tree and its products can be damaged from many diseases and pests. The most dangerous are the bacterium *Pseudomonas savastanoi*, that produce tubercules forms on the branches and stems, the fungus *Cycloconium oleaginum* that damage the leaves and fruits and *Verticillum dahlie* that is harmful for the root apparatus and the growth of the plants. Among phytophagous, most harmful are the olive fruit fly (*Bactrocera olea* Gmelin), the olive moth (*Prays oleae* Bernard) and black scale *(Saissetia oleae* Olivier). Olive fruit fly is the major pest and can cause severe economic damage to olive production, which effect oil extraction and table use. Olive is susceptible to several viruses, more than 70% of cultivated olive plants seem to be affected by latent viruses. At present, about 20 viruses have been isolated from olive (Martelli, 1998). More common symptoms, as described in olive plants, are twisted and narrow leaf lamina and poor growth and deformed fruits that has been demonstrated due to the presence of strawberry latent ringspot virus (SLRV) (Marte *et al*., 1985; Pasquini *et al*., 2002).

Olive trees were multiplied by using different explants including ovule (spheroblast) or sucker and subsequently leafy stem cutting and grafting on seedlings or clonal stocks. Vegetative reproduction potential varies, which is dependent on genotype, e.g. easy to rooting and recalcitrant to root initiation (Hartmann and Kester 1968). Micropropagation of the olive cultivar was successful on OM medium (Rugini, 1984) and subsequently several other researchers sligthly modified the culture medium by adding different growth substances (Fiorino and Leva, 1986; Cozza et al., 1997; Mencuccini et al., 1997) or rooting conditions (Mencuccini, 2003). The micropropagated materials can be used to screen for resistance to biotic and abiotic stress (Sasanelli et al., 2000; Bartolozzi et al., 2001) and for genetic improvement activity (Rugini et. al, 1999). Olive callus has been established from different olive tissues such as shoot (Lavee and Messer, 1969), fruit mesocarp (Lavee, 1977), hypocotyl seeds (Bao et al., 1980). Shoots organogenesis was obtained from seedling explants (Gilad and Lavee, 1974; Cañas and Benbadis, 1988; Rugini, 1988) and adventitious buds from petioles cultivars with plantlets development (Mencuccini and Rugini, 1992). In olive, somatic embryogenesis was induced from immature zygotic embryos (Rugini, 1988; Leva et al., 1995), cotyledons and radicles from mature embryos (Orinos and Mitrakos, 1991; Mitrakos et al., 1992; Shibli et al., 2001), petioles, excised from adventive buds derived from *in vitro* growing shoots of cultivars Canino and Moraiolo (Rugini and Caricato, 1995) and explants of young lateral shoots of *in vitro* growing cultures (Mencuccini and Pollacci, 2002).

Olive plants regeneration via somatic embryogenesis and DNA recombinant technique, both have enabled us to obtain transgenic olive cultivars. Currently, the genes inserted in olive plants are *rol*A,B,C and *osmotin* (Rugini et al., 2000) and transgenic plants are in field under observation.

In this chapter, we have described protocols for somatic embryogenesis from mature and immature zygotic embryos, and mature tissue of olive cultivars, histological studies and plantlet regeneration.

2. MATERIALS

2.1. Embryogenesis from mature and immature zygotic embryos, seedlings and mature tissue explants:

1. Sterile water, Commercial bleach (NaClO commercial product with 5.5% of chlorine)
2. Flow hood, Petri dishes, pipettes, forceps, scalpel, parafilm
3. Dissecting microscope
4. Mature and immature fruits (75 days after full bloom and use at once or store them at 14-15^0 C for 2-3 months before use)
5. Zygotic embryos (germinated and non germinated)
6. *In vitro* shoots of mature cultivar proliferated on OM medium
7. Aventitious buds from lef petioles of cultivar
8. Media (see Table 1)

Table 1: Basic culture media

Constituents	**OM**	**MS**	**OMe**
Macro elements	mg/l	mg/l	mg/l
KNO_3	1100	1900	950
NH_4NO_3	412	1650	720
$Ca(NO_3)_2 \cdot 4H_20$	600	-	-
KCl	500	-	-
$CaCl_2 \cdot 2H_20$	440	440	166
$MgSO_4 \cdot 7H_20$	1500	370	92,5
KH_2PO_4	340	170	68
Micro elements			
$FeSO_4 \cdot 7H_20$	27.8	27.8	27.8
Na_2EDTA	37.5	37.5	37.5
$MnSO_4 \cdot 4H_20$	22.3	22.3	22.3

H_3BO_3	12.4	6.2	12.4
$ZnSO_4 \cdot 7H_20$	14.3	8.6	14.3
$NaMoO_4 \cdot 2H_20$	0.25	0.25	0.25
$CuSO_4 \cdot 5H_20$	0.25	0.025	0.25
$CoCl_2 \cdot 6H_20$	0.025	0.025	0.025
KI	0.83	0.83	0.83
Vitamins			
myo-Inositol	100	100	100
Glycine	2	2	2
Thiamine·HCl	0.5	0.1	0.5
Pyridoxin·HCl	0.5	0.5	0.5
Nicotinic acid	5	0.5	5
Biotin	0.05	-	0.05
Folic acid	0.5	-	0.5
Amino acid			
L-Glutamine	2190	-	
Sugars			
Mannitol	30000	30000	30000
Solidifying agent			
Agar	6000	6000	6000
Pectin of must wine	10000		
Supplements			
NAA	-		-
IBA			0.05
Zeatin	2-4		-
2iP			0.1
BA			0.1
Thidiazuron		0.05-2	-
GA_3	5-10		
Cefotaxime			200
Caseine hydrolisate			1.000

*The pH of all media are adjusted to 5.8

3. METHOD

The success of inducing somatic embryogenesis depends on origin of material and age of tissues. Somatic embryos have been obtained from both immature and mature zygotic embryo explants, with or without callus interposition and from mature tissues of olive cultivars.

3.1 Somatic Embryogenesis from immature zygotic embryo

3.1.1 Initiation of Embryogenic Cultures

1. Break the stones and remove the seeds from stony endocarp.
2. Surface-sterilize seeds with 10% sodium hypochlorite (NaClO) commercial product (5.5% chlorine) for 10 minutes.
3. Wash seeds 2-3 times in sterile water.
4. Soak for at least 24 hr in sterile water at room temperature
5. Remove with the scalpel the zygotic embryos by longitudinal or transversal cut of the endosperm.
6. Place embryos horizontally on half strengthen MS medium (Table 1) supplemented with 0.5-2.5μM BA, 2% sucrose and agar in a Petri dishes and wrap the plates with a double layer of parafilm.
7. Place the cultures in the dark at 23°C for one month.

3.1.2. Maintenance of callus culture and maturation of Somatic Embryos

1. Subculture the embryogenic callus to the same medium reducing BA concentration (normally the callus looses the capability to produce new embryos after one subculture).
2. The embryos are visible after 5-6 weeks of initial culture, directly from the initial explant (60-70%), and also from callus (20-30%). The remainder normally is not possible to identify the origin with the stereo-microscopic examination.
3. Secondary embryogenesis is normally observed from the epidermal tissue of neoformed embryos or teratoma.

3.1.3. Embryo germination and conversion to plant

1. Somatic embryos separated from callus or from original tissues can germinate quickly on OM solid medium plus 0.5-1 mg/l zeatin or in OMe liquid medium plus 0.3 mg/l zeatin, and to convert into plantlets.
2. When the second pair of leaves are formed, transfer them to jiffy-pots and place in the greenhouse under high relative humidity

Note: Embryogenic cultures have also been induced from cotyledons of 126-day-old zygotic embryos of 'Chalkidikis' (Pritsa and Voyatzis, 1999), from cotyledon segments from mature zygotic embryos of wild olive (O. europaea var. sylvestris),

from the radicle of mature embryos of (Rugini and Tarini, 1986; Mitrakos et al., 1992; Rugini et al., 1995; Shibli et al., 2001) and from mature zygotic embryos (Orinos and Mitrakos, 1991; Mitrakos et al., 1992). However no standard medium can be used since the success is highly genotypic dependent and often not repeatable, contrary to immature zygotic embryos.

3.2 Somatic embryogenesis from mature tissue explants

Induction of somatic embryogenesis from tissues of elite olive has been rather difficult to obtain. Up-to-now it has been reported for three cultivars, 'Canino', 'Moraiolo' and "Dolce Agogia" (Rugini and Caricato, 1995; Mencuccini and Pollacci, 2002). The type of initial of explant and genotype are essential for the success. Up to now two types of explants have been used: a) leaflets from adventitious buds, 2-3 mm long (double regeneration) (Fig 1 *left*), and b) 3-10 mm long young shoots, after bud sprout from uninodal explants on OM medium.

3.2.1. Initiation of Embryogenic Cultures

1. Collect leaf petioles from *in vitro* olive cultivar from micropropagated shoots with frequent subcultures (not more than 20-25 days intervals) on OM medium.
2. Place the petioles in Petri dish (25x90 mm) containing 20 ml half-strength MS medium (Table 1) and seal with parafilm with the aim to regenerate adventitios buds.
3. Dissect the leaflets with petiole from 1-3 mm long neoformed adventitious buds, regenerated from petioles, and place them individually in 25 multiwell plate, each well containing 3 ml OMe medium and place the cultures in the dark at 23°C.

3.2.2. Formation of Embryonal Callus Mass

After about 4 weeks, morphogenic callus mass is produced from petioles. Transfer it to Petri dishes containing 5 ml OMe liquid medium (Table 1) sufficient to soaking a filter paper (Whatman No 3). Every 3-week interval, add fresh medium and remove the equivalent part of the resulted spent medium by pipetting it. After about 20 days proembryo masses recognizable because it looks like a callus with yellowish smooth surface (Fig 1 *right*), continue to enlarge differentiate somatic embryos. If the callus results too aboundant reduce or remove completely the hormones from the medium.

3.2.3. Production of cyclic secondary embryogenesis and embryo Maturation

Place the neoformed embryos on agar OMe medium (table 1) for producing cyclic secondary embryogenesis both from normal and abnormal (teratoma) embryos.

3.2.4. Embryo Germination

Culture single embryos in multiwells (12 wells containing 2 ml OMe liquid medium supplemented with 0.3 mg/l zeatin per 5-6 embryos) and place on a gyratory shaker, 80 rpm, in a growth chamber at 23°C in the light photoperiod (16 h light). Conversion rate declines with time. Embryo germination is difficult, although the hypocotyl elongation generally occurs. On contrary, epicotyl development rarely occurs. However, 1-week cold treatment (4°C) increases often the subsequent phase of embryos germination.

3.2.5. Synthetic seeds

Micheli et al. (2002) reported first time production of olive synthetic seeds by using somatic embryos of cv. Canino.

1. Use 2-4 mm long somatic embryos
2. Immerse them in a 2.5% sodium alginate solution (encapsulation matrix) and then drop them in a complexing solution of 100 mM $CaCl_2$, for 30 min.
3. After hardening, rinse the capsules twice for 10 min in distilled water to wash away calcium chloride residues.
4. In order to insert an artificial endosperm into the beads, use half strength OM medium solution containing sucrose (87.6 μM) without any growth regulator and agar.
5. For germination, sow them in Petri dishes containing half strength OM medium containing 9.2 μM zeatin.
6. Transfer the germinated seedlings on Jiffy-7 pots with high humidity and under continuous light flux

3.2.6. *In vitro* preservation

Embryogenic ‘Canino’ cultures, consisting of Pro-Embryo-Mass and somatic embryos at various developmental stages are highly suitable for cryopreservation by vitrification (Lambardi et al., 2000). After incubation in vitrification solution, high percent (38%) of cryopreserved embryogenic cultures survive. Moreover, the recovered embryogenic tissue show enhanced proliferative and morphogenic activity.

3.3 Histology of somatic embryogenesis.

In order to identify conditions for improving the development of secondary embryos into normal plantlets, different culture conditions are studied, as well as histological analysis is done. Secondary embryogenesis is induced under conditions differ in medium composition and temperature.

The tested media are standard hormone-free OMe (medium A), medium OMe containing low plant growth regulators, i.e. 0.05 mg/l 2i-P plus 0.05 mg/l BA and 0.005 mg/l IBA and double concentration of K and P (medium B), and medium OMe supplemented with 0.3mg/l zeatin only plus double concentration of Calcium (medium C) (see Table 1). The cultures are either combined with a short-term low temperature (4°C for 7 days) treatment or maintained at 23±1°C for the entire period. After one month of culture, under continuous darkeness, transfer secondary embryos into liquid OMe medium for germination, and after 20 days samples are taken for histological examination (Altamura et al, 1992).

Under the tested conditions, the highest number of normal and well-developed somatic embryos is obtained on hormone-free medium (figure 2A). Independent of the culture medium, low temperature treatment is relevant to induce regular embryo polarity, however, irrelevant to reduce morpho-structual anomalies when compared with cultures maintained at 23°C. In fact, even with exposure to low temperature exposure, somatic embryos with more than two cotyledons are obtained on all media, and the development of globular adventive embryoids on cotyledons (figure 2B-C). Another anomaly is the callusing of the hypocotyl (figure 2D-E). These anomalies occur in hormone-free medium (figure2B-D), but more frequently on the media supplemented with hormones (figure 2E-H). Moreover, some somatic embryos growing on medium B show drastic reduction in cotyledonary expansion and adventive embryoid formation on rudimentary cotyledons. The adventive embryoids develop up to the cotyledonary stage (figure 2F). Another major problem is enhanced callusing of hypocotyls (figure 2G), and of the cotyledonary rudiments on medium C (figure 2H). The point to be noted is that independent of medium composition, the primary root always develops, and regular differentiation of root apex and lack of adventive embryogenesis (figures 2A-H and 3A).

The histological analysis show that the shoot apex of the abnormal embryos obtained under hormone-free conditions may exhibit strong alteration in the formation of leaf primordia, with localised events of cell lysis (figure 3B). On the cotyledons, the adventive embryoids are present at various developmental stages, from globular (figures 3B-D) to heart stage (figure 3E). Also, caulogenesis (shoot formation) occur infrequently from the cotyledons (figure 3F).

Somatic embryos cultured on medium B, adventive embryogenesis occur on the cotyledons was more extended and irregular than under hormone-free condition (figures 3C and 3G, in comparison). Furthermore, also the histological structure of the cotyledons change due to increased xylem formation (figure 3H). The embryos also show anomalous proliferation of cells along the hypocotyl (figure 3I), with formation of new globular embryoids on it, and occurrence of cell lysis (figure 3J). In the embryos cultured on medium C, the cotyledon structure is badly disturbed by adventive embryoid formation, and they are located deep in the callused mesophyll (figure 3K). Forthermore, fusion events also occur and are so pronounced to strongly affect adventive embyoid morphology (figure 3L).

3.3.1. Derived Plants

1. Transfer germinated somatic embryos (Figure 4) to Jiffy-7 pots and place them under high humidity (95 % RH) and 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux in a growth room at 23 ± 1 °C for two months, before transplanting them to pots (8 cm diam.) containing a peat moss substrate held under standard glasshouse conditions.
2. Break dormancy by spraying with 400 mg/l GA_3 solution.

3.3.2. Field trials

Plants derived from somatic embryogenesis (Figure 5) show a tap root and a relative long juvenile period, however not exceeding 3-4 years, 2-3 years superior than corresponding micropropagated plants by axillary bud stimulation, and phenotype not different than mother plants, although molecular analysis is needed to ascertain somaclonal variation.

4. CONCLUSION AND FUTURE PROSPECTIVE

Regardless of available efficient protocols for the induction of somatic embryos in olive tree, the possibility of somatic embryos develop into healthy plants is still low. Therefore, the germination rate of somatic embryos needs to be increased for commercial application for large-scale plant multiplication, cryopreservation and encapsulation. Since somatic embryos start from adult cells through an indirect process (i.e., formed from callus produced by the adult cells), they may express new traits due to somaclonal variation. Thus, the stabilization of the embryogenic process, as well as the canalization of embryos into a regular plant development program could represent a valid tool for using the possibly induced somaclonal variation for biotechnological applications (Lambardi et al., 1999; 2002).

The instability of somatic embryogenesis under the standard conditions, as described earlier, has frequently led to abnormal embryo structures responsible for the altered plant development, the same as secondary embryo production (Benelli

et al., 2001). The latter event would, however, be utilized. In fact, the capability to form secondary embryos is the way to obtain somatic embryonic clones useful for early selection to various conditions. Furthermore, secondary embryos are suitable target for *Agrobacterium*-mediated transformation, especially by particle bombardment method. Nowadays, the latter seems to be an important strategy of forest biotechnology (Altamura, 2002). In olive tree, secondary embryogenic process is not yet completely controlled for obtaining healthy plants. Further attention is needed to overcome frequent structural anomalies in secondary embryos, such as the lack of embryo polarity, the proliferation of adventive embryoids at the primary root pole, and embryo fusion (Benelli et al., 2001).

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6. REFERENCES

Altamura M.M. (2002). Somatic and androgenic embryos in Angiosperm. Abstract of Lectures and Free Communications of the XLVIII Convegno del Gruppo Embriologico Italiano, Grottammare (AP) 4-7 June 2002: 23-25

Altamura M.M., A. Cersomino, C. Majoli, and M. Crespan (1992). Histological study of embryogenesis and organogenesis from anthers of *Vitis rupestris* du Lot cultured *in vitro*. Protoplasma 171: 134-141.

Bao Z.H., Y.F. Ma, J.F. Liu, K.J. Wang, P.F. Zhang, D.X. Ni and W.Q. Yang (1980). Induction of plantlets from the hypocotyl of *Olea europaea* L. *in vitro*. Acta Bot. Sin. 2:96-97. (Chinese).

Bartolozzi F., M. Mencuccini and. G. Fontanazza (2001). Induction of frost tolerance in olive plants *in vitro* by cold acclimation and sucrose increase. Plant Cell, Tissue and Organ Culture 67 (3): 299-302.

Benelli, C., Fabbri, A., Grassi, S., Lambardi, M. & Rugini, E. (2001) Histology of somatic embryogenesis in mature tissue of olive (*Olea europaea* L.). *Journal of Horticultural Science Biotech* **76**, 112-119.

Blazquez J. M. (1996). The origin and expansion of olive cultivation. In: International Olive Oil Council (ed) World Olive Encyclopedia. IOOC, Madrid, pp.19-20.

Cañas, L.A. and A. Benbadis (1988). Plant regeneration from cotyledon fragments of the olive tree (*Olea europaea* L.). Plant Sci. 54: 65-74.

Gilad F., S. Lavee (1974). Callus formation and organogenesis from varius parts of developing olive embryos. Abstract Book 'III International Congress on Plant Tissue and Cell Culture'. Leicester, p. 87.

Hartmann H.T. and D. Kester (1968). Plant propagation. Prentice Hall, pp. 222-230.

Heywood, V. H. (1978). Flowering Plants of the World. Oxford, London, Melbourne: Oxford University Press.

Lambardi M., S. Amorosi, G. Caricato, C. Benelli, C. Branca and E. Rugini (1999). Microprojectile-DNA delivery in somatic embryos of olive (*Olea europaea* L.). Acta Horticulturae 474: 505-509.

Lambardi M., C. Benelli, A. De Carlo, A. Fabbri, S. Grassi and P.T. Lynch (2002). Medium and long-term in vitro conservation of olive germplasm (*Olea europaea* L.). Acta Horticulturae 586: 109-112.

Lavee S. (1977) . The growth potential of olive fruit mesocarp *in vitro* (*Olea europaea* L.). Acta Horticulturare, 78: 115-12.

Lavee S. and G. Messer (1969) - The effect of growth-regulating substances and light on olive callus growth in vitro. J. Exp. Bot. 20: 604-614.

Martelli, G.P. (1998) Enfermedades infecciosas y certificacion del olivo: panorama general. Phytoma Espana 102, 180-186.

Mencuccini M. (2003). Effect of medium darkening on *in vitro* rooting capability and rooting seasonality of olive (*Olea europaea* L.) cultivars. Scientia Horticulturae vol 97/2: 129-139.

Mencuccini M., M. Micheli, A. Standardi (1997). Micropropagazione dell'olivo: effetto di alcune citochinine sulla proliferazione. Italus Hortus vol. 4 n. 7: 32-37.

Micheli M., P. Dell'Orco, M. Mencuccini, and A. Standardi (2002). Preliminary studies on the synthetic seed and encapsulation technologies of olive vitro-derived olive explants. Acta Hort. 586: 911-914.

Mencuccini M., P. Pollacci (2002). Rigenerazione di embrioni somatici da cultivar di olivo e loro impiego nella costituzione del seme sintetico e nella trasformazione genetica. Convegno Internazionale di Olivicoltura, Spoleto 22-23 Aprile, pp. 417-422.

Mencuccini, M. and E. Rugini (1993). *In vitro* shoot regeneration from olive cultivars tissues. Plant Cell, Tissue and Organ Culture 32: 283-288.

Mitrakos K., A. Alexaki, P. Papadimitriou (1992). Dependence of olive morphogenesis on callus origin and age. J. Plant Physiol. 139: 269-273.

Orinos, T. and K. Mitrakos (1991). Rhizogenesis and somatic embryogenesis in calli from wild olive (*Olea europaea* var. *sylvestris* (Miller) Lehr) mature zygotic embryos. Plant Cell Tissue and Organ Culture 27: 183-187.

Pasquini, G., L. Baldoni, L. Ferretti, G. Pannelli, F. Faggioli, M. Barba (2002). Evaluation of the strawberry latent ringspot virus (SLRSV) in some olive cultivars. Atti Convegno Internazionale Olivicoltura, Spoleto, Italy, pp. 462-465.

Pritsa T.S. and D.G. Voyiatzis (1999). The *in vitro* morphogenetic capacity of olive embryos, as affected by their developmental stage and the L-arginine and L-glutamine concentration in the nutrient substrate. Acta Horticulturae 474: 87-90.

Rugini E. (1984). In vitro propagation of some olive (*Olea europaea* sativa L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Sci. Hortic. 24: 123-134.

Rugini E. and E. Fedeli (1990). Olive (*Olea europaea* L.) as an oilseed crop. In: Y.P.S. Bajaj (ed.) Legumes and oilseed crops I. Biotecnology in Agriculture and Forestry, vol. 10 Springer , Berlin Heidelberg New York, pp. 593-641.

Rugini E. and S. Lavee (1992). Olive. In: F.A. Hammerschlag, R.E. Litz (eds.) Biotechnology of Periennal Fruit crops. Biotecnologi In Agriculture, vol. 8 C·A·B International, Wallingford, pp. 371-382.

Rugini E., P. Gutierrez-Pesce, P.L. Spampinato, A. Ciaramiello and C. D'Ambrosio (1999). New perspective for biotecnologies in olive breeding: morphogensis, *in vitro* selection and gene transformation. Acta Horticulturae. 474: 107-110.

Rugini, E. and P. Tarini (1986). Somatic embryogenesis in olive (*Olea europaea* L.). In: MoetHennessy (ed.), Proceedings Conference Fruit Tree Biotechnology. Paris (France), p.62.

Rugini E., A. Pezza, M. Muganu, and G. Caricato (1995). Somatic embryogenesis in olive (*Olea europaea* L.). In: Y.P.S. Bajaj (ed), Somatic Embryogenesis and Synthetic Seed I. Biotechnology in Agriculture and Forestry, Vol. 30. Springer, Berlin Heidelberg New York, pp. 404-414.

Rugini E., R. Biasi, R. Muleo (2000). Olive (*Olea europaea* var. sativa) Transformation. In: Molecular Biology of Woody Plants Vol. 2. S.M. Jain and S.C. Minocha (eds), Kluwer Academic Publishers, pp 245-279.

Sasanelli N., N. D'Addabbo, P. Dell'Orco and M. Mencuccini (2000). The *in vitro* use of olive explants in screening trials for resistance to the root-knot nematode, Meloidogyne incognita. Nematropica, 30: 101-106.

Shibli R.A., M. Shatnawi, M. Abu-Ein and K.H. El-Juboory (2001). Somatic embryogenesis and plant recovery from callus of 'Nabali' Olive (*Olea europaea* L.). Scientia Horticulturae 88: 243-256.

Figure 1 - Adventive buds from petioles of *in vitro* grown cv Canino (*left*); Proembryonic mass originated from petioles of adventive buds, obtained by a "double regeneration system" (*right*).

Figure 2 – Stereomicroscope images of olive tree secondary embryos cultured on different media and exposed to low temperature. A-D, medium A; E-F, medium B, G-H, medium C. Description of embryo morphology in the text.

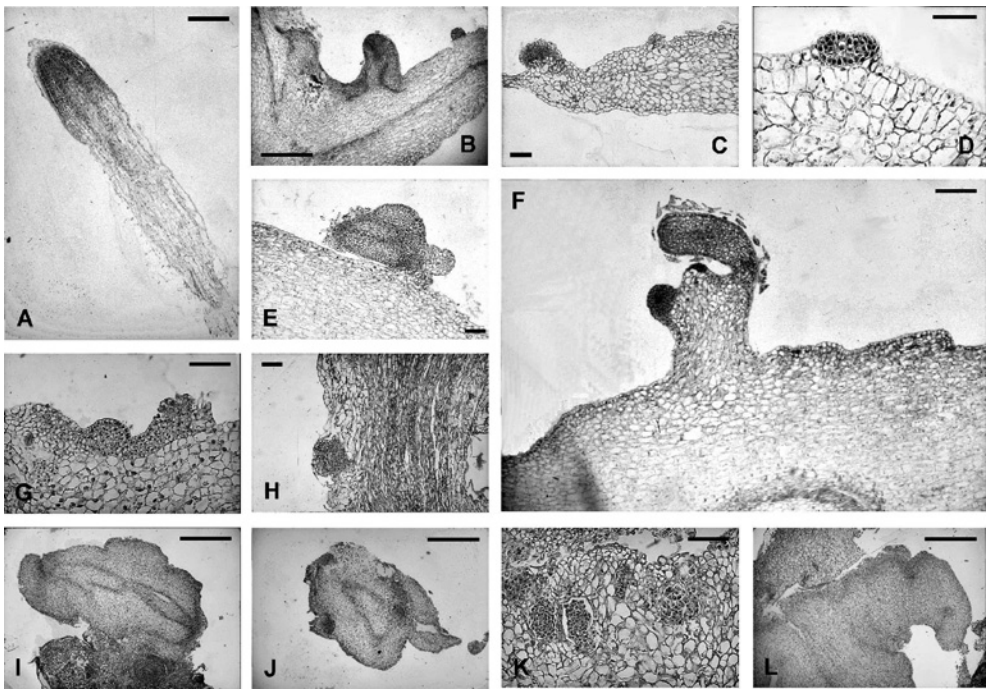

Figure 3 – Histological analysis of olive tree secondary embryos cultured on different media and exposed to low temperature. A-F, medium A (bars: A= 200µm; B= 500 µm; C=100 µm; D= 250 µm; E=100 µm; F= 200 µm); G-J, medium B (bars: G= 200 µm; H= 100 µm; I=500 µm; J=500 µm); K-L, medium C (bars: K= 200 µm; L= 500 µm). Description of embryo histology in the text.

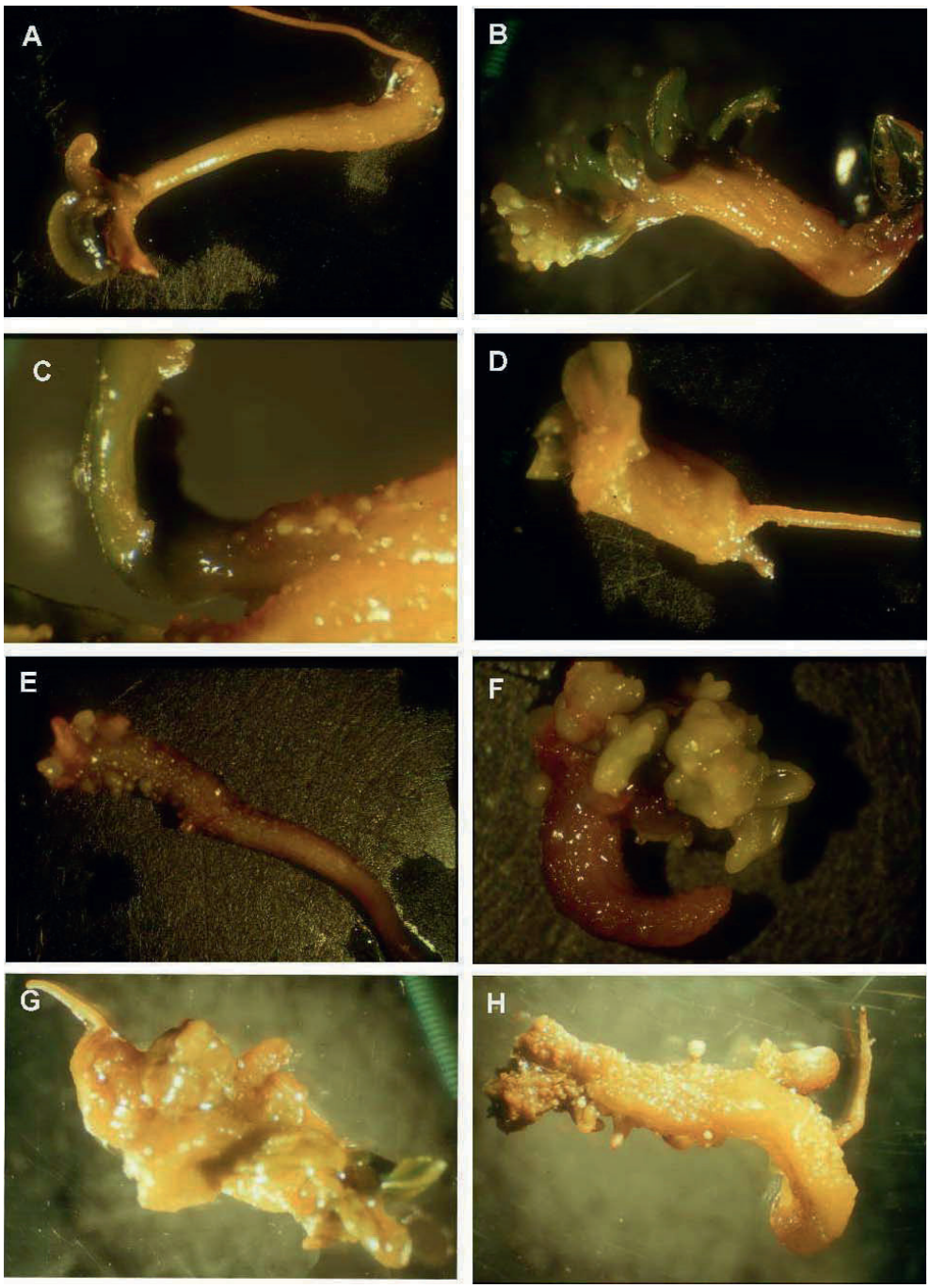

Figure 4 - Cyclic somatic embryogenesis showing different developmental stages of somatic embryos.

Figure 5 - Field grown 4-year-old olive tree cv Canino derived from somatic embryogenesis (left); Root system of a potted plant obtained from somatic embryo of cv Canino (note the presence of tap root) (right).

PROTOCOL OF SOMATIC EMBRYOGENESIS: *DALBERGIA SISSOO* ROXB. (SISSOO)

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1. INTRODUCTION

Trees are not only valued for timber but also for a range of other natural products such as fibres, alkaloids, tannins and resins. Tree improvement by conventional breeding is a slow process because of the long juvenile period and high heterozygosity of trees.

Dalbergia sissoo Roxb. (sissoo) is one of the most important tree legume of the Indian subcontinent and is valued mainly for its wood. The propagation of *D. sissoo* through seeds is unreliable due to poor germination and death of young seedlings under natural environmental conditions (Anonymous 1989). The economical and ecological importance of leguminous trees necessitates the application of tissue culture techniques for their clonal multiplication. Rapid clonal propagation of elite trees is of importance.

In vitro plant regeneration in *D. sissoo* has been achieved from callus cultures derived from hypocotyl segments (Sharma and Chandra 1988), from cell suspension cultures (Kumar *et al*. 1991), from nodal segments (Gulati and Jaiwal 1996), from cotyledonary nodes derived from axenic seedlings (Pradhan *et al*. 1998), from mature and semi-mature cotyledon (Singh *et al*. 2002), and from encapsulated nodal segments (Chand and Singh 2003). Das *et al* (1997) reported somatic embryogenesis in *D. sissoo* from semi-mature zygotic embryos, but conversion of somatic embryos into plantlets was not reported. Singh and Chand (2003) reported a reliable method for regeneration of plantlets through somatic

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embryogenesis from callus cultures derived from semi-mature cotyledon explants of *D. sissoo*. *In vitro* somatic embryogenesis offers great potential in tree improvement programme since it is used for efficient cloning and as an appropriate regeneration system for gene transfer techniques (Roberts *et al*. 1995 and Torne *et al*. 2001). In recent years, somatic embryos have been used in developing synthetic seeds, in shortening breeding cycle and in genetic transformation studies.

This chapter is devoted to somatic embryogenesis protocol for plant regeneration of *D. sissoo.* The details of procedures including explant preparation, callus induction, somatic embryos development, maturation and germination, and acclimatization are described.

2. PROTOCOL OF SOMATIC EMBRYOGENESIS IN SISSOO

2.1. Explant

1. Wash the green pods (40-50 days after anthesis) thoroughly under running tap water for 30 min., followed by treatment with tween-20 solution (10 drops/100 ml distilled water, v/v) for 15 min. Rinse with distilled water, followed by savlon antiseptic solution treatment (1 ml/100 ml distilled water, v/v) for 15 min. and wash 3-4 times with distilled water.
2. Rinse the pods with 70% ethanol for 1 min and wash with sterile distilled water and then surface sterilize with 0.1% freshly prepared aqueous mercuric chloride solution for 20 min., followed by 4-5 times washing with sterile distilled water under laminar flow chamber.
3. After surface sterilization, isolate seeds from the pods. Excise cotyledons from seeds.

2.2. Medium preparation

1. The pH of the medium is adjusted to 5.76 ±0.02 using 0.1 N NaOH or 0.1 N HCl prior to adding 0.8% (w/v) agar-agar (Bacteriological grade, Hi-media, India).
2. The culture tubes containing nutrient medium are capped with non-absorbent cotton plugs wrapped with one layer of cheesecloth.

2.3. Callus induction

1. Under asceptic conditions, culture cotyledon pieces (5x5 mm) on Murashige and Skoog's (1962) medium containing auxin and cytokinin (Table 1). Initially, callus formation is started from cut end of explant. After first subculture, rapid proliferation of callus is started and whole explants is converted into callus.
2. Maintain the cultures under a 16/8 h light/dark photoperiod, temperature 25 ±2°C, and light intensity of 40 μmole $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.
3. Transfer the cultures to a fresh medium at 4 weeks intervals.

2.4. Maintenance of callus

Transfer the callus to the same medium for one more passage for multiplication before transfer on somatic embryo induction medium. On this medium, semi-compact callus is proliferated.

2.5. Somatic embryos induction

1. Transfer the callus clumps (250 ±25 mg) on half-strength Murashige and Skoog (1/2-MS) medium supplemented with 0.68 mM L-glutamine. Somatic embryos are developed on callus surface and occasionally, directly from cotyledon explants without intervening callus phase after 35 days of culture. During direct somatic embryogenesis, initially, globular somatic embryos are developed over the entire surface of cotyledon explants and latter on develop into heart, torpedo and cotyledonary stages of somatic embryos (Fig. 1). Somatic embryos developed from callus cultures or directly from explants are multiplied in subsequent subcultures on the same medium. A large number of somatic embryos, mostly cotyledonary stage, are developed on the surface of callus (Fig. 2). Sometimes, somatic embryos first develop at torpedo or cotyledonary stage on the surface of the callus. This indicates that early development of somatic embryos (globular to torpedo stage) occurs inside the callus tissues and further development occurs after emergence of the embryos.
2. Maintain the cultures in the culture room at similar physical conditions as described for callus induction.

2.6. Maintenance of somatic embryogenic cultures

For long-term maintenance, transfer the embryogenic cultures on ½- strength Murashige and Skoog medium without plant growth regulator. On this medium, various developmental stages of somatic embryos are obtained through primary somatic embryogenesis. The secondary somatic embryogenesis does not occur during long-tern maintenance.

3. MATURATION AND GERMINATION OF SOMATIC EMBRYOS

3.1. Maturation

1. Isolate somatic embryos from callus cultures or directly from cotyledon explants.
2. Transfer isolated somatic embryos to ½- strength Murashige and Skoog medium enriched with 10% sucrose for 15 days and then transfer to ½-MS medium with 2% sucrose (Table 1).

3.2 Germination

Transfer mature cotyledonary somatic embryos on ½- strength Murashige and Skoog medium without plant growth regulator. Germination of somatic embryos is characterized by simultaneous emergence of shoots and roots (Fig. 3).

4. TRANSFER OF PLANTLETS TO POTS

1. Transfer plantlets with shoots and roots [20-day-old and 25-30mm in height] to ½ and ¼- liquid MS medium with 2% sucrose subsequently, each for 10 days.
2. For hardening, transfer plantlets to plastic pots containing autoclaved mixture of peat moss and compost (1:1), and cover with transparent polythene bags poked with many small pores for maintaining 85-95% humidity.
3. Now, transfer acclimatized plants to earthen pots containing autoclaved mixture of peat moss: compost: soil (1:1:1) (Fig. 4).

5. CONCLUSION AND FUTURE PROPECTS

The increasing demand of wood based industries and the decline of world's forests are major hindrance for forestry operation to raise forest productivity.

Natural forest plantations are hindered by their high genetic diversity and the lack of phenotype uniformity. Recent studies have shown that it is possible to induce somatic embryogenesis and plant regeneration from callus cultures derived from semi-mature cotyledon explants of *D. sissoo*. Somatic embryogenesis technology coupled with automated micropropagation system allows rapid multiplication of selected high value genotypes. Such studies are very useful for genetic transformation and might be helpful for producing synthetic seeds. For practical application of this technology, it is important to improve germination rate of somatic embryos. In the protocol presented here, germination of somatic embryos has been improved by raising sucrose concentration up to 10% in maturation medium. The presence of sucrose might serve as a signal for the synthesis of storage proteins, resulting in improved quality and germinability of somatic embryos. Sucrose might act as a regulatory factor in addition to serving as a source of carbon, energy and as an osmoticum during maturation of somatic embryos. The protocol for plant regeneration from *D. sissoo* cotyledons through somatic embryogenesis presented herein could be useful for obtaining true-to-type plants and for large scale plantation of this timber-yielding tree.

6. REFERENCES

Anonymous, 1989. The wealth of India: a dictionary of India raw materials and industrial products. Council of Scientific and Industrial Research, New Delhi, 4: 7-11

Chand, S. & A.K. Singh, 2003. Plant regeneration from encapsulated nodal segments of *Dalbergia sissoo* Roxb., a timber-yielding leguminous tree species. J Plant Physiol (in press).

Das, P., S. Samanatray, A.V. Roberts & G.R. Rout, 1997. *In vitro* somatic embryogenesis of *Dalbergia sissoo* Roxb.- a multipurpose timber-yielding tree. Plant Cell Rep. 16: 578-582.

Gulati, A. & P.K. Jaiwal, 1996. Micropropagation of *Dalbergia sissoo* from nodal explants of mature trees. Biol Plant. 38: 169-175

Kumar, A., P. Tandon & A. Sharma, 1991. Morphogenic response of cultured cells of cambial origin of a mature tree *Dalbergia sissoo* Roxb. Plant Cell Rep. 9: 703-706.

Murashige, T. & F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant. 15: 473-497.

Pradhan, C., S. Kar, S. Pattnaik & P.K. Chand, 1998. Propagation of Dalbergia sissoo Roxb. through in vitro shoot proliferation from cotyledonary nodes. Plant Cell Rep. 18: 122-126.

Roberts, A.V., K. Yokoya, S. Walker & J. Mottley, 1995. Somatic embryogenesis in Rosa spp. In: Jain, S., P. Gupta & R. Newton (eds) Somatic embryogenesis in woody plants, Vol. 2, pp. 277-2789, Kluwer Academic Publishers, The Netherlands

Sharma, S. & N. Chandra, 1988. Organogenesis and plant formation in vitro in Dalbergia sissoo Roxb. J Plant Physiol. 132: 145-147.

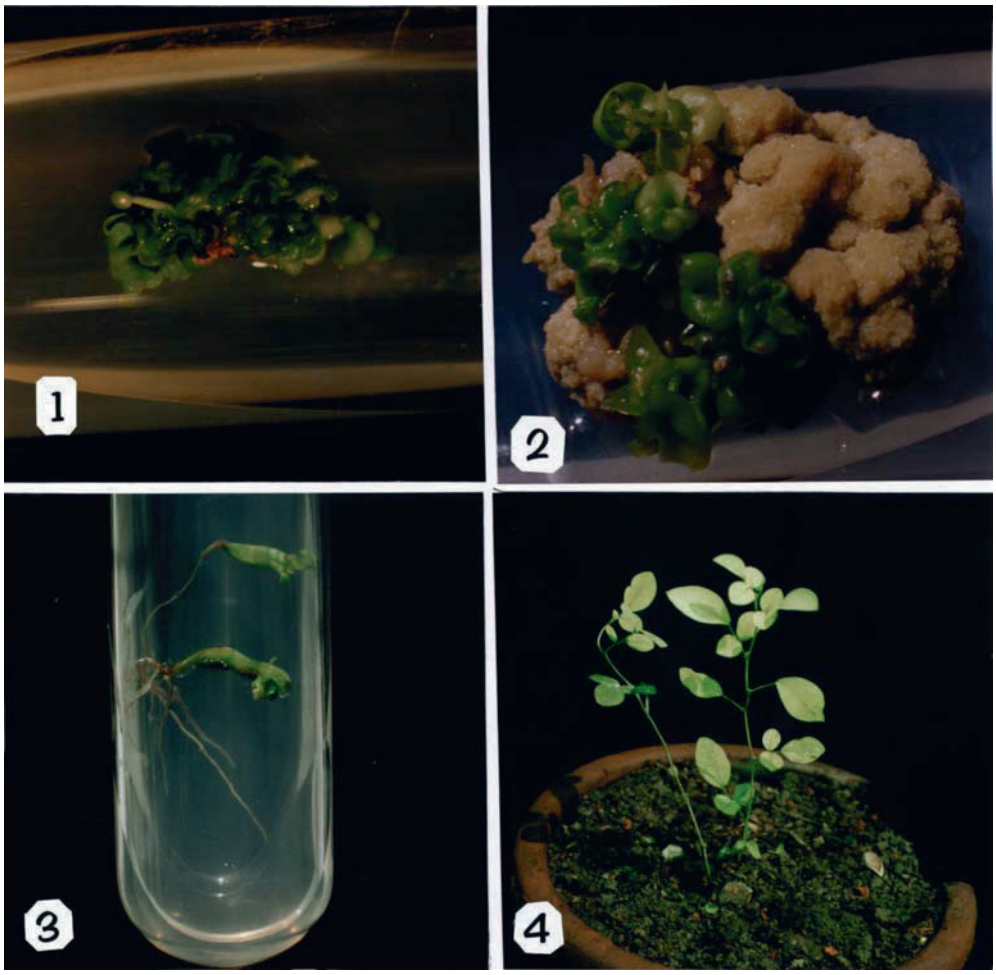
Singh, A.K. & S. Chand, 2003. Somatic embryogenesis and efficient plant regeneration from cotyledon explants of a timber-yielding leguminous tree- Dalbergia sissoo Roxb. J Plant Physiol. 160: 415-421.

Singh, A.K., S. Chand, S. Pattnaik & P.K. Chand, 2002. Adventitious shoot organogenesis and plant regeneration from cotyledons of Dalbergia sissoo Roxb., a timber-yielding tree legume. Plant Cell Tissue Organ Cult. 68: 203-209.

Torne, J.M., L. Moysset, M. Santos & E. Simon, 2001. Effect of light quality on somatic embryogenesis in Araujia sericifera. Physiol. Plant. 111:405-411.

Table 1. Composition of the different media used for different stages of somatic embryogenesis in ***D. sissoo***.

Ingredients	Callus induction medim mg/L	Somatic embryo induction medium mg/L	Maturation medium mg/L	Germination medium mg/L
NH_4NO_3	1650	1650	825	825
KNO_3	1900	1900	950	950
KH_2PO_4	170	170	85	85
$MgSO_4.7H_2O$	370	370	185	185
$CaCl_2.2H_2O$	440	440	220	220
KI	0.83	0.83	0.42	0.42
H_3BO_3	6.2	6.2	3.1	3.1
$MnSO_4.H_2O$	22.3	22.3	11.2	11.2
$ZnSO_4.7H_2O$	8.6	8.6	4.3	4.3
$Na_2MoO_4.2H_2O$	37.5	37.5	18.7	18.7
$CuSO_4.5H_2O$	0.025	0.025	0.012	0.012
$CoCl_2$	0.025	0.025	0.012	0.012
$FeSO_4.3H_2O$	27.8	27.8	13.9	13.9
Na_2EDTA	37.5	37.5	18.7	18.7
Nicotinic Acid	0.5	0.5	0.25	0.25
Pyridoxine-HCl	0.5	0.5	0.25	0.25
Thiamine-HCl	0.1	0.1	0.05	0.05
Myo-inositol	100	100	50	50
Glycine	2.0	2.0	1.0	1.0
L-Glutamine	-	0.68 mM	-	-
Sucrose	3%	3%	10%	2%
2,4-D	9.04 µM	-	-	-
Kn	0.46 µM	-	-	-
pH	5.76 ±0.02	5.76 ±0.02	5.76 ±0.02	5.76 ±0.02



Figures: 1. Somatic embryo development without intervening callus phage. 2. Cluster of somatic embryos developed over the surface of callus tissues. 3. Germination of somatic embryos showing shoots and roots differentiation. 4. Plantlet in pot.

PROTOCOL OF SOMATIC EMBRYOGENESIS: PEDUNCULATE OAK (*QUERCUS ROBUR* L.) AND SESSILE OAK (*QUERCUS PETRAEA* /MATT./ LIEBL.)

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1. INTRODUCTION

Oaks are important broadleaved forest tree species, widely distributed in southern, western and eastern part of Europe. The altitudinal range of oaks extends from lowland to the mountains. In Europe, proportion of oak forests is high and oaks represent about 9% of forested area. In some European countries oaks cover 20 – 30% of the forested land.

Oak is widely planted and there is an increasing interest to use it in reforestation of lowland and hilly regions. Oaks are resistant to wind and snow and as deciduous trees are also more resistant to air pollution. Oaks have an important economic value. Oaks provide the fine hardwood valued because of its great strength and durability. Wood of oak is hard and heavy and has a pronounced growth-ring figure. Oak wood is used for furniture, house and ship constructions and as a surface veneer.

Under the influence of different ecological conditions, oak trees differentiated physiologically into various ecotypes. Within the species various provenances occur in different regions. Oak provenances, when trees are grown under identical conditions, show differences in growth,

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timber production, wood quality and disease resistance. To meet the need for high quality cultivars, oak improvement is in progress.

At present , oak is propagated mostly by seeds, however, good seed harvests are not frequent and oak seeds are difficult to store for longer time than one year. So far, forest nurseries are oriented toward production of planting stock from seeds and vegetative methods of oak propagation by cuttings are difficult. However, vegetative propagation of oak could provide an adequate plant supply when there is a shortage of seeds. *In vitro* propagation is important because of its potential to propagate commerciallly valuable genotypes. Forest tree improvement programs might be accelerated by application of new biotechnology techniques. Forest yield and wood quality could be enlarged significantly by *in vitro* propagation of selected genotypes with the fast growth rates, disease resistance and valuable wood quality.

In vitro propagation of oak can be used for the production of plants with desirable genetic traits. A system based on *in vitro* multiplication of shoots from axillary buds has been developed (Chalupa 1979, 1981, 1983, 1984, 1985) and can be used for fast *in vitro* propagation of selected oak genotypes. Somatic embryogenesis has also a great potential to be used for fast clonal propagation of oaks. Regeneration of oaks via somatic embryogenesis is considered to be an efficient approach for improvement of growth rates and disease resistance of oak. Somatic embryogenesis can be used for propagation of more productive and resistant trees.

2. INDUCTION OF EMBRYOGENIC TISSUE

Induction of oak embryogenic cultures can be achieved from immature and mature zygotic embryos and from leaf and stem segments. The embryogenic potential of explants varies and is dependent on the age and genotype of explants. Somatic embryos develop either from subcultured embryogenic tissue or directly on explants.

2.1. Induction of embryogenic cultures from immature and mature zygotic embryos

Oak embryogenic cultures are frequently initiated from immature and mature zygotic embryos. The induction of embryogenic tissue is stimulated on WPM or MS medium supplemented with cytokinin and auxin. Acorn sterilization consists of 70% ethanol dip followed by a 20 min immersion in calcium hypochloride solution (7.5% w/v). The acorns are then rinsed twice in sterile distilled water. Following the surface sterilization, zygotic embryos are removed using aseptic procedures and are placed on agar nutrient medium.

The induction medium consists of WPM or MS medium. The basal medium is supplemented with glutamine (200mg . l^{-1}) and casein hydrolysate (200 – 500mg . l^{-1}) and 2% (w/v) sucrose. Growth regulators added to culture medium include 1mg.l^{-1} 6-benzylaminopurine (BA) plus 2mg . l^{-1} indole-3-butyric acid (IBA) or 2mg . l^{-1} naphtalene acetic acid (NAA). Difco Bacto–agar (0.7% w/v) is used to solidify the medium. The pH is adjusted to 5.7 with 1N NaOH before autoclaving at 121º C. Glutamine is filter–sterilized and added to the cooled medium. 30ml of agar nutrient medium is poured in 100 ml glass flasks. Cultures are grown in dark in growth cabinets at 24 ± 1º C. In most cases the explants are cultured for five week culture period and then transferred on fresh medium.

The initiation of embryogenic cultures from zygotic embryos is related to the developmental stage of zygotic embryos. Explants from immature zygotic embryos collected during June and July give rise to embryogenic tissues in high frequency (52 – 70 %) with the optimal induction time between 6 to 10 weeks after anthesis. Immature zygotic embryos cultured in dark produce white embryogenic tissues within 5 – 7 weeks. Embryogenic cultures are subcultured to a fresh medium every 4 – 5 weeks..

2.2 Induction of embryogenic cultures from leaf and stem segments

Initiation of embryogenic tissue from leaf segments and from internodal stem segments of oak seedlings can be induced on WPM or MS medium supplemented with cytokinin (BA 1mg . l^{-1}) and auxin (NAA 3 – 4 mg . l^{-1}). The basal medium is supplemented with casein hydrolysate (500 mg . l^{-1}) and glutamine (200 mg . l^{-1}). Young seedlings (4 to 5-week-old) are used as source of explants. Shoots are excised from seedlings and are sterilized in 70% ethanol for 10 seconds followed by 20 minutes immersion in calcium hypochloride (7.5% w/v) solution. The leaves and stem segments are then rinsed twice in sterile distilled water. Following the surface sterilization, uppermost expanding leaves are cut transversally and the basal leaf halves with the petiole (2 mm) attached are placed with the abaxial surface touching the nutrient medium. Stem segments (4–6 mm) excised from seedlings are placed horizontally on the nutrient medium. After 1–3 days on the medium, the explants are transferred to fresh nutrient medium.

Within 4–6 weeks, callus tissues develop in the explants of leaf and internodal segments. For induction of embryogenic tissue, multi-stage initiation treatment is required. Development of embryogenic tissue is stimulated by lowering of plant growth regulators in nutrient medium. Embryogenic tissue is induced after transfer of original explants with callus tissue on nutrient media containing a low concentration of growth regulators (NAA 0.1 mg.1^{-1}, BA 0.1 mg.1^{-1}). Within 2–3 months after transfer on new nutrient media, embryogenic structures develop on the surface of white nodular callus that developed on the callus tissues produced on original explants. Embryogenic cultures produced from leaf and internodal segments are maintained by secondary embryogenesis.

The initiation of oak embryogenic cultures is greatly dependent on the type of explants used and related to the developmental stage of explant. Stage of development of cultured leaf and stem segments is important for somatic embryogenesis initiation frequency. Young leaves produce embryogenic tissues in higher frequency. A strong genotypic effect of used explants on embryogenic tissue formation was observed.

3. MAINTENANCE OF EMBRYOGENIC TISSUE

Oak embryogenic cultures are maintained by secondary embryogenesis. Repetitive somatic embryogenesis of oak cultures is frequent. Somatic embryos are transferred and cultured on WPM or MS media containing a low concentration of growth regulators (BA 0.1 $mg.1^{-1}$ plus IBA 0.1 $mg.1^{-1}$) or media lacking growth regulators. Secondary somatic embryos are formed mainly on the root pole and main axis of mature embryos. Adequate nitrogen levels support formation of secondary somatic embryos. Mature dicotyledonary somatic embryos produce in high percentages secondary somatic embryos. Both multicellular and unicellular pathways are possible origins of secondary embryogenesis, depending on the applied hormones. Different embryogenic lines are established and maintained by repetitive embryogenesis in multiplication medium containing 0.1 $mg.1^{-1}$ BA plus 0.05 $mg.1^{-1}$ NAA.

Oak embryogenic cultures of zygotic origin are maintain by repetitive embryogenesis on BA containing media. Repetitive embryogenesis is the efficient method of secondary embryo production and gives somatic embryogenesis a great potential for oak mass propagation.

4. MATURATION

A maturation stage of oak somatic embryos is important for their germination. During maturation stage the structured and functional shoot meristem is established and it is an important prerequisite for the germination and plant development from somatic embryos. The evolution of a flat shoot meristem into a convex dome-shaped meristem and the starch accumulation are important processes during maturation of oak somatic embryos. A maturation stage has to be accomplished prior to the germination stage.

The maturation of oak somatic embryos is stimulated by application of osmoticum (7% sorbitol or 7-8% sucrose) and abscisic acid treatments. Oak somatic embryos matured for 5–7 weeks on WPM or on half

concentration of MS medium supplemented with higher concentration of sucrose (8%) or sorbitol (7%) plus ABA (3 mg . l^{-1}) exhibit higher germination and conversion into plants. Desiccation of somatic embryos inside sterile dishes for 2–3 weeks and exposure to cold (2º C for 2–3 weeks) improve maturation processes and increase the frequency of somatic embryo germination and conversion to plants.

5. GERMINATION AND TRANSFER TO SOIL

After maturation treatmens, somatic embryos are germinated in 100 ml flasks containing 30 ml of germination medium (WPM or ½ MS medium supplemented with 0.1 $mg.1^{-1}$ BA and 2% sucrose). The germination and conversion capacity of mature somatic embryos are closely associated with the maturation treatment. Somatic embryos matured on nutrient medium (WPM or ½ MS) containing higher concentration of osmoticum (sucrose 8% or sorbitol 7%) plus ABA (3.0 $mg.1^{-1}$) germinated in high frequencies (35–62%). High germination and conversion of mature somatic embryos is associated with higher content of endogenous IAA and increased endogenous levels of cytokinins and low content of ABA in mature somatic embryos . The conversion capacity of mature somatic embryos is also under genetic control. Different germination responses of various oak somatic embryogenic cell lines can be often observed.

Well–developed plantlets regenerated from somatic embryos are transplanted into containers with potting mixture (agroperlite and peat, 1:1 v/v) and are grown at 25° C under high air humidity (RH 85–90%) and long photoperiod (16 – 20 h) to stimulate root and height growth of plants. After 2 – 3 months, the high air humidity is gradually reduced to normal values. After hardening off, the acclimated somatic plants are transferred outdoors in the nursery.

6. CONCLUDING REMARKS

The breeding of oak species is a slow and difficult process, because juvenile non–flowering stage last a long time. Clonal *in vitro* propagation of selected genotypes can bring a fast improvement of oak tree quality.

Forest yield can be enhanced significantly by large – scale multiplication of selected oak genotypes with improved growth rates, valuable wood quality and disease resistance.

Propagation of oak via somatic embryogenesis is a promising method for fast clonal propagation of selected genotypes. Significant progress has ben achieved in recent years in oak propagation by somatic embryogenesis. Experiments indicate that oak tissue is highly embryogenic. Repetitive somatic embryogenesis in oak cultures is frequent, and embryogenic tissues retain embryogenic potential for a long time.

The low frequency of somatic embryo conversion into plantlets is the main present problem of oak regeneration by somatic embryogenesis. The development of somatic embryos is often blocked after the formation of cotyledons. A maturation stage of oak somatic embryos is important for their germination. Improvements of somatic embryo maturation and germination processes are necessary for the high plant production. More research is needed to improve processes of maturation and conversion of somatic embryos to plants.

7. REFERENCES

Chalupa, V., 1979. *In vitro* propagation of some broad – leaved forest trees. – Commun. Inst. Forest. Czech. 11 : 159 – 170.

Chalupa, V., 1981. Clonal propagation of broadleaved forest trees *in vitro*. – Commun. Inst. Forest. Czech. 12 : 255 – 271.

Chalupa, V., 1983. Micropropagation of conifer and broadleaved forest trees. – Commun. Inst. Forest. Czech. 13 : 7 – 39.

Chalupa, V., 1984. *In vitro* propagation of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). – Biol. Plant. 26 : 374 – 377.

Chalupa, V., 1985. *In vitro* propagation of *Larix, Picea, Pinus, Quercus*, *Fagus* and other species using adenine – type cytokinins and thidiazuron. – Commun. Inst. Forest. Czech. 14: 65 – 90.

Chalupa, V., 1987. Somatic embryogenesis and plant regeneration in *Picea, Quercus, Betula*, *Tilia, Robinia, Fagus* and *Aesculus*. – Commun Inst. Forest. Czech. 15 : 133 – 148.

Chalupa, V., 1989. Plant regeneration by somatic embryogenesis in Norway spruce (*Picea abies* / L. / Karst.) and sessile oak (*Quercus petraea* / Matt. / Liebl. – Commun. Inst. Forest. Czech. 16 : 135 – 149.

Chalupa, V., 1990a. Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). – Plant Cell Reports 9 : 398 – 401.

Chalupa, V., 1990b. Somatic embryogenesis and plant regeneration in *Quercus petraea* (Matt.) Liebl., *Tilia platyphyllos* Scop. and *Aesculus hippocastanum* L. – Lesnictví (Forestry) 36 : 599 – 604.

Chalupa, V., 1992. Somatic embryogenesis and plant regeneration in *Quercus robur* L. and *Quercus rubra* L. Lesnictví – Forestry 38: 475 – 481.

Chalupa, V., 1993. Vegetative propagation of oak (*Quercus robur* and *Q. petraea*) by cutting and tissue culture. – Ann. Sci. For. 50, Suppl. 1 : 295 – 307.

Chalupa, V., 1995. Somatic embryogenesis in oak (*Quercus* spp.) – In : S. M. Jain, P. K. Gupta, R. J. Newton (eds.) : Somatic embryogenesis in woody plants, Vol. 2, Kluwer Acad. Publ. Dordrecht, Boston, London : 67 – 87.

Chalupa, V., 2000. *In vitro* propagation of mature trees of pedunculate oak (*Quercus robur* L.). - Jour. Forest. Sci., 46: 537 – 542.

Cuenca, B.; San-Jose, M. T.; Ballester, A.; Vieitez, A.M., 1999: Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Rep. 18: 538 – 543.

Cvikrová, M.; Malá, J.; Hrubcová, M.; Vágner, M., 1998. Abscisic acid, polyamines and phenolic acids in sessile oak somatic embryos in relation to their conversion potential. Plant Physiol. Biochem. 36: 247 – 255.

Endemann, M.; Wilhelm, E., 1999. Factors influencing the induction and viability of somatic embryos of *Quercus robur* L. Biol. Plant. 42: 499 504.

Gingas, V.M.; Lineberger, R.D., 1989. Asexual embryogenesis and plant regeneration in Quercus. Plant Cell. Tiss. Organ. Cult. 17: 191 – 203.

Jörgensen, J., 1993. Embryogenesis in *Quercus petraea.* Ann. Sci. For. 50, Suppl. 1: 344 – 350.

Lloyd, G.; McCown, B., 1980. Commercially feasible micropropagation of mountain laurel *Kalmia latifolia*, by use of shoot tip culture. Proc. Int. Plant Propagators Soc. 30: 421 – 427.

Murashige, T.; Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473 – 497.

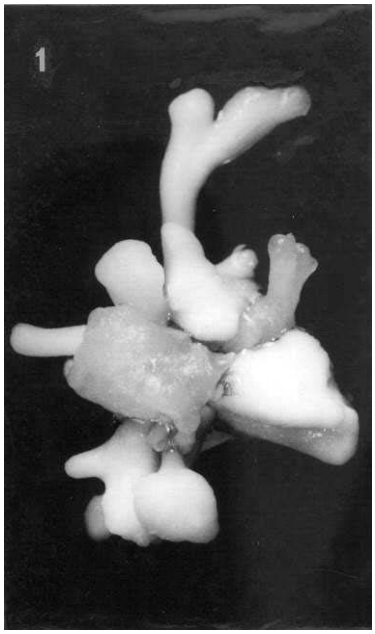

Fig. 1. Somatic embryoids arising from the embryogenic culture.

Fig. 2. Germinating mature somatic embryo.

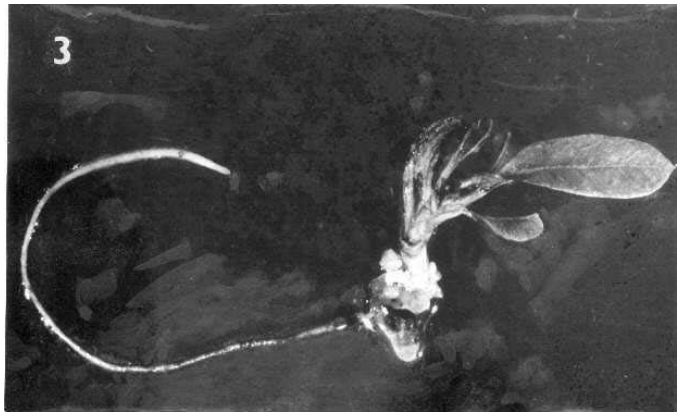

Fig. 3. Plantlet with expanding leaves and growing root, regenerated from somatic embryo.

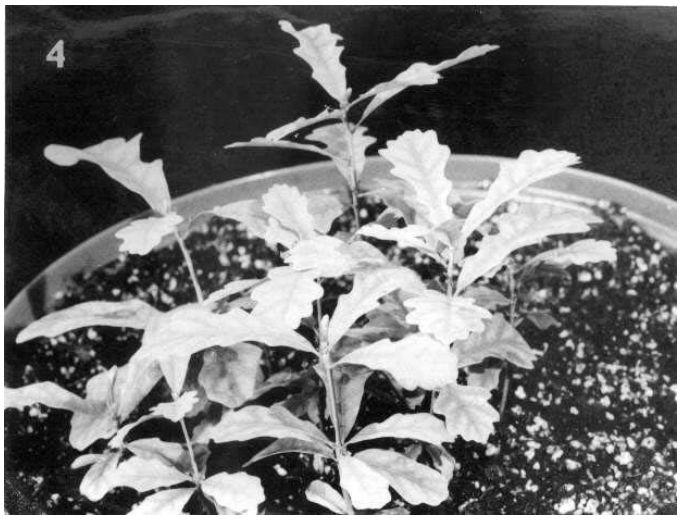

Fig. 4. Plants originated from somatic embryos, growing in a greenhouse.

PROTOCOL OF SOMATIC EMBRYOGENESIS: TAMARILLO (*CYPHOMANDRA BETACEA* (CAV.) SENDTN.)

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1. INTRODUCTION

Cyphomandra betacea, commonly known as tamarillo or tree tomato, is a soft wooded, fast growing, evergreen shrub or small tree seldom reaching 5 m. The species, a member of the *Solanaceae* family, is native of median altitude Andean regions, in South America where it has been under cultivation for a long time and has become one of the most popular local fruits (Dawes and Pringle 1983; http://www.crfg.org/pubs/ff/tamarillo.html). From South America it has spread to Central America and West Indies and, later on, to the Portuguese islands of Azores and Madeira and to Southern Europe (Atkinson and Gardner 1993; Hooker 1899). By the end of the XIX century it had reached Australia and New Zealand (Slack 1976; Symon 1981). This last country is nowadays the main producer and exporter of tamarillo and also is the main place where most progress in crop improvement has occurred (Dawes and Pringle 1983). Depending on the cultivar, tamarillo fruits are red, orange or yellow egg-shaped berries with 2-3 inches long and 2 inches in diameter (Fouque 1973; Hooker 1899). The fruits of the red cultivar are most popular due to their more striking appearance and better flavour (Carloto et al. 1999; Slack 1976). Tamarillo crop has high nutritional value and contains relatively high contents of protein and fibres, rich in vitamins B6, C and E, provitamin A, citric acid and mineral elements (iron and potassium); and low in carbohydrates and calories (Cacciopo 1984; McCane and Widdowson 1992).

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Although tamarillo can be grown in a variety of soils and climates, there are some limitations to its cultivation. In temperate regions this plant is usually grown as an outdoor ornamental although some attempts have been made to explore this species commercially due to the high prices attained by its fruits in market places (10 – 15 euros/Kg). The plant requires a period of low temperatures along the year but very low temperatures, occurring in winter and spring, and autumn frosts are important limitations to a large-scale cultivation of tamarillos (Lopes et al. 2000).

Propagation of tamarillo can be achieved from seeds or from cuttings. Seeds germinate easily and have been used for the development of new cultivars (Slack 1976). However, if the objective is to maintain the genetic characteristics of the cultivars they could not be used. Since young cuttings are soft and difficult to manipulate mature cuttings (30-40 cm in length and 1 –2.5 cm diameter) should be used for rooting. Micropropagation techniques can also be used with advantage over conventional methods. Among these techniques somatic embryogenesis has several potential advantages such as large-scale production (Thorpe and Stasolla 2001), regeneration of transgenic plants from transformed cells (Dandekar 1995) and possibility of artificial seed production (Deverno 1995). It also provides a model system for the study of plant development (Leyser and Day, 2003). In the case of tamarillo, somatic embryogenesis associated with genetic transformation may play a crucial role in developing new cultivars more tolerant against biotic and abiotic factors.

2. EXPLANT SOURCE, STERILIZATION AND CULTURE

In tamarillo, somatic embryogenesis or embryogenic calli can be induced from different source of explants (Guimarães et al. 1988, Guimarães et al. 1996; Lopes et al. 2000) such as: mature zygotic embryos, hypocotyl segments, internodes and young leaves (from seedlings or from shoots of *in vitro* propagated adult plants) using different approaches. Recently we found (data not published) that explants from yellow cultivars produce embryogenic calli more readily than from red cultivars and calli are also more stable in culture. Mature embryos or young leaves isolated from seedlings of this cultivar are routinely being used in embryogenic studies. Following desiccation, seeds can be stored in plastic recipients at room temperature for extended periods of time before use.

Cultures are initiated with seed sterilization for 20 min in 7.5% calcium hypochlorite solution followed by 3 rinses with double distilled water. Leave seeds for over night in sterile water. Induce embryogenic callus and somatic embryos by using whole isolated zygotic embryos or leaf sections taken from 6-week-old seedlings. Zygotic

embryos are cultured on MS (Murashige and Skoog, 1962) medium containing one of the auxins: naphthaleneacetic acid (NAA), picloram or 2,4-dichlorophenoxyacetic acid (2,4-D). Seedlings for leaf sections are obtained by culturing aseptically isolated zygotic embryos on MS medium containing 1% sucrose, under a 16h light daily regime. Leaf sections are cut into 4-6 pieces after removing the main vein, which are immediately platted, adaxial surface down, on MS induction medium added with 2,4-D or Picloram. Leaf sections and internode segments from shoots obtained through micropropagation of adult plants, are also used for the induction of embryogenic calli. In this case, nodal segments from adult plants are first established in MS medium containing 0.5mg/l benzylaminopurine (BA). Axillary shoot proliferation is achieved in the same medium. Leaves and internodal segments (5 mm long) from the resulting axillary shoots are taken after one month growth and cultured in media containing combinations of 2,4-D or Picloram with kinetin (Kn). For somatic embryogenesis induction or embryogenic calli production cultures are kept in the dark at 25ºC. The pH of all media is adjusted to 5.7 before autoclaving at 121ºC. Difco Bacto-agar (0.8% w/v) is added to all media. Normally, two embryos or one leaf section or internode segment are inoculated per test tube containing 15 ml culture media. Embryogenic callus, 80-100 mg (fresh weight), is sub-cultured (4-6 weeks) to the fresh culture media.

3. SOMATIC EMBRYOGENESIS INDUCTION

Mature zygotic embryos of tamarillo are cultured in the induction media containing different NAA concentrations (0.1 to 10 mg/l) and 3% sucrose. After 3-4 weeks, callus is produced and 2-3 weeks later, somatic embryos develop from calli (Fig. 1a) (Guimarães et al. 1996). Calli develop mainly from the hypocotyledonary region, the best results are obtained when 2.0 mg/l NAA is used. Under these conditions, about 80% explants produce calli, out of which 42% show somatic embryo formation. The inclusion of higher sucrose levels (9%) in the induction media strongly increases somatic embryo formation raising the efficiency of somatic embryogenesis induction to 85% (Guimarães et al. 1996). The number of somatic embryos per induced explant is generally low and, in most cases, less than 10 somatic embryos are produced. During development most somatic embryos pass through different morphological phases similar to those occurring during zygotic embryogenesis (globular, heart-shaped, torpedo and cotyledonary). However, morphological abnormalities such as fused cotyledons, altered number of cotyledons and precocious germination are commonly seen, especially when low levels of sucrose are used. Calli induced in the presence of NAA are unable to maintain their embryogenic potential and rapidly lose ability to further differentiate somatic embryos. Under these conditions, induced somatic embryos are able to proceed with their development up to the cotyledonary

stage in the same culture medium. This process of somatic embryogenesis induction has been called “one step somatic embryogenesis” (Sharp et al. 1980) and has also been observed in other species studied in our lab such as *Feijoa sellowiana* (Canhoto and Cruz 1996) and *Myrtus communis* (Canhoto et al. 1999a).

4. INDUCTION AND MAINTENACE OF EMBRYOGENIC CALLI

When zygotic embryos, leaf sections from six-week-old seedlings or from one-month-old shoots were cultured in the presence of the auxins 2,4-D or Picloram a slow growing callus was induced after 4 to 6 weeks of culture (Lopes et al. 2000 ; Maia 2002). By the 8th to 10th week of culture whitish clusters of embryogenic cells (Fig. 1b and c) were formed in some areas of the callus, which kept on proliferating. These cultures have been called “indirect somatic embryogenesis” (Sharp et al. 1980) and this type of embryogenesis, occurring in a large number of angiosperms and gymnosperms, has been recently reviewed (Raemakers et al. 1999). In the following sections we describe the particular conditions used for the induction and maintenance of embryogenic calli for each of the different explants tested.

Zygotic embryos: A wide range of 2,4-D concentration (1 to 25 mg/l) is able to induce embryogenic calli. Best results (33%) are obtained with 10 mg/l of the auxin. Since we have found that 2,4-D concentrations of 5 mg/l or higher have some deleterious effect on further callus growth and embryo development, 2,0 mg/l of 2,4-D is currently used in our experiments. Calli maintenance is done with the transfer of small portions (80–100 mg) of embryogenic calli to the same culture medium by monthly transfer. Embryogenic calli can also be transferred to liquid medium (Fig. 1d) of the same composition with subcultures made at 3 weeks interval. Similar results are observed when Picloram is used instead of 2,4-D.

Leaf sections from seedlings: Explants are cultured on a medium containing 5.0 mg/l Picloram and 3% sucrose. Small whitish embryogenic areas can be seen at the leaf surface, after 6 weeks of culture, together with fast growing non-embryogenic calli. In these conditions about 40% of the explants produced embryogenic calli (Lopes et al. 2000). Increasing the level of sucrose in the culture medium to 9% allowed that induction rates over 70% were attained. Lower Picloram concentrations (1.0 or 2.0 mg/l) also can induce embryogenic calli but at lower frequencies (15 and 27%, respectively). A medium containing 5.0 mg/l Picloram and 9% sucrose is currently used to maintain embryogenic. Subcultures are made at 6-week intervals.

Leaf sections and internodes from shoots: *in vitro* shoots (Fig. 1e) from field-growing adult plants are established and multiplied according with a protocol developed by

Barghchi (1998). Among them, the best results are obtained with internodes (35% of the explants showing embryogenic calli) cultured on a medium containing 5.0 mg/l 2,4-D, 0.5 mg/l (Kn) and 9% sucrose (Maia 2002). Comparatively, leaf sections produce embryogenic calli at lower frequencies, with best results (25% explants producing embryogenic calli) being achieved when the Kn concentration is raised from 0,5 to 1,0 mg/l. Assays of embryogenic calli of internodal origin show that the medium containing 2.0 mg/l 2,4-D produce the best proliferation rate, with a 5x increase of the original fresh weight after 8 weeks of culture (Maia 2002). Accordingly this medium is being used to maintain calli of internodal origin.

Embryogenic callus formation and maintenance offers a great potential for large-scale production and for genetic transformation (Merkle et al. 1995). Embryogenic calli of tamarillo can be maintained in the conditions described for several years without loss of the embryogenic potential. However, our experiments have also shown that embryogenic calli become very unstable in culture, especially those maintained for periods longer than one year. RAPD analysis showed polymorphisms among embryogenic calli of the same origin (Lopes et al. 2000). Chromosome abnormalities, including the occurrence of tetraploid plantlets, are observed by chromosome counting (data not published). Studies at our lab have also shown that plantlets obtained from 5-year-old embryogenic calli display more abnormalities than those regenerated from younger embryogenic calli (one year or younger). Differences are also observed in the multiplication rate among several callus lines and among cultivars

Independently of the conditions and explant source, embryogenic calli do not produce embryos on a medium containing auxin. Subsequent embryo development only occurs when the embryogenic calli are transferred to a medium without auxin as described in the next section.

5. SOMATIC EMBRYO DEVELOPMENT

Embryogenic calli are formed from densely stained isodiametric meristematic cells (Fig. 1c). When pieces of embryogenic calli (80 to 100 mg), originate from leaves, zygotic embryos or internodes, are transferred to a medium (liquid or solidified) containing 2% sucrose (reduced level) and without growth regulators, the embryogenic masses develop into somatic embryos morphologically identical to those obtained by “one step somatic embryogenesis”. Under these conditions, morphologically abnormal somatic embryos are also found. Somatic embryo development is not synchronized and different phases can be found in the same callus.

However, after three to four weeks on this medium most of the embryos are at the cotyledonary stage and ready to conversion. The effect of several growth regulators during somatic embryo development shows that abscisic acid (ABA) and Kn do not improve somatic embryo quality (Guimarães et al. 1996). However, reduced levels (0.1mg/l) of gibberellic acid (GA_3) strongly improved somatic embryo development (Guimarães et al. 1996).

The inclusion of GA_3 in the development medium also reduces time for somatic embryo conversion. The number of somatic embryos per embryogenic callus is dependent of several factors such as the initial explant, genotype, callus lines, maintenance medium and the period of callus maintenance in culture. However, values as high as 140 embryos per 100 mg (fresh weight) of embryogenic calli can be obtained after 4 weeks in the development medium. Particularly relevant is the fact that embryogenic calli obtained from zygotic embryos produce a higher number of somatic embryos than calli of internode origin (Maia 2002).

Although an auxin-free medium is necessary for somatic embryo development from embryogenic calli, use of auxin polar transport inhibitors has shown that endogenous auxin is required for normal somatic embryo development. In fact, the presence of auxin-polar transport inhibitors such as triiodobenzoic acid (TIBA) or chlorophenoxy methyl propionic acid (CFA) in the development medium sharply decreases the number of somatic embryos attaining the torpedo stage (Lopes et al. 2000). Furthermore, some of the embryos show fused cotyledons indicating a disturbance during transition from radial to bilateral symmetry (Hadfi et al. 1998; Liu et al. 1993).

6. SOMATIC EMBRYO CONVERSION

For somatic embryo conversion cotyledonary embryos are isolated and transferred to the conversion medium. Five somatic embryos are cultured on test tubes containing the same medium as for development: MS plus 2% sucrose. Cultures are kept at 25ºC under 16h photoperiod of 96 $\mu mol.m^{-2}\ s^{-1}$ photosynthetically active radiation provided by cool white fluorescent lamps. The results show low frequency rate (37% inoculated embryos) are of conversion into plantlets (Fig. 1f). The inclusion of GA_3 (0.1 mg/l) or charcoal (15g/l) don't improve somatic embryo conversion rate. The way in which somatic embryos are formed in the presence of NAA, 2,4-D or Picloram, also don't influences somatic embryo conversion. Although the somatic embryos cultured on the germination medium are morphologically normal, they must have some kind of abnormalities that impair somatic embryo conversion such as poor meristem differentiation, defficiencies in

the process of embryo maturation or abnormalities in chromosome number and strucuture. Preliminary histochemical and ultrastructural studies have shown that cotyledonary cells of the somatic embryos show lower levels of proteins and lipids as compared to zygotic embryos. In some cases, conversion of the embryos occur by shoot development with out the concomitant root development. However, adventitious roots often arise at the base of these shoots that makes plant regeneration possible.

7. ACCLIMATISATION

Following root formation, the plantlets are transferred to the greenhouse (Fig. 1g). Agar is removed gently from roots with tap water. The plantlets are immersed for 1min in 0.6g/l fungicide (Benlate) solution. Transfer plantlets in "Melfert" bags (220 ml) containing vermiculite and "Osmocote" slow release fertilizer (0.6 g/plantlet). Place plantlets in containers (60 x 40 cm) and cover them with plastic sheet to maintain high humidity environment. Containers are placed on an irrigation sheet in the greenhouse. Gradually reduce humidity by raising the plastic sheets.

Allow plants to grow for a period of 3 months in the greenhouse. The survival rate of the plants (Fig. 1h) is 55%. Transfer plantlets to the field and observe growth and fruit production. Our experience shows plants perform well in terms of growth and fruit production.

During the initial phases of plant development some plantlets show morphological abnormalities, further growth recovered the normal phenotype. High levels of plant mortality during this phase are probably related with abnormal plant development rather than with the growing conditions. In fact, similar experiments carried out with plantlets obtained by axillary shoot proliferation showed a survival rate of about 100%.

Figure 2 resumes the protocols used in our labotatory for somatic embryogenesis induction and plant regeneration in tamarillo.

8. CONCLUDING REMARKS

Somatic embryos of tamarillo can be induced from various explant types under different culture conditions. With this protocol, a large number of plantlets can be obtained. However, some problems still persist that reduce the success of the technique. One of the major problems is the occurrence of genetic alterations in embryogenic callus cultures, especially older than one year, that could be prevented

with cryopreservation or encapsulation of young embryogenic calli (Sakai 1995; Tremblay et al. in this book). Alternatively, modify the maintenance medium by reducing auxin that may prevent or minimise genetic alterations in among regenerated plants derived from callus, maintained on the modified medium. Factors leading to abnormal somatic embryo development should be determined, e.g. events leading to genetic modifications. Anomalous somatic embryo formation is a common feature in numerous woody species (Canhoto et al. 1999b) and the factors controlling embryo development must be better understood. The process of somatic embryo maturation in tamarillo is being monitored by histochemical and ultrastrucutural analysis. Our results demonstrate that several somatic embryo cells of the cotyledons and hypocotyl are often vacuolated and have reduced number of lipid and protein bodies. In contrast, cells of the cotyledonary zygotic embryos are completely filled with those type of reserve organelles. Poor somatic embryo maturation can also impair further somatic embryo conversion into normal plantlets (Thorpe and Stasolla, 2001).

In some of the media tested, embryogenic and non-embryogenic calli can be obtained from the same explant, and their cultures are maintained as embryogenic and non-embryogenic lines. Important insights about the process of somatic embryo induction and development can be obtained from biochemical and molecular studies that are being carried out with this material. Previous work have helped us to isolate and characterize a cDNA specific of non-embryogenic calli of tamarillo (Faro et al. 2003).

9. REFERENCES

Atkinson, R.G. & R.C. Gardner, 1993. Regeneration of transgenic tamarillo plants. Plant Cell Rep 12:347-351.

Barghchi, M., 1998. Regeneration, plant improvement and virus elimination of tamarillo [*Cyphomandra betacea* (Cav.) Sendt.]. In: M.R. Davey *et al*. (Eds.). Tree Biotechnology – Towards the Millennium, pp. 173-185. Nottingham University Press. Nottingham.

Cacciopo, O., 1984. La feijoa. Reda. Roma.

Canhoto, J.M. & G.S Cruz, 1996. *Feijoa sellowiana* Berg (PineappleGuava). In: Y.P.S. Bajaj (Ed.). Biotechnology in Agriculture and Forestry, vol. 35, pp.155-171. Trees IV. Springer-Verlag. Berlin.

Canhoto, J.M., M.L. Lopes & G.S. Cruz, 1999a. Somatic embryogenesis and plant regeneration in Myrtle (*Myrtaceae*). Plant Cell Tiss. Org. Cult. 57:13-21.

Canhoto, J.M., M.L. Lopes & G.S. Cruz, 1999b. Somatic embryogenesis in myrtaceous plants. In: S.M. Jain *et al*. (Eds.). Somatic embryogenesis in woody plants, vol. 4, pp. 293-340. Kluwer Academic Publishers. Dordrecht.

Carloto, J.M., M.L. Lopes, G.S. Cruz & J.M. Canhoto, 1999. O tamarilho (in Portuguese). Lavoura Moderna 4:8-9.

Dandekar, L.L., 1995. Genetic transformation of angiosperms. In: S.M. Jain *et al*. (Eds.). Somatic embryogenesis in woody plants, vol. 1, pp. 193-226. Kluwer Academic Publishers. Dordrecht.

Dawes, S.N. & G.L Pringle, 1983. Subtropical fruit from South and Central America. In: G.S. Wratt *et al*. (Eds.). Plant Breeding in New Zealand, pp. 123-138. Butterworths. Wellington.

Deverno, L.L., 1995. An evaluation of somaclonal variation during somatic embryogenesis. In: S.M. Jain *et al*. (Eds.). Somatic embryogenesis in woody plants, vol. 1, pp. 361-377. Kluwer Academic Publishers. Dordrecht.

Faro, M.R., M.L. Lopes, J.M. Canhoto, A. Cheung & G.S. Cruz, 2003. Identification and molecular characterisation of a non-embryogenic calli protein in *Cyphomandra betacea* (Cav.) Sendt. Abstracts of the 7th International Botanical Microscopy Meeting – Plant Cell Biology (Lisbon).

Fouque, A., 1973. Solanacées. Fruits 28:41-42.

Guimarães, M.L., G.S. Cruz & J.M. Montezuma-de-Carvalho, 1988. Somatic embryogenesis and plant regeneration in *Cyphomandra betacea* (Cav.) Sendt. Plant Cell Tiss. Org. Cult. 15:161-167.

Guimarães, M.L., M.C. Tomé & G.S Cruz, 1996. *Cyphomandra betacea* (Cav.) Sendt. (Tamarillo). In: Y.P.S. Bajaj (Ed.). Biotechnology in Agriculture and Forestry, vol. 35, pp.120-137. Trees IV. Springer-Verlag. Berlin.

Hadfi, K., V. Speth & G. Neuhaus, 1998. Auxin-induced developmental patterns in *Brassica juncea* embryos. Development 125 :879-887.

Hooker, J.D., 1899. *Cyphomandra betacea*. Curtis's Bot. Mag 55:7682.

http://www.crfg.org/pubs/ff/tamarillo.html (California Rare Fruit Growers, Inc. Retrieved October 13, 1997 from the World Wide Web).

Leyser, O. & S. Day, 2003. Mechanisms in Plant Development. Blackwell Publishing. Oxford.

Liu, C.-M, Xu, Z.-H. & Chua, N.-H., 1993. Auxin polar transport is essential for the establishment of bilateral symmetry during early plant embryogenesis. Plant Cell 5:621-630.

Lopes, M.L., M.R. Ferreira, J.M. Carloto, G.S. Cruz & J.M. Canhoto, 2000. Somatic embryogenesis induction in tamarillo (*Cyphomandra betacea*). In: S.M. Jain *et al*. (Eds.). Somatic embryogenesis in woody plants, vol. 6, pp. 433-455. Kluwer Academic Publishers. Dordrecht.

Maia, J.M., 2002. Tamarillo plant regeneration through somatic embryogenesis from adult micropropagated shoots. Master Thesis. University of Coimbra.

McCane, J. & D.A Widdowson, 1992. In: Fruit and nut. Suppl. to the composition of foods, 5th edn, pp. 74-77. Holland, Unwin & Buss, London.

Merkle, S.A., W.A. Parrot. & B.D. Flinn, 1995. Morphogenic aspects of somatic embryogenesis. In: T.A. Thorpe (Ed.). *In vitro* embryogenesis in plants, pp. 155-203. Kluwer Academic Publishers. Dordrecht.

Murashige, T. & F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-497.

Raemakers, K., E. Jacobsen & R. Visser, 1995. Proliferative somatic embryogenesis in woody species. In: S.M. Jain *et al*. (Eds.). Somatic embryogenesis in woody plants, vol. 4, pp. 29-59. Kluwer Academic Publishers. Dordrecht.

Sakai, A., 1995. Cryopreservation for germplasm collection in woody plants. In: S.M. Jain *et al*. (Eds.). Somatic embryogenesis in woody plants, vol. 6, pp. 433-455. Kluwer Academic Publishers. Dordrecht.

Sharp, W.R., M.R. Sondhal, A.E. Evans, L.A. Caldas & S.B. Maraffa, 1980. The physiology of in vitro asexual embryogenesis. Horticultural Reviews 2 :268-310.

Slack, J.M., 1976. Growing tamarillos. Agric. Gaz 86:2-4.

Symon, D.E., 1981. The solanaceous genera, *Browallia*, *Capsicum*, *Cestrum*, *Cyphomandra*, *Hyoscyamus*, *Lycopersicon*, *Nierembergia*, *Physalis*, *Petunia*, *Salpichroa* and *Withania*, naturalized in Australia. J. Adelaide Bot. Gard 3:133-166.

Thorpe, T.A., & C. Stasolla, 2001. Somatic embryogenesis. In: W.Y. Bhojwani *et al*. (Eds). Current Trends in the Embryology of Angiosperms, pp. 279-336. Kluwer Academic Publishers. Dordrecht.

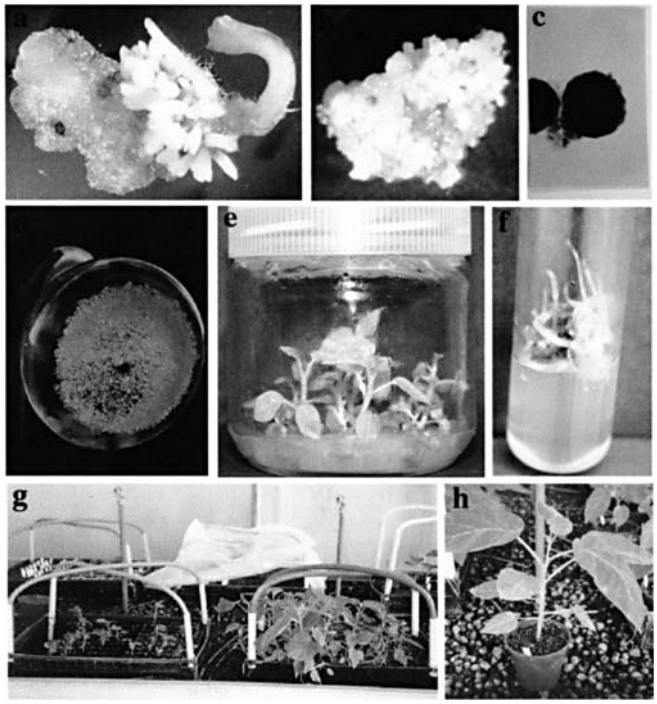

Figure 1. Somatic embryogenesis in tamarillo : (a) Somatic embryos produced in a medium containing NAA. Embrygenic calli (b) and embrygenic masses (c) obtained through the culture of leaf sections in a 2,4-D containing medium. (d) Embryogenic calli maintained in liquid medium. (e) Axillary shoot proliferation. (f) Somatic embryo conversion. (g) Plantlet acclimatation. (h) Plant of tamarillo regenerated by somatic embryogenesis and ready to go to field conditions.

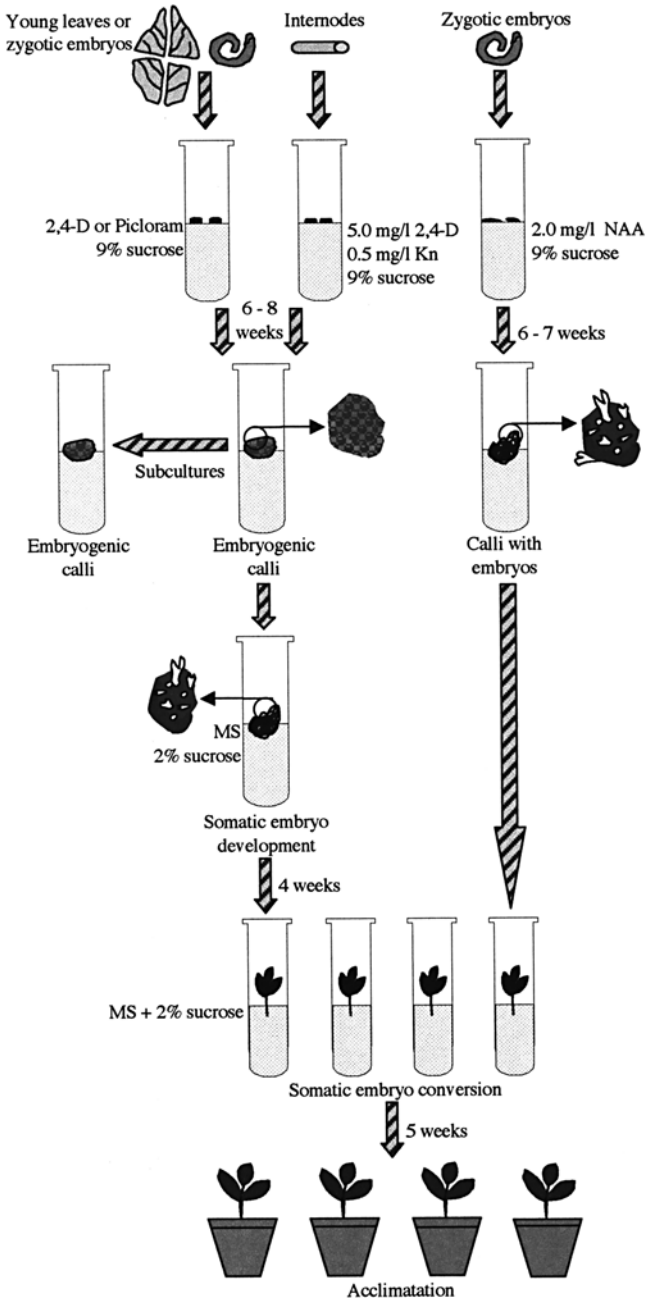

Figure 2. Schematic representation of the protocols for somatic embryogenesis induction in tamarillo

PROTOCOL OF SOMATIC EMBRYOGENESIS: EUROPEAN CHESTNUT (*CASTANEA SATIVA* MILL.)

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1. INTRODUCTION

European chestnut (*Castanea sativa* Mill.) is an important tree species for both landscaping and multifunctional plantations. Besides their main role in nut production in grafted orchards, the trees are found in coppices for production of small pieces of wood or in high forests supplying a variety of valuable timber highly resistant to decay (Fernández-López 200x). Interesting by-products are mushrooms and tannins for leather processing. Up to 500,000 tonnes of chestnuts are produced each year around the world from four commercially important species of the genus, a large part coming from European chestnut. Nowadays chestnuts are grown not only in the traditional Mediterranean areas of cultivation such as Spain, Turkey and Italy, but also in South America, Australia, and New Zealand.

However, this tree species is threatened by pollution, social and economic changes, and by two major fungal diseases: ink disease (*Phytophthora ssp*.) and chestnut blight (*Cryphonectria parasitica* (Murr.) Barr.). With its high nut and timber quality the European chestnut is an important breeding partner in hybridisation programmes with *Castanea crenata* Sieb. & Zucc. is undertaken to produce blight-resistant trees.

As in other recalcitrant, large-seeded temperate tree species, storage of seeds is limited and rooting is a non-trivial task, making somatic embryogenesis an interesting alternative for clonal propagation of superior

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genotypes. Cryostored somatic embryos may serve the end of preserving the germplasm of old landraces as well as new superior varieties. In addition, somatic embryogenesis is a potential tool for regenerating transgenic trees. Carraway *et al.* (1994) transferred neomycin phosphotransferase and ß-glucuronidase genes into American chestnut embryogenic cultures via particle bombardment. However, the application of somatic embryogenesis for the improvement of chestnut is still limited as a result of problems with low initiation frequencies, maintenance of embryogenic cell lines and low conversion rates.

2. INDUCTION OF EMBRYOGENIC TISSUE

Induction of sweet chestnut somatic embryos has been described for juvenile reproductive tissues and for leaves of *in vitro* stock shoot cultures. Besides the physiological status of the explant tissue the genotype also greatly influences the induction frequency.

2.1. Induction of somatic embryos from ovaries, ovules and zygotic embryos (Sauer & Wilhelm, in press):

Spikes of burs are removed and burs are surface-sterilised (2 x 10 minutes) with a commercial bleach (containing 2.5 % NaOCl) in an ultrasonic bath with an intermittent washing step using 70 % ethanol for 15 s (Wilhelm 1997). Burs are rinsed twice in a sterile solution of 300 mM Na_2HPO_4 + 42 mM citric acid + 100 mM KI + soluble starch (5 g/l), then transferred to a sterile solution of starch (5 g/l) and rinsed twice in sterile distilled water (Dirks *et al.* 1991). By employing these disinfection and washing procedures, losses of not more than 5 % are observed. The cupule is removed aseptically and nuts are excised. Induction is possible from ovaries (longitudinal sections), ovules or zygotic embryos in a developmental window between the second and tenth week postanthesis.

To induce somatic embryos, explants are placed in Petri dishes containing 25 ml basal medium with 5 µM 2,4-D plus 0.5 µM BA, and 2 ml/l Preservative for Plant Tissue Culture Media (PPM™, Plant Cell Technology, Inc., Washington, DC), supplemented with 3 % sucrose and gelled with 0.8 % agar. The pH of the medium is adjusted to 5.5 – 5.6 prior to autoclaving at 121 °C for 20 min. To avoid the inhibitory influence of

polyphenols, the explants are transferred to fresh induction medium within the first week. After 3 weeks explants are transferred to basal medium with 0.89 µM BA. As basal media P24 (Teasdale 1992) or GD (Gresshoff and Doy 1972) are suitable. Embryogenic tissues usually can be observed after 3 weeks. Cultures are incubated in the growth chamber at 24 °C (+/- 2 °C) under white fluorescent light at 50 µmol m^{-2} s^{-1} at a 16h photoperiod. The induction frequency when using ovaries ranges from 2.0 to 19.1 % and somatic embryos are observed in tissue collected 2 to 8 weeks postanthesis, while the yield is 0.8 to 7.8 % when ovules are used as starting tissue 3 to 9 weeks postanthesis. Zygotic embryos collected 5 to 10 weeks postanthesis form somatic embryos at a rate of 10.5 to 57.1 %.

2.2. Induction of somatic embryos from leaf explants (Corredoira *et al.* 2001):

Starting material is the 2-3 uppermost unfurled expanding leaves originated from in vitro shoots multiplied in tissue culture.

Explants are cultured on solid MS medium supplemented with 1 mg/l BA and 1 mg/l NAA, and incubated under a 16h photoperiod and 25 °C light/20 °C dark temperatures. After eight weeks somatic embryos form on the leaf explants at an induction rate of 0.5 % and are transferred to maintenance medium.

3. MAINTENANCE OF EMBRYOGENIC TISSUE

In general, low salt concentrations, as found in WPM, MS with half strength of macronutrients, GD and P24, as well as low hormone levels are thought to be beneficial for chestnut somatic embryo proliferation (Vieitez 1995). Repetitive somatic embryogenesis occurs as direct secondary embryogenesis in the hypocotyl region or as indirect proliferation via a nodular callus. As described by Carraway *et al*. (1997) explants produce a mixture of callus types and continuous selection of embryogenic cells is necessary.

3.1. Somatic embryo cultures derived from ovaries, ovules and zygotic embryos are maintained on solid medium with 0.89 µM BA (0.5 - 0.7 g of tissue per plate). Hormone-free basal medium is suitable for maintenance

as well (Sauer, 2001). Omitting BA from multiplication medium has no significant effect on fresh weight increase and no differences in multiplication of somatic embryos are observed. While during long-term culture of SE on hormone-free medium the beneficial effect of BA on cell division may be missing, embryo quality should nonetheless improve in the absence of BA, as described by Zegzouty and Favre (1999), and less somaclonal variation should occur. We recommend adding BA to the multiplication medium once within four to five subculture cycles. Subculture intervals are 5 weeks. Cultures are incubated in the growth chamber under the same conditions as for induction.

3.2. For maintenance of somatic embryos from leaf explants by repetitive embryogenesis, solid MS medium (half-strength macronutrients; 3 g/l sucrose; 8 g/l agar) plus 0.1 mg/l BA and 0.1 mg/l NAA is employed. Subculture intervals are 6 weeks. (Ballester *et al.* 2001)

3.3. Cold storage of embryogenic tissue: as induction from zygotic embryos is restricted to a certain season and handling of the cultures is enormously time-consuming, we looked for a method of storing embryogenic tissue for some months without subculture. Seven days after the last subculture material is transferred to an environment of 10 °C under dim light in which it may be stored for at least three months. To avoid losses due to excessive condensation, Petri dishes are stored vertically, so that water gathers in the lower part of the dish.

4. MATURATION

During the maturation phase, the embryos must accumulate nutrient reserves. Translucent somatic embryos regularly fail to germinate, in contrast to embryos with thickened opaque cotyledons. Various types of stress, such as use of osmotic compounds, desiccation and the application of ABA have been investigated as switches for accumulation of storage products (Wilhelm 2000). Increasing the agar concentration up to 1.1 % or application of an osmoticum such as 6 % sorbitol (Sauer 2001) or 3 % maltose (Corredoira *et al.* 2003) enhances embryo maturation and conversion.

Maturation treatment 1 (Sauer & Wilhelm in press): clusters of somatic embryos, 0.5 - 0.7 g per Petri dish, are placed on maintenance medium

solidified with 1.1 % agar instead of the standard concentration of 0.8 %. After 5 weeks cotyledonary-stage embryos (size > 3 mm) are collected and subjected to the chilling treatment or transferred to germination medium directly.

Maturation treatment 2 (Sauer 2001): instead of the water stress in maturation treatment 1, an osmoticum is applied: standard maintenance medium is supplemented with 6% sorbitol.

Maturation treatment 3 (Corredoira *et al*. 2003): cotyledonary embryos are cultured individually on maturation medium consisting of PGR-free basal medium supplemented with 3 % maltose.

5. GERMINATION AND TRANSFER TO SOIL

As pregermination treatments, desiccation and/or chilling are applied in order to brake dormancy. Lipid and protein reserves are mobilised to enable root and shoot growth. Most often radicles germinate first, while epicotyls form only after delay or fail to germinate. Conversion rates range from 6 % (maturation treatments 1 and 3) to 13 % (maturation treatment 2) when combined with a chilling treatment.

Chilling treatment: after 4-5 weeks on maturation medium, somatic embryos are cultured on maintenance medium and stored at 4 °C in the dark for 2 months.

For germination, somatic embryos are cultured in Petri dishes on basal medium with 0.1 µM indole-3-butyric acid (IBA) and 0.89 µM BA. After 5-6 weeks the seedlings are transferred to test tubes or Magenta GA-7 vessels containing PGR-free medium supplemented with 1 % m/v activated charcoal.

Plants with well developed shoots and roots are transplanted into pots containing a 1:1 perlite : soil mixture and acclimatised in a growth chamber under artificial light at a 14h photoperiod, a temperature of 20 - 22 °C and a relative humidity of 97 %. After 4 weeks the plants are kept under daylight conditions at 20 to 25 °C in the greenhouse.

5. CONCLUDING REMARKS

The first records on somatic embryogenesis in *Castanea sativa* date back to Piagnani *et al.* in 1990, and Leva *et al.* in 1993, but conversion into plantlets was not achieved before 2000 (Sauer & Wilhlem). For the genus *Castanea*, somatic embryogenesis has been reported for *C. mollissima* Blume x *C. dentata* (Marsh.) Borkh. hybrids (Skirvin 1981), *C. dentata* (Merkle 1991, Xing *et al*. 1999) and *C. sativa* x *C. crenata* Sieb. & Zucc. hybrids (Vieitez *et al*. 1990). Regeneration of plants via somatic embryogenesis in *C. sativa* x *C. crenata* hybrids has already been achieved (Vieitez *et al*. 1992).

A main focus for the future is on the development of propagation systems for initiating somatic embryos from mature trees, in order to establish cell lines with known phenotypes. The induction from juvenile leaves of sweet chestnut and the protocols for somatic embryogenesis from mature tissues of cork oak (Hernandez *et al*. 2003), pedunculate oak (Cuenca 1999) and elm (Conde *et al.* 2003) are promising starting points.

In the face of problems with somaclonal variation, a system for cryopreservation of somatic embryos has to be addressed next. Holliday *et al.* (2000) have defined a protocol for cryostorage of embryogenic cultures of American chestnut, and Pence (1992) showed that zygotic embryo axes of European chestnut survived and elongated after exposure to liquid nitrogen.

REFERENCES

Ballester, A., L. Bourrain, E. Corredoira, J. Concalves, C. Lê, M. E. Miranda-Fontaína, M. San-José, U. Sauer, A. M. Vieitez, & E. Wilhelm, 2001. Improving chestnut micropropagation through axillary shoot development and somatic embryogenesis. For. Snow Landsc. Res. 76,3: 460-467.

Carraway, D.T., H.D. Wilde, S.A. Merkle, 1994. Somatic embryogenesis and gene transfer in American chestnut. – J. amer. Chestnut Foundation 8:29-33.

Carraway, D.T., S.A. Merkle, 1997. Plantlet regeneration from somatic embryos of American chestnut. - Can. J. Forest. Res. 27: 1805-1812.

Conde P., J. Loureiro, C. Santos, 2003. Somatic embryogenesis and plant regeneration from leaves of Ulmus minor Mill. Plant Cell Rep. 13 preprint.

Corredoira, E., A. Ballester & A.M. Vieitez, 2003. Proliferation, maturation and germination of *Castanea sativa* Mill. somatic embryos originated from leaf explants. Ann. Bot. 92: 129-136.

Corredoira, E., A.M. Vieitez, A. Ballester, 2001. Somatic embryogenesis from leaf explants of chestnut. Abstract COST G4, Monte Veritá, Switzerland.

Cuenca, B., M.C. San-José, M.T. Martínez, A. Ballester, A.M. Vieitez, 1999. Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Rep. 18: 538-543.

Dirks, R., M. van Buggenum, C. Tulmans, R. de Vogel, 1991. Use of Iodide Ions for Chemical Reduction of the Oxidative Agent H_2O_2 and Hypochlorites after Application as Decontaminating Agents for Plant Tissue. - In: I. Negrutiu, G.B. Gharti Chhetri (Eds.). A Laboratory Guide for Cellular and Molecular Plant Biology. 1.10:100-104. Birkhäuser Verlag. Basel.

Fernández J., R. Alía, 1999. Chestnut (*Castanea sativa).* European long-term conservation strategies. Noble Hardwoods Network. Report of the third meeting, 13-16 June, Sagadi, Estonia: 21-27.

Gresshoff, P.M., C.H. Doy, 1972. Development and differentiation of haploid *Lycopersicon esculentum* (tomato). - Planta 107:161-170.

Hernandez, I., C. Celestino, J. Alegre, M. Toribio, 2003. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis. I. Factors affecting the induction in leaves from mature cork oak trees. Plant Cell Rep. 21(8): 759-64.

Holliday, C., S. Merkle, 2000. Preservation of American chestnut germplasm by cryostorage of embryogenic cultures. J. amer. Chestnut Foundation 14: 46-52.

Leva, A.R., F.P. Nicese, N. Vignozzi, A. Benelli, 1993. Embriogenesi somatica in castagno. In: E. Antognozzi (ed.). Proceedings of the International Congress on Chestnut, pp. 187-190. Spoleto, Italy

Merkle, S.A., A.T. Wiecko, B.A. Watson-Pauley, 1991. Somatic embryogenesis in American chestnut. - Can. J. For .Res. 21: 1698-1701.

Pence, V.C., 1992. Desiccation ad the survival of *Aesculus, Castanea,* and *Quercus* embryo axes through cryopreservation. Cryobiol. 29:391-399.

Piagnani, C. & T. Eccher, 1990. Somatic embryogensis in chestnut. Acta Horticulturae 280: 159-161.

Sauer U. & E. Wilhelm, 2000. Somatic embryogenesis in European chestnut. Abstract COST G4: Multidisciplinary chestnut research. Saloniki, Greece.

Sauer, U., 2001. Improving maturation and germination conditions for SE of European chestnut. Abstract COST G4, Monte Veritá, Switzerland.

Sauer, U. & E. Wilhelm. Somatic embryogenesis from ovaries, developing ovules and immature zygotic embryos and improved embryo development of Castanea sativa. Biologia Plantarum. (In press).

Skirvin, R.M., 1981. Fruit crops. - In: Conger B.V. (ed.): Cloning agricultural plants via in vitro techniques. Pp. 51-139. CRS Press, Boca Raton.

Teasdale, R., 1992. Formulation of plant culture media and applications therefore.- International Publication No. WO 92/07460, Patent No. Europe: 92902531.0, Forbio PTY Ltd., Queensland Australia.

Vieitez, F.J., M.C. San-Jose, A. Ballester, A.M. Vieitez, 1990. Somatic embryogenesis in cultured immature zygotic embryos in chestnut. - J. Plant Physiol. 136: 253-256.

Vieitez, F.J., A. Ballester, A.M. Vieitez, 1992. Regeneration of chestnut via somatic embryogenesis. - Abstract. International Chestnut Conference, Morgantown, WV, 1992:11.

Vieitez, F.J., 1995. Somatic embryogenesis in chestnut. In: S. Jain, P. Gupta (Eds). Somatic Embryogenesis in Woody Plants, vol. 2, pp. 375-407. Kluwer Academic Publishers, The Netherlands.

Wilhelm, E., 1997. Process for disinfecting plants and plant tissues. Verfahren zur Desinfektion von Pflanzengut bzw. Pflanzengewebsgut, Patentschrift AT 403 006. PCT WO 96/23528.

Wilhelm, E., 2000. Somatic embryogenesis in oak (*Quercus spp*.). In Vitro Cell. Dev.Biol.-Plant 36: 349-357.

Xing, Z., W.A. Powell, C.A. Maynard, 1999. Development and germination of American chestnut somatic embryos. - Plant Cell Tiss. Org. Cult. 57:47-55.

Zegzouti, R. & J.M. Favre, 1999. Histogenesis of secondary enbryogenesis in Quercus robur: Preliminary results. - In: Espinel S., Ritter E. (ed.): Proc. of Applications of Biotechnology to Forest Genetics. Biofor 99. Pp. 227-230. Vitoria-Gasteiz, Spain.

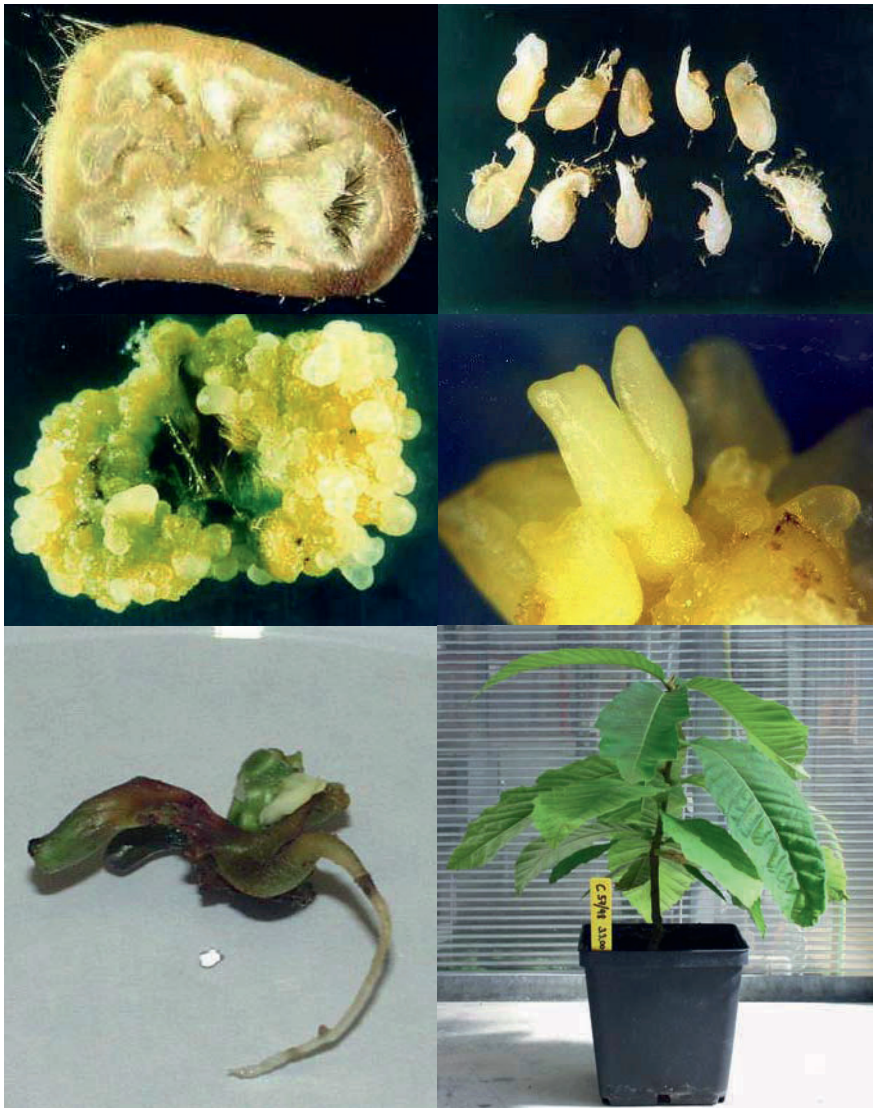

Figure 1. Somatic embryogenesis in European chestnut: a) explant type: ovary b) explant type: ovules c) mixture of callus types produced from ovary on induction medium d) secondary embryos produced on maturation medium e) germinated somatic embryo f) plantlet regenerated from somatic embryo under greenhouse conditions.

PROTOCOL OF SOMATIC EMBRYOGENESIS IN *ACACIA ARABICA* (Lamk.) Willd.

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1.INTRODUCTION

The genus *Acacia* (Greek Akaia, derived from akis, 'a thorn') belongs to family Leguminosae: Mimosoideae and widely distributed throughout the tropics and subtropics. *Acacia arabica* (Babul) is found naturally in the Deccan part of India and tropical Africa and is naturalized in all parts of India. It is an economically valued timber tree, and is used for agricultural implements and fuel wood, while the leaves are used for fodder. This species has great ecological value because it helps in controlling erosion and improving soil fertility. However, these valuable timber species are at the verge of extinction as germplasm conservation is difficult because of high intrafruit seed abortion and inbreeding depression.

The woody plants, particularly, legume tree species have a low genetic and physiological ability for adventitious root formation when propagated from cutting which limit their commercial production; cutting is also labour intensive and many species are difficult to root under normal condition. Tree legumes (*Acacia* species) are recalcitrant with regard to seed germination. *In vitro* culture technique is an alternative method for propagation and conservation and it will

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help in understanding the basic biology of the plant under controlled environmental condition.

In vitro propagation of different *Acacia* species has been reported by many researchers by using nodal explants or shoot tip cultures from *in vitro* grown seedlings, juvenile greenhouse plants or adult field-grown material (Duhoux and Davies, 1985; Zhao *et al*., 1990; Dewan *et al*., 1992; Badji *et al*., 1993; Huang *et al*., 1994 ; Kaur *et al*., 1998). So far, somatic embryogenesis has been achieved in *Acacia catechu* (Rout *et al*., 1995), *Acacia arabica* (Nanda and Rout, 2003) and *Acacia farnesiana* (Ortiz *et al*., 2000). In this article, we have described a protocol on somatic embryogenesis in *Acacia Arabica*.

2. INDUCTION OF SOMATIC EMBRYOGENIC CALLUS

2.1. EXPLANT

In this protocol, we have used explant, which was taken from an elite *Acacia arabia* tree growing in the Regional Plant Resource Centre, Bhubaneswar, India. Green pods are collected (45 days after anthesis), and after collection, washed with 2% (v/v) "Teepol" detergent (Qualigen, India) for 10 min and rinsed with autoclaved distilled water four to five times. Seed coat is removed and immature zygotic embryos are excised and placed into the induction medium (Table 1).

2.2. MEDIUM

The pH of the induction media is adjusted with 5.7 with 0.1 N NaOH or HCl prior to the addition of agar 0.8% (w/v) (Qualigen, India). Routinely, 20 ml molten medium is dispensed into culture glass tubes (25 x 150 mm), which are plugged with non-absorbent cotton wrapped in one layer of cheesecloth. Media are autoclaved at 104 kPa for 15 min.

2.3. EXPLANT CULTURE

The immature zygotic embryos are placed into the induction culture medium (Table 1). After inoculation, the cultures are incubated at a 16-h photoperiod,

light intensity 55µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (Phillips, India) at 25 ± 2^0 C.

The initial primary embryogenic callus is yellowish, nodular and friable (Figure 1A). The medium having 2,4-D helps to induce somatic embryogenesis. The response to the induction of embryogenic callus is higher in the medium supplemented with BA + 2,4-D. After 3 – 4 weeks, the embryogenic calli (150 ± 20 mg) are subcultured on fresh medium for proliferation and maintenance.

3. DETERMINATION OF SOMATIC EMBRYOGENIC CALLUS

At every 2-week interval, the embryogenic callus is fixed in FAA (Formalin / glacial acetic acid / ethanol, 5: 5: 90, v/v) for 48 h; dehydrate through a graded ethanol-xylol series; and embed in paraffin wax. The tissues are sectioned at 10 µm thickness, double-stained with 1% (w/v) safranin and 0.1% (w/v) toludine blue and examine under a light microscope. The histological examinations reveal thickening of cell wall and various developmental stages of somatic embryos with shoot and root meristems. The embryos are loosely attached to the mother callus with a short suspensor-like structure at the basal end, which subsequently develop a root system.

4. MAINTENANCE OF EMBRYOGENIC CALLI

Embryogenic calli are separated from the primary culture (induction medium) and subculture on the maintenance medium [(Table 1) (Figure 1B-C)]. The cultures are incubated at a 16-h photoperiod, light intensity 55 µmol m^{-2} s^{-1} provided by cool-white fluorescent lamps (Phillips, India) at 25 ± 2^0 C. Numerous globular and bipolar somatic embryos develop on the surface of the primary embryogenic calli within 2 weeks of culture. In some cases, secondary somatic embryosgenesis develop by regular subculture in the same fresh media. The highest average number of somatic embryos per 150 mg embryogenic callus is 72.6 after 8 weeks of culture on the maintenance medium (Table 1). In general, somatic embryos develop one or two cotyledons, although some have unequal cotyledons and others fused multiple cotyledons. To avoid the

blackening the embryogenic calli, cultures are sub-cultured regularly at 4-week interval.

5. MATURATION AND GERMINATION

The maturation medium contains half strength MS salts, vitamins supplemented with 0.1–0.25 mg/L abscisic acid (ABA), 0.01 – 0.05 mg/L BA and 2% (w/v) sucrose. The medium is solidified with 0.8% (w/v) agar (Qualigen, India). All the media are steam sterilized at 104 kPa for 15 min. The isolated and group of embryos are inoculated into the maturation and germination medium (Figures 1D - E). The highest percentage germination rate (67.8%) is obtained within 2 weeks of culture. The somatic embryos produce green cotyledons and a plumule and radicle without showing secondary callus. Further, the germinated embryos are transferred to semi-solid half-strength MS medium without growth regulators for better growth. After 3 weeks, the embryos develop complete plantlets.

6. ACCLIMATIZATION AND TRANSFER TO SOIL

About 1-month-old somatic seedlings are transferred to sterilized soil mixture at the ratio of 1:1 (Cow-dung: Sand: Soil) and keep in the greenhouse at 30 ± 2^0 C with 85% relative humidity (Figure 1F). Plants are watered at every alternate day. Nearly, 40 % plants survive in the greenhouse.

7. GENETIC FIDELITY STUDY

Several strategies can be used to assess the genetic fidelity of *in vitro* derived clones, but most have limitations. Karyological analysis, for example, can't reveal alterations in specific genes or small chromosomal rearrangements (Isabel *et al*., 1993). Isozyme markers provide a convenient method for detecting genetic changes, but are subject to ontogenic variations. They are also limited in number, and only DNA regions coding for soluble proteins can be sampled. Using polymerase chain reaction (PCR) in conjunction with short primers of arbitrary sequence (Williams *et al*., 1990), randomly amplified polymorphic

DNA (RAPD) markers were recently shown to be sensitive for detecting variations among individuals (Isabel *et al*., 1993; 1996).

Leaves (500 mg) collected from somatic embryo derived plants individually and also field grown mother plants are ground to a fine powder in liquid nitrogen. The fine powder is resuspended in preheated 10 ml DNA extraction buffer (2% CTAB (w/v), 0.2% β-mercaptoethanol (v/v), 100 mM Tris-HCl pH- 8.0, 2 mM EDTA, 1.4 M NaCl) and incubated for 2hrs at 60^0 C. After incubation, equal amount of isoamyl alcohol and chloroform (24:1) are added and centrifuge at 10,000 rpm for 20 min. The supernatant is collected and precipitated using chilled isopropanol. The crude DNA pellet is obtained by adding chilled ethanol. The DNA pellet is resuspended in 200 µl to 300 µl of Tris-EDTA (10 mM- 1mM). Subsequently, it is treated with 3 µl RNAse (10 mg/ml) and incubated for one hour at 37^0 C. DNA purification is done twice by using equilibrated phenol. The pure DNA is precipitated using chilled ethanol and then dried. About 200 – 300 µl Tris-EDTA buffer (TE) is added in pure DNA pellet for dissolving it and it is kept for randomly amplified polymorphic DNA (RAPD) analysis.

Polymerase Chain Reactions (PCR) are carried out in a final volume of 25µl containing 20ng template DNA, 100 µM each deoxynucleotide triphosphate, 20ng of decanucleotide primers (M/S Operon Technology, Inc., Alameda, CA 94501, USA), 1.5 mM $MgCl_2$, 1x Taq buffer (10 mM Tris-HCl (pH-9.0), 50 mM KCl, 0.01% gelatin) and 0.5 U Taq DNA polymerase (M/S Bangalore Genei, India). Amplification is done in a PTC 100 thermal cycler (M J Research, USA) programmed for a preliminary 4 min denaturation step at 94^0 C, followed by 45 cycles of denaturation at 94^0 C for 1 min, annealing at 37^0 C for 1 min and extension at 72^0 C for 2 min, finally at 72^0 C for 10 min. Amplification products are separated alongside a molecular weight marker (1kb ladder, MBI Fermentas, USA) by electrophoresis on 1.2 % (w/v) agarose gels run in 0.5 X TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs are scanned through Gel Doc System (Gel Doc. 2000, BioRad, USA) and the amplification product sizes are evaluated using the software Quantity one (BioRad, USA). The amplification bands obtained through RAPD analysis show that the somatic seedlings are similar

with mother plants grown in the field. Forty different decamers tested, the amplification products that are monomorphic across all somatic seedlings of *Acacia arabica*. The size of the monomorphic DNA fragments, produced by these primers ranged from 0.1 to 2.0 Kbp. No polymorphism or changes in the amplified DNAs are detected after amplification by PCR within somatic embryo derived plants.

8. CONCLUSION AND FUTURE PROSPECTS

Maturation and germination of somatic embryos are important factors for the application of somatic embryos in improvement of tree species. The poor germination rate of somatic embryos has hampered their large-scale application in tree improvement. A*cacia arabica* has some difficulties in production of plantlets. This protocol requires more refinement for the improvement of germination rate of somatic embryos. In some tree species, the development of plantlets were triggered with the alterations of the culture conditions *e.g.* with the GA_3 or ABA or cold treatment (4 ^{0} C) (Attree *et al*., 1991; Rout *et al*., 1995; Ortiz *et al*., 2000; Nanda and Rout, 2003) as well as alteration of osmoticum in the medium (Garcia – Martin *et al*., 2001; Garin *et al*., 2000). Micropropagation via somatic embryogenesis can be helpful in conservation of genotype and commercialization. The clonal propagation of a tree species via somatic embryogenesis would be highly desirable provided the genetic fidelity of somatic seedlings is maintained. Currently, molecular tools are available to determine genetic variability among regenerated plants. Bioreactors would certainly be useful to produce somatic embryos in large numbers under controlled conditions, and that would lead to automation of somatic embryo production. It is also helpful for large-scale multiplication in a bioreactor. Further investigations are being carried out to improve germination rate of somatic embryos and plant production.

9. REFERENCES

Attree, S.M., D. Moore, V.K.Sowhney. & L.C. Fowke, 1991. Enhanced maturation anddesiccation tolerance of White spruce (*Picea glauca* Moench. Voss) somatic

embryos effects of a nonplasmolysing water stress and absicsic acid. Ann. Bot., 68: 519-525.

Badji, S., Mairone, Y., Ndiaye, I., Merlin, G., Danthu, F., Neville P. & Colonna, J.P. (1993) *In vitro* propagation of the gum Arabic tree (*Acacia senegal* (L.) Willd.. I. Developing a rapid method for producing plants). Plant Cell Rep., 12: 629-633.

Dewan, A., Kanan, N. & Gupta S.C (1992) *In vitro* micropropagation of *Acacia nilotica* subsp *indica* Brenan via. Cotyledonary node. Plant Cell Rep., 12: 18-21.

Duhoux, B. & Davies, U (1985) Shoot production from cotyledonary buds of *Acacia albida* and influence of sucrose on rhizogenesis. J. Plant Physiol., 121: 175-180.

Garcia-Martin, G., M.E.GonzalezBenito & J.A.Manzanera. 2001. *Quercus suber* L. somatic embryo germination and plant conversion: Pretreatments and germination conditions. In Vitro Cellular and Developmental Biol. –Plant, 37 (91): 190-198.

Gartin, E., M.M.Berniercardou, N.Isabel, K. Klimaszawska & A.Plourde. 2000. Effect of sugars, aminoacids and culture technique on maturation of somatic embryos of *Pinus strobes* on medium with two gellan gum concentrations. Plant Cell. Tiss. Org. Cult., 62 (1): 27-37.

Huang, F.H., Al-Khayri, J.M & Gbur, E.E (1994) Micropropagation of *Acacia mearnsii*. *In vitro* Cell Dev. Biol., 30: 70-74.

Isabel, N., Tremblay, L., Michaud, M., Tremblay, F.M. & Bousquet, J (1993) RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis – derived populations of *Picea mariana* (Mill.) B.S.P. Theor. Appl. Genet., 86: 81-87.

Isabel, N., Tremblay, L., Michaud, M., Tremblay, F.M. & Bousquet, J (1996) Occurrence of somaclonal variations among somatic embryo-derived white spruces (*Picea glauca*, Pinaceae). Amer. J. Bot., 83: 1121-1130.

Kaur, K., Verma, B. & Kant, U (1998) Plants obtained from the khair tree (*Acacia catechu* Willd.) using mature nodal segments. Plant Cell Rep., 17: 427-429.

Murashige, T. & F.Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant., 15: 473-479.

Nanda, R.M & G.R.Rout, 2003. *In vitro* somatic embryogenesis and plant regeneration in *Acacia arabica*. Plant Cell, Tiss. Org. Cult., 73: 131-135.

Ortiz, B.O.C., M.E.Reyes & E.P.M. Balch. (2000) Somatic embryogenesis and plant regeneration in *Acacia farnesiana* and *A. achaffneri*. *In Vitro* Cellular and Developmental Biology-Plant, 36 (4): 268-272.

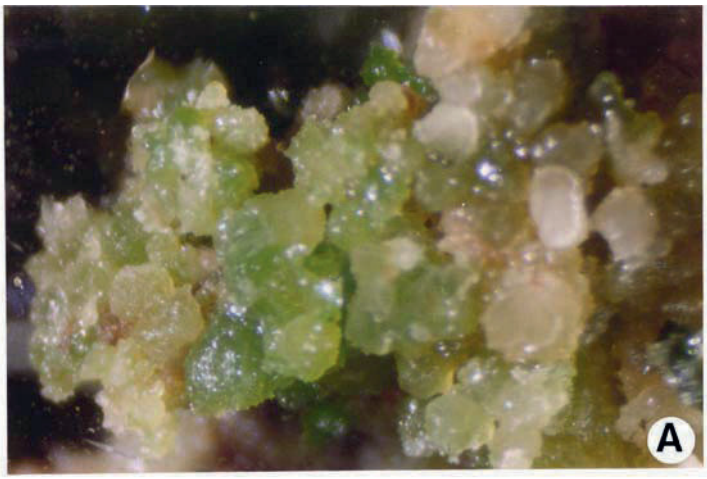
Rout, G.R., S. Samantaray & P. Das, 1995. Somatic embryogenesis and planregeneration from callus culture of *Acacia catechu*- a multipurpose leguminous tree. Plant Cell. Tiss. Org. Cult., 4: 238-245.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. & Tingey, S.V (1990) DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res., 18: 6531-6535.

Zhao, X.Y., Yao, D.Y. & Harris, P.J.C (1990) In vitro regeneration of plantlets from explants and callus of *A.salicina*. Nitrogen Fixing Tree Res. Rep., 8: 113-115.

Table. 1. Modified MS media along with growth regulators used for the different stages of somatic embryogenesis in *Acacia arabica*.

	Induction medium (mg/L)	Maintenance medium (mg/L)	Maturation & Germination medium (mg/L)
NH_4NO_3	1650	1650	825
KNO_3	1900	1900	950
KH_2PO_4	170	170	85
$CaCl_2.2H_2O$	440	440	220
$MgSO_4.7H_2O$	370	370	185
H_3BO_3	6.2	6.2	3.1
KI	0.83	0.83	0.42
$MnSO_4.4H_2O$	22.3	22.3	11.2
$ZnSO_4.4H_2O$	8.6	8.6	4.3
$Na_2MoO_4.2H_2O$	0.25	0.25	0.12
$CuSO_45H_2O$	0.025	0.025	0.012
$CoCl_2.6H_2O$	0.025	0.025	0.012
Na_2 EDTA	37.2	37.2	18.5
$FeSO_4.7H_2O$	27.8	27.8	14.0
Glycine	20.0	20.0	10.0
Nicotinic acid	0.5	0.5	0.25
Pyridoxine-HCl	0.5	0.5	0.25
Thiamine-HCl	0.1	0.1	0.05
Myo-inositol	100	100	100
Sucrose	3%	3%	2%
BA	2.0	1.5	0.01-0.05
2,4-D	1.5	1.5	----
ABA	---	---	0.1 – 0.25
Agar	0.8%	0.8%	0.8%
pH	5.8	5.8	5.8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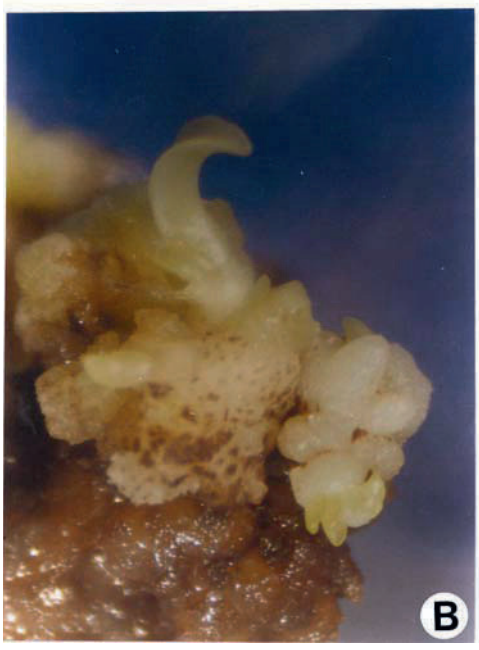
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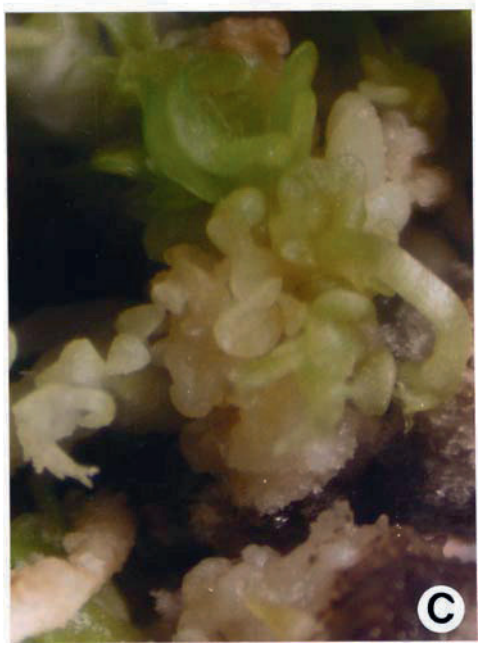
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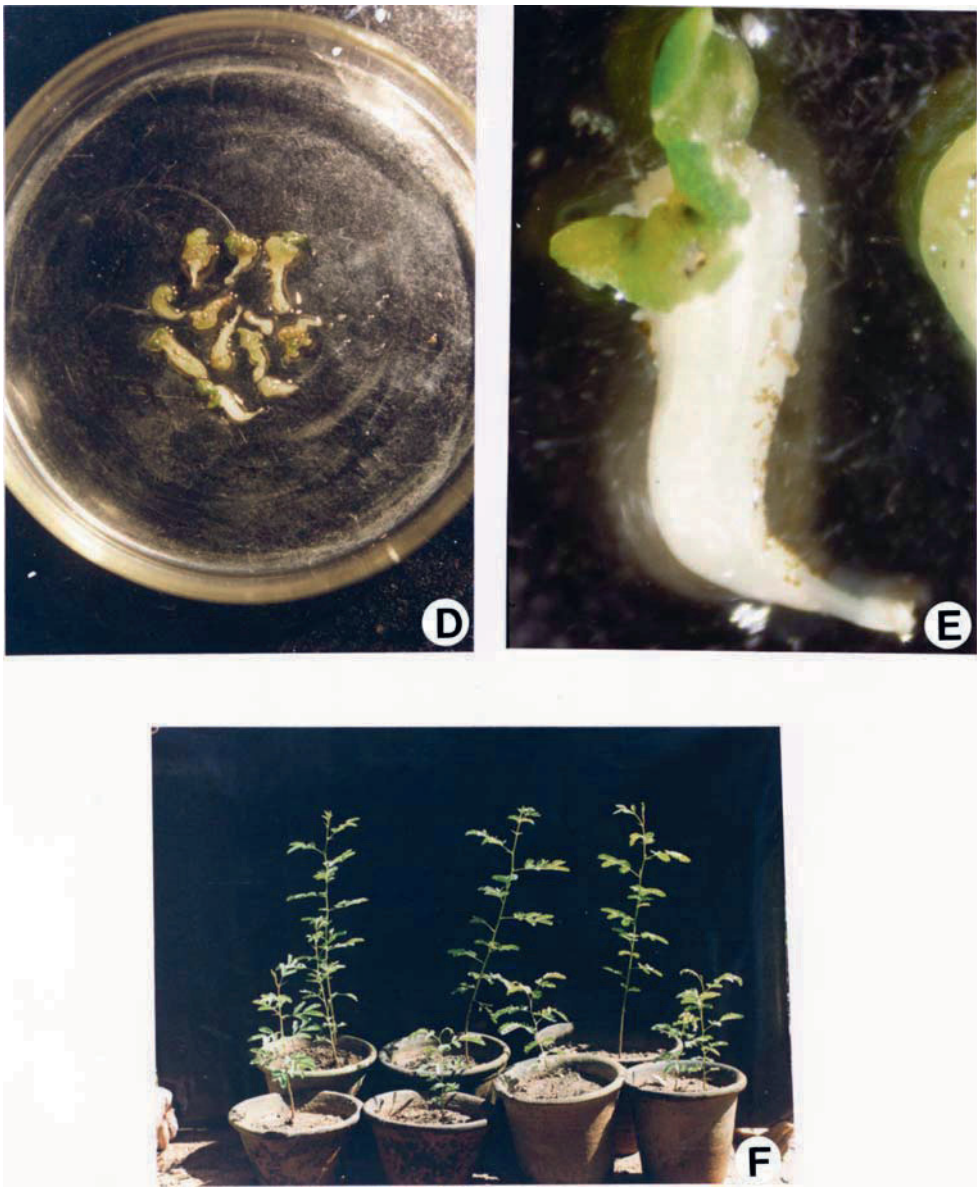

Figure 1. Somatic Embryogenesis in *Acacia arabica*: (A) Development of embryogenic tissue on induction medium. (B & C) Development of group of somatic embryos on maintance medium. (D) Somatic embryos germinated in the maturation and germination medium. (E) Germinated somatic embryo cultured on germination medium. (F) Somatic embryo derived plants growing under greenhouse conditions.

PROTOCOL FOR HAZELNUT SOMATIC EMBRYOGENESIS

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1. INTRODUCTION

Somatic embryogenesis is currently recognized not only as an important pathway to plant regeneration, but also as a model system for the study of early regulatory and differentiation events in plants. The embryogenesis model established from hazelnut (*Corylus avellana* L.*)* tissues offers considerable use for basic research, because it is direct, omitting the callus phase; growth and differentiation processes occur at different times and culture conditions do not need to be changed to promote each phase of development. The use of asexual embryogenesis (Ammirato, 1989) in plants may be considered as a proven method for exploiting totipotency in plants. Thus, in our current research, embryogenesis is considered not only as a parasexual method for plant modification and improvement but also as an ideal experimental process for investigation of plant differentiation as well as a mechanism for the expression of totipotency in plants.

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 413–426.

The induction of somatic embryogenesis in hazelnut callus cultures was reported for the first time in 1975 (Radojevic *et al.* 1975). This process was further improved by using cotyledonary nodes (Pérez *et al.* 1983). A few years later Berros *et al.* (1992) described embryonic axes from immature zygotic embryos as the most suitable embryogenic explant.

Types of inocula and somatic embryogenesis pathways

Asexual somatic embryogenesis induction and regeneration (Fig. 1) was accomplished in *Corylus avellana* L. from several types of organs and cultivars such as var. Casina (I.E.A.P. Villaviciosa. Asturias. Spain) and var. Gironell (IRTA, Tarragona, Spain) [Figs. 2 and 3]. Earlier results indicated that the ability to regenerate plantlets *via* somatic embryogenesis was absolutely dependent on the physiological stage which is linked to the chronological stage. In spite of the variable plant growth regulator combinations utilized, every embryogenic response obtained was performed on immature tissues taken from juvenile phases (Fig. 2); and the induction was dependent on the cultivar and specific genotype used. Our experience (Fig. 3) revealed that embryogenesis in hazelnut is successfully induced when young tissues, which remain not totally differentiated, are reprogrammed by breaking or disrupting their normal differentiating pathway and physiological stage; in these effects, the role played by exogenous regulators, at suitable concentrations, could be the activator of multipotent cells. The tests carried out reveal the existence of a critical period defined as immature fruit stage (Table 1) in which embryogenesis can be obtained with ease to yield a desirable embryogenesis response of 20-40%, that is at the cotyledonary stage when both meristems and the initials of the procambial bundle are not totally defined. However when both meristems are completed and the apical-basal pattern is imposed, the ability of seed tissues to reenter the embryogenic induction programmes greatly decreases.

Plantlet regeneration *via* somatic embryogenesis was successfully accomplished using developing fruits from open-pollinated flowers of *Corylus avellana* L. collected every fifteen days, between July and September (Table 1). Three types of inocula, both immature and mature, were isolated: embryonic axes, proximal and distal cotyledonary portions,

discerned by proximity to the axis position. Resemblances observed through histological procedures in the different developmental stages of induced embryos and zygotic ones confirmed the embryogenic competence of these tissues (Berros *et al.* 1992; 1995).

2. SOMATIC EMBRYOGENESIS INDUCTION

Explants

The most competent inocula for somatic embryogenesis induction are mature and immature zygotic embryos (Radojevic et al.,1975), cotyledonary portions from mature seeds and cotyledonary nodes from mature seedlings (Perez et al.al., Berros et al.,1995).

Medium

The basal induction medium (Table 2) contained the mineral components of Murashige and Skoog's (1962) medium (MS) with vitamins and organic supplements. According to previously-published results (Radojevic et al.1975;Radojevic,1980; Pérez et al. 1983 and Berros et al. 1992), different combinations of regulators could be used: IM 1-6 (Table 2). As reported for other woody species (Tulecke *et al*. 1988, Wang 1988, Krogstrup 1990), induction and development of hazelnut somatic embryos were feasible in the presence of BA but not when Kin alone was used. The addition of 2,4-D to the media along with a low BA concentration increased embryogenic induction in relation to media lacking 2,4-D. These results indicate that, in hazelnut, a combination of cytokinins and auxins may be the most effective inducers of the embryogenic pathway. In spite of embryogenic induction being maximized when 2,4-D was used, two undesirable effects were produced: a decrease in the induction percentage when the number of transferences increased and the formation of a high number of anomalous embryos. Higher concentrations or the accumulation of several doses of 2,4-D may produce alterations in the pre-existing polarity of the inocula utilized; this being one of the most important factors in the differentiation process. Changes in polarity can inhibit factors such as cellular proliferation and embryogenic evolution.

Considering the results obtained under different combinations of plant growth regulators, it is possible to conclude that: in hazelnut, zygotic embryo tissues present an elevated embryogenic/organogenic competence, and a combination of growth regulators condition the induction of one or various development programs. In particular, embryogenic induction was specially favored with BA application. Our results showed that 2,4-D may be the best inducer of embryogenesis from zygotic tissues. However, higher embryogenic induction obtained from cotyledonary leaves in the presence of BA shows that this phythormone is the best inducer of repetitive embryogenesis in this species. In addition, the decline of embryogenic percentages cultured into MS base ?medium? showed that the maintenance of embryogenic cultures depends on the continuous application of BA.

Culture conditions

The medium was distributed in culture vessels (125 ml) containing 25 ml of medium. Six inocula were layered on the induction medium. Growth conditions were 25±2ºC under a 16-h photoperiod (20 μmol m^{-2} s^{-1}) provided by cool-white fluorescent lamps. After 40 days on the cited media, embryogenesis induction was assessed (Table 3) as the percentage of inocula that showed cotyledonary embryos, while the embryogenic features of callus were determined by observing the different developmental embryonic stages at the end of the next subculture.

3. MAINTENANCE OF EMBRYOGENIC TISSUES

Somatic embryos multiplication (Fig. 1a, b) could normally be obtained with ease by culturing embryonic clusters constituted from 4-6 somatic embryos or cotyledonary leaves on the fresh maintenance medium (MM) indicated (Table 2). Embryonic clusters multiply successfully in all media (MM 1, MM 2 and MM 3), but the best rate of continuous proliferation is obtained on MM 2 medium. Lines with a reduced embryogenic ability are better manipulated on MM 3 medium.. While on the latter medium cultures could be multiplied for a long period of time,on MM2 medium

after two-three subcultures callusing interferes with somatic embryogenesis.

Cotyledonary leaves (Fig. 1c, d) isolated from embryonic clusters with different development times (20, 60, 100 and 120 days) facilitate the appearance of secondary embryogenesis on MM 2 media.

(At the beginning the text is written in the past tense, as in a normal scientific paper, but here it is in the present tense, more typical of a protocol. I think you should be more consistent, either one way or the other.)

4. MATURATION AND GERMINATION

Although in some woody species maturation and germination of somatic embryos has been obtained on media lacking phythormones (Roberts *et al*. 1990 a, b; Harry and Thorpe 1991) in most cases the application of a specific treatment is necessary. In this phase, difficulties are frequently encountered due mainly to the great embryogenesis potential of the cultured tissues (Fig. 1b). When one secondary embryo appears, the main problems are: a) heterogeneity of embryo development and, b) control of the embryo multiplication process. The efficiency of maturation treatments is normally evaluated by the decrease in percentage of repetitive embryogenesis and embryo root development. Inhibition rate of embryo maturation is quantified as the percent mean number of white opaque embryos developed without manifestation of precocious germination. Embryos with precocious germination are characterized by the presence of cotyledonary leaves along immature shoots. The true maturation effect is validated through the percentage of germinated embryos on the next subculture. The normal germination rate is quantified as the percentage of emblings with simultaneous root and green shoot development (Fig. 1d).

Isolated cotyledonary embryos or clusters formed by 4-6 embryos approximately 2-3 mm long from 40-day cultures are considered the optimal embryo stage for germination. Frequently, direct germination on MS medium without regulator (Table 2, Mt 1) reveals the immaturity of the embryo stage by abnormal shoot or root manifestation. Maturation of cultures takes place in 125 ml vessels containing 25 ml full strength MS

medium with 3% sucrose and 4μM abscisic acid (ABA) (Table 2, Mt 2) for two weeks. To promote the future germination rate, embryos can be stored on gelled full strength MS medium at 4ºC in the dark for one month (Table 2, Mt 3).

For germination, isolated embryos are placed vertically on solid half-strength MS medium with a light intensity of 30 μmol m^{-2} s^{-1} and 16 h photoperiod (Table 2, G1). Filter-sterilized GA_3 solution to a final concentration of 0,725 μM or 1,45 μM (Table 2, G2) increases the yield of plantlets.

5. TRANSFER TO SOIL

Plantlets regenerated from somatic embryos (Fig. 1d) are transferred to a pot with a soil mix composed of peat and perlite (1:1). Plantlets are developed in the growth chamber under a similar regime of temperature and light as the former cultures. Humidity is maintained by covering the potted plants with plastic bags. Holes of increasing size are made in the bags to reduce humidity levels gradually until the bags are removed after 30 days (Fig.a). After acclimatization, the plants are placed in the greenhouse. The percentage of surviving plants 12 weeks after being transferred to soil is 80 % and these grow as normal plants (Fig. 4b).

6. CONCLUSIONS AND FUTURE PROSPECTS

The technique for direct somatic embryogenesis from immature embryos is fully developed, and applicable to any genetic transformation programme. The use of mature explants greatly reduces the yield of emblings. Therefore, further research is needed mainly on the following three aspects:

1. Synchronisation of responses
2. Maturation of somatic embryos
3. Level of embling production

Indirect morphogenesis, including both caulogenesis and somatic embryogenesis, has been exploited to a lesser extent. Bearing in mind the

detailed knowledge about the physiological (Berros et al.,1994),cytological (Radojevic et al., 1979) and molecular bases (Berros et al.,1994) of morphogenesis and development in hazelnut, while no publications exist on indirect caulogenesis, when reviewing the indirect embryogenesis protocols, it is clear that in the near future these alternatives could be commercially exploited if agricultural demands support the effort. Due to the great ease of performing somatic embryogenesis (Rodriguez et al.,1989,2000), studies on transformation and cryopreservation (gonzales and Perez,1994) are in progress.

Acknowledgments

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REFERENCES

Ammirato, P. 1989. Recent progress in somatic embryogenesis. *Newsletter* (isn't there more to the journal name?) 57;2-16.

Berros, B., Albuerne, M. & Rodríguez, R. 1994. Biochemical and morphological markers in hazelnut asexual embryogenesis. In: J. Pardos, M. Ahuja & R. Rossello (Eds). *Biotechnology of Trees*, *Investigación Agraria, Sistemas y Recursos Forestales 4*, pp. 113-124. INIA, Madrid.

Berros, B., Rey, M., Albuerne, M. & Rodríguez, R. 1992. Characterization of somatic embryo induction in seed of filbert: effect of maturation. *Acta Hort.* 351;341-352.

Berros, B., Rey, M., Díaz-Sala, C., Albuerne, M. & Rodríguez, R. 1995. Somatic embryogenesis in hazelnut (*Corylus* species). In: Y.P.S. Bajaj (Ed). *Biotechnology in Agriculture and Forestry*, pp. 318-334. Springer-Verlag, Berlin.

Berros, B. & Rodríguez, R. 1994. Polyamine and leaf maturity modulate hazelnut somatic embryogenesis. *Biol. Plant.* 36;S 377.

Evans, D., Sharp, W. & Flick, C. 1981. Growth and behaviour of cell cultures. Embryogenesis and organogenesis. In: T.A. Thorpe (Ed). *Plant Tissue Culture: Methods and Applications in Agriculture*, pp. 45-113. Academic Press, New York.

Krogstrup, P. 1990. Effect of culture densities on cell proliferation and regeneration from embryogenic cell suspension of *Picea sitchensis*. *Plant Sci.* 72;115-123.

Gonzales, B. & Perez, C. 1994. Cryopreservation of embryogenic axes of two cultivars (*Corylus avellana* L.). *Cryo. Lett*. 15; 41-46.

Harry, I. & Thorpe, T. 1991. Somatic embryogenesis and plant regeneration from mature zygotic embryos of red spruce. *Bot.Gaz.* 152;446-452.

Murashige, M. & Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15;473-497.

Nomura, K. & Komamine, A. 1986. Molecular mechanisms of somatic embryogenesis. *Oxford Sur Plant Mol. Cell Biol.* 3;456-466.

Pérez, C., Fernández, B. & Rodríguez, R. 1983. *In vitro* plantlet regeneration through asexual embryogenesis in cotyledonary segments of *Corylus avellana* L. *Plant Cell Rep.* 2;226-228.

Pérez, C., Rodríguez, R. & Sánchez-Tamés, R. 1986. Regulation of asexual embryogenesis in filbert cotyledonary nodes. Morphological variability. *Plant Sci.* 45;59-64.

Radojevic, Lj. 1980. Embryogenese somatique et androgenese chez certaines especes ligneuses. *Bull. Soc.Bot. Fr.* 127;99-107.

Radojevic, Lj., Kovoor, J. & Zylberberg, L. 1979. Anatomical and histochemical study of embryogenic callus tissues of *Corylus avellana* L. and *Paulownia tomensa* Steud. *Rev. Cytol. Biol. Veget.Bot.* 2,155-167.

Radojevic, Lj., Vujicic, R. & Neskovic, M. 1975. Embryogenesis in tissue culture of *Corylus avellana* L. *Z Pflanzenphysiol Bd* 77,33-41.

Roberts, D., Flinn, B., Webb, D., Webster, F. & Sutton, C. 1990a. Abscisic acid and indole-3-butyric acid regulation of maturation and accumulation of storage proteins in somatic embryos of interior spruce. *Physiol. Plant* 78;355-360.

Roberts, D., Sutton, B. & Flinn, B. 1990b. Synchronous and high frequency germination of interior spruce somatic embryos following partial drying at high relative humidity. *Can. J. Bot.* 68;1086-1090.

Rodríguez, R., Rodríguez, A., González, A. & Perez, C. 1989. Hazelnut (*Corylus avellana* L.). In: Y.P.S. Bajaj (Ed). *Biotechnology in agriculture and forestry 5*, pp. 127-160. Springer-Verlag, Berlin.

Rodríguez, R., Berros, B., Centeno, M., Rovira, M., Rodríguez, A. & Radojevic, Lj. 2000. Applied and basic studies on somatic embryogenesis in hazel (*Corylus avellana* L) In: S.M. Jain, P. Gupta & R. Newton (Eds). *Somatic Embryogenesis in woody plants*, pp. 291-390. Kluwer Academic Publishers, Dordrecht.

Tulecke, W., McGranahan, G. & Ahmadi, H. 1988. Regeneration by somatic embryogenesis of triploid plants from endosperm of walnut, *Juglans regia* L. *Plant Sci* 7;301-304.

Vujicic, R., Radojevic, Lj. & Kovoor, A. 1983. Effect of some nucleic acid base analogues and inhibitors of protein synthesis on orderly arrangement of ribosomes in callus tissue of *Corylus avellana*. *Biochem. Physiol. Pflanzen* 178; 61-66.

Vujicic, R., Radojevic, Lj. & Neskovic, M. 1976. Orderly arrangement of ribosomes in the embryogenic callus tissue of *Corylus avellana* L. *The Journal of Cell Biol.* 69;686-692.

Wang, S.R. 1988. Somatic embryogenesis in woody species. *Hortic. Rev.* 10;153-181.

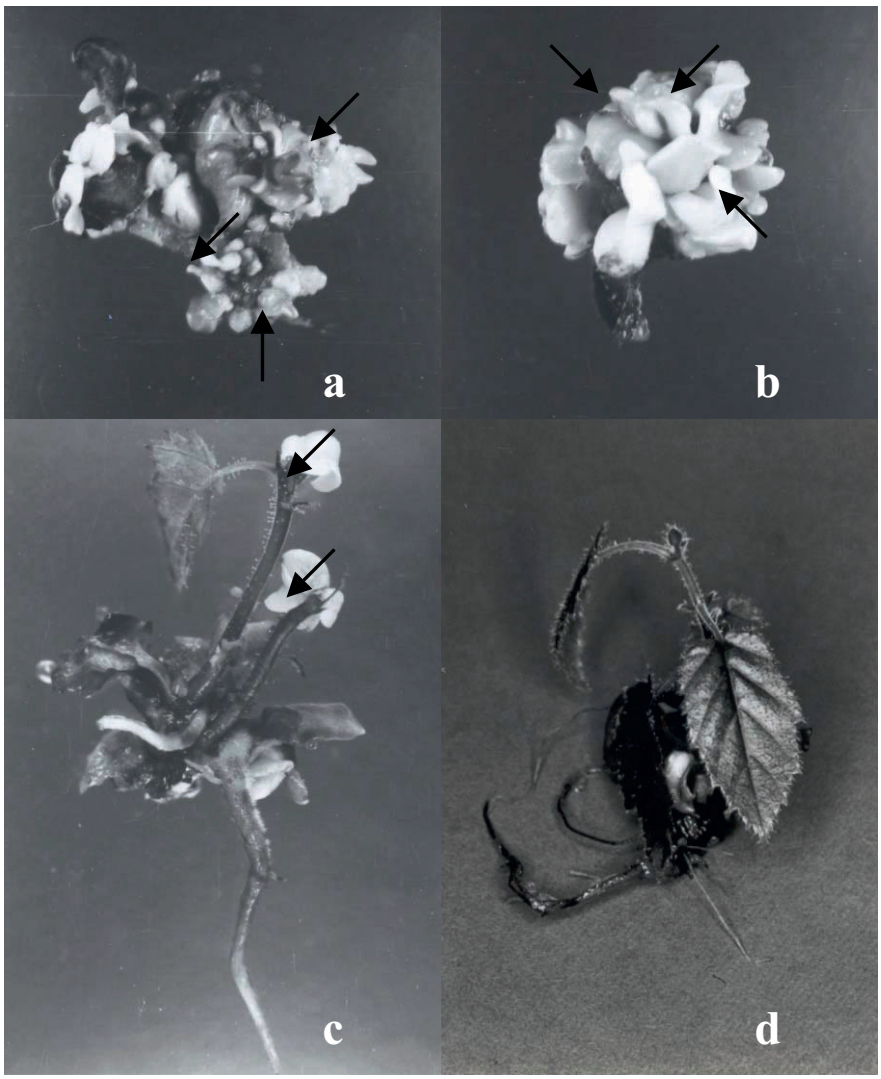

Figure 1. *Corylus avellana* L. somatic embryogenesis phases: a) induction phase. Note the different embryo stages (arrows), b) maintenance of somatic embryogenesis responses (arrows) by cotyledonary leaf subculture, c) immature somatic embryo germination. Note the presence of cotyledonary leaves (arrows) and d) plantlet formation (regeneration) after mature somatic embryo germination.

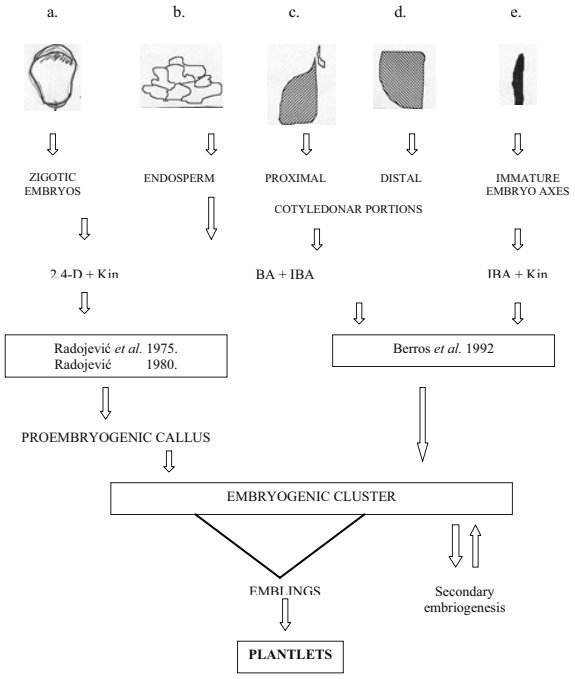

Figure 2. Alternatives to somatic embryogenesis from immature tissues.

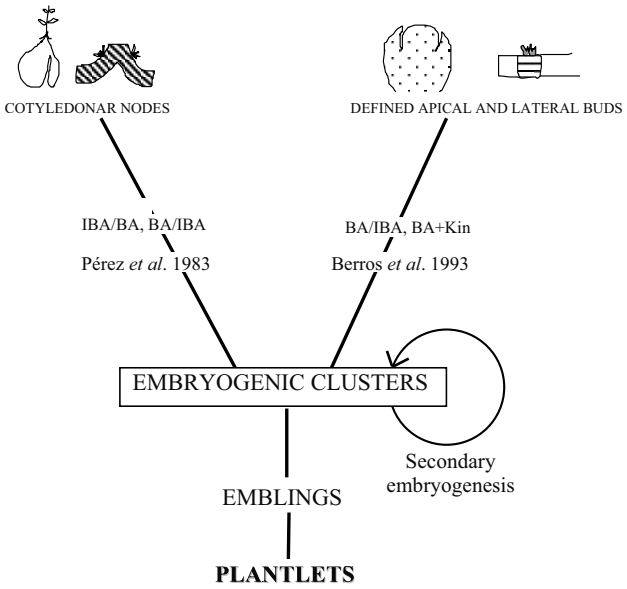

Figure 3. Alternatives to somatic embryogenesis from young tissues.

Figure 4. Hazelnut plant regeneration from somatic embryos: a) 30 days old after transfer to soil and b) 1 year old normal plants.

Table 1. Characterization of hazelnut fruit development phases in relation to embryogenesis induction.

STAGE	PREFRUIT		IMMATURE FRUIT		MATURE FRUIT	
Date	May 29	June22	July 16	August 2	August 20	September 8
Fruit size (mm)	3-6	6-8	8-10	10-12	12	12
Calycine bracts	greenish joined until the prefruit		basally adhered to the fruit		dun and separable with ease from the pericarp	
Carpelar tissue/pericarp	carpelar tissue	not lignified	lignified	lignified and separable with ease from the pericarp		
Carpelar tissue/mesocarp	carpelar tissue	spongy	reabsorbed			
Carpelar tissue/testa	carpelar tissue	pulpy and thick		thin and adhered to the cotyledon		
Zigotic embryo stages	preembryogenic stages		cotyledonary stages		foliar meristems and root cap	
Cellular stages	cellular divisions and cell growth		cellular growth and accumulation of reserves		apparent cellular differentiation	
Pattern of development	radial pattern (formation)		longitudinal pattern (formation & expression)		maintenance of pattern (expresion pattern)	
Embryogenesis induction (%)	low somatic embryo induction: 20,0		high somatic embryo induction: 46,2		low somatic embryo induction: 14,5	

Table 2. Composition of media for hazelnut somatic embryogenesis.

BASAL MEDIUM	MM
Macronutrients	
NH_4NO_3	20.6
KNO_3	18.8
$CaCl_2.2H_2O$	3.0
$MgSO_4.7H_2O$	1.5
KH_2PO_4	1.2
NaEDTA	0.1
Micronutrients	
$FeSO_4.7H_2O$	0.1
KI	5.5
H_3BO_3	100
$MnSO_4.4H_2O$	100
$ZnSO_4.7H_2O$	29.9
$NaMoO_4.2H_2O$	1.0
$CuSO_4.5H_2O$	0.1
$CoCl_2.6H_2O$	0.1
Vitamins and organic supplements	
Inositol	555.1
Nicotinic acid	4.06
Pyridoxine HCl	2.43
Thiamine HCl	0.30
Glycine	26.60
Sucrose 3% (w/v) Agar 7 % (w/v)	
Final pH adjusted to 5.8 before autoclaving	
INDUCTION MEDIUM (IM)	**µM**
IM 1. BA	5
IM 2. BA + IBA	5 + 0.5
IM 3. BA + 2,4-D	0.5 + 0.05
IM 4. BA + 2,4-D	0.5 + 0.005
IM 5. BA + 2,4-D	0.5 + 0.45
IM 6. 2,4-D + Kin	0.5 + 0.5
MAINTENANCE MEDIUM (MM)	**µM**
MM 1. Basal medium deprived of growth regulator	
MM 2. BA	5
MM 3. BA + 2,4-D	5 + 0.05
MATURATION (Mt)	**µM**
Mt 1. Basal medium deprived of growth regulator	
Mt 2. ABA	4
Mt 3. Stored at 4 ˚C in the dark for 1 month	
GERMINATION (G)	**µM**
G 1. Under a light intensity of 30 µmol m^{-2} s^{-1}16 h photoperiod	
G 2. GA_3	0.725 or 1.45

Table 3. Embryogenic responses quantified according to the minimal induction period and percentage of inoculum responses.

Ontogenic Stage	Tissues	Induction Media	Induction Period (days)	Embryogenesis (%)
Prefruit	Endosperm	IM 1 – 6	60-140	20.0
Immature	Embryonic Axe	IM 1 – 6	20-40	20.0-46.2
Fruit	Proximal Cotyledonar Portions	IM 1 – 6	20-40	14.3-16.0
	Distal Cotyledonar Portions	IM 1 – 6	20-40	0.0-4.0
Mature Fruit	Proximal Cotyledonar Portions	IM 1 – 6	40	0.0-8.2
	Distal Cotyledonar Portions	IM 1 – 6	40	0.0-14.5

PROTOCOL OF SOMATIC EMBRYOGENESIS: Ocotea catharinensis Mez. (Lauraceae)

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1. INTRODUCTION

The Atlantic Forest located in the South of Brazil is considered the most endangered ecosystem in the world with only 5% of the original forest remaining. *Ocotea catharinensis* as many other species of the Lauraceae has economic importance for its highly valued hard wood and for the production of essential oils and important biologically active compounds such as the lignans and neolignans (Sakita and Yatagai, 1992). The erratic flowering, limited seed viability and slow growth are the main limitations to commercial production and artificial regeneration (Carvalho, 1994). The high frequency somatic embryogenesis system developed for this species consists of the following phases: induction of embryogenic cell aggregates from the root end of excised mature zygotic embryos, differentiation of cell aggregates to the globular and early cotyledonary stage embryoids, embryoid maturation and germination after embryo desiccation. The partial desiccation treatments increased the germination rates but the frequencies of embryo conversion into complete plants were still low (Viana, 1998). The system developed has been deployed in phytochemical, biochemical, cytochemical and *in vitro* conservation studies (Lordello, 1996; Catarina, 2001; Catarina et al., 2003).

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 427–443.

2. EMBRYOGENIC CULTURE INITIATION AND MAINTENANCE

2.1. Zygotic embryo culture and induction of somatic embryogenesis

Materials and equipment required: mature fruits**,** sterile distilled water**,** 40% (v/v) commercial bleach, 0.1% (v/v) Tween 20, flow hood, sterilized filter paper, Petri dishes, forceps and scalpel, 76 x 76 mm sterilized transparent polypropilene film, rubber bands, culture media (Table 1).

Protocol 1

1. Collect mature fruits at the end of the growing season and store them in the dark at 4-8 °C until use.
2. Wash the fruits under running tap water for 10 min.
3. Place the fruits in a 100-ml beaker, cover them with 50 ml of 40% v/v commercial bleach containing 1-2 drops of Tween-20 detergent and agitate for 20 min.
4. Rinse the fruits five times with sterile distilled water in a flow hood.
5. Remove the fruit and the seed coat with scalpel and forceps and isolate the zygotic embryo (1-2 mm long) from the cotyledon.
6. Place one zygotic embryo-explant/culture tube (25 x 150 mm) horizontally into the induction medium (M1).
7. Seal the culture tubes with polypropylene film and incubate cultures at 25±2°C, under a 16-h photoperiod and photon flux of 20-23 μmol m^{-2} s^{-1} for eight weeks.

Protocol 2

Follow the same steps as in protocol 1 but using the induction medium M2 (Table 1).

Yellow masses of friable callus (Figure 1A) consisting of cell aggregates as well as globular structures and somatic embryos at early cotyledonary stage proliferated from the radicle end of mature zygotic embryos within eight weeks of culture on the induction medium. The average frequency of embryogenic tissue formation in *O. catharinensis* is 13%.

2. 2. Culture medium composition

The composition of the media employed in all protocols of the somatic embryogenic system of *O. catharinensis* is shown in Table 1. The media used were MS (Murashige and Skoog, 1962), WPM (Woody Plant Medium) (Lloyd and McCown, 1981) or Gamborg B5 (Gamborg et al., 1968). Add all hormones before autoclaving. Adjust media to pH 5.8 before autoclaving. Sterilize media by autoclaving for 18 min at 110 kPa, 121 °C.

2.3. Multiplication of embryogenic cultures at the cell aggregate stage on semi-solid medium

1. Remove samples (each of 80 mg fresh weight) of the yellow friable callus produced according to the protocols described in item 3 above and transfer into the multiplication medium M3.
2. Seal the culture tubes with polypropylene film and incubate cultures at 25±2°C, under a 16-h photoperiod and photon flux of 20-23 μmol m^{-2} s^{-1} for four weeks.
3. Subculture in four-week intervals.
4. Determine the growth increase (g.i.) of the cultures as the increase of fresh weight within one-week intervals [g.i. = (final weight minus initial weight) divided by initial weight].
5. Determine the cell viability of the cultures within one-week intervals as described in item 6 bellow.

The yellow friable callus (Figure 1A) reaches maximum fresh weight increase (4-fold the initial fresh weight) after four weeks of culture. The maximum cell viability of the cultures is achieved in the second week of culture.

2.4. Initiation of cell suspensions from embryogenic cell aggregates

Materials and equipment required: 125 ml conical flasks, horizontal rotatory shaker, graduate 15 ml centrifuge tubes, centrifuge.

1. Collect samples (each of 1 g fresh weight) of the yellow friable callus produced by four-week-old cultures as described in item 4 and transfer into the medium M4.
2. Seal the culture flasks with polypropylene film.
3. Incubate the cultures at 25±2°C, under a 16-h photoperiod and photon flux of 20-23 μmol m^{-2} s^{-1} for four weeks, on a rotatory shaker at 100 r.p.m.
4. Subculture in four-week intervals transferring 5 ml aliquots of the cell suspension to 20 ml fresh medium/flask.
5. Determine the growth increase (g.i.) of the cultures using either the packed cell volume method or as the increase of fresh weight within

one-week intervals [g.i. = (final weight minus initial weight) divided by initial weight].

6. Determine the cell viability of the cultures within one-week intervals as described in item 6 bellow.

The cell suspension culture consists of proembryogenic structures and has a yellow color. The maximum fresh weight increase (1.5-fold the initial fresh weight) occurs at the fourth week of culture. The maximum cell viability of the cultures is achieved in the second week of culture.

2.5. Viability test of embryogenic cultures at the cell aggregate stage

Materials and equipment required: 0.6% (w/v) 2,3,5-triphenyl tetrazolium chloride (TTC) in 0.05 M Na_2HPO_4/KH_2PO_4 buffer pH 7.4, 95% (v/v) ethanol, waterbath, centrifuge, spetrophotometer,

1. Pour the content of one flask containing the cell suspension developed in 2.3 into a centrifuge tube.
2. Centrifuge the content at 2000 *g* for 5-10 minutes.
3. Remove samples of cell aggregates (each of 50 mg fresh weight) and transfer to test tubes.
4. Add 3 ml of the TCC solution to the cells.
5. Seal the test tubes and incubate overnight at 25°C in the dark.
6. Add 7 ml of 95% ethanol to the tubes in 5 and incubated them for 7 min in a boiling water bath.
7. Centrifuge the suspension twice at 2000 *g* during 6 minutes.
8. Determine the absorbance of the supernatant at 490 nm.
9. Express the viability as $absorbance_{490\ nm}$/mg fresh weight.

The cell viability of cultures grown on semi-solid or liquid medium can be assessed weekly (using samples of cell aggregates with the same fresh weight) and the comparison of the values of $absorbance_{490\ nm}$/mg fresh weight shows a pogressive decline after the second week of culture. This protocol was adapted from Benson (1994) and measures spectrophotometrically the red pigment (formazan) produced by the reduction of TTC by the dehydrogenase activity of viable cells in a known fresh weight.

2.6. Maintenance of embryogenic cultures at early cotyledonary stage by repetitive embryogenesis

1. Collect samples (each of 12 early cotyledonary somatic embryos) produced from zygotic embryos according to described in 2.1 and transfer to medium M5.
2. Seal the culture tubes with polypropylene film and incubate the cultures at 25±2°C, under a 16-h photoperiod and photon flux of 20-23 μmol m^{-2} s^{-1} for four weeks.
3. Subculture in four-week intervals.
4. Determine the growth increase (g.i.) of the cultures as the increase of fresh weight and dry weight after 4 weeks [g.i. = (final weight minus initial weight) divided by initial weight].
5. Determine the percentages of somatic embryos at globular, early cotyledonary, cotyledonary and mature stages.

The growth increase of the cultures is 25.2-fold the initial fresh weight and 20.2-fold the initial dry weight after the fourth week of culture. The early cotyledonary somatic embryos undergo repetitive embryogenesis (Figure 1B) and develop throughout the culture period producing embryos at different developmental stages (Figure 1C). On the basis of their size and morphology the embryos can be classified as globular (≤ 2 mm), early cotyledonary (2-3 mm), cotyledonary (3.1-4.9 mm) and mature (≥ 5 mm). After 4-week culture the percentages of somatic embryos at cotyledonary and mature stage increase to 28% and 10% respectively (Catarina et al., 2003).

3. EMBRYO DEVELOPMENT

During maturation, the somatic embryos of *O. catharinensis* expand due to water uptake and accumulate higher levels of soluble sugars and starch. Mature somatic embryos present higher water content than those at the globular stage (Catarina et al., 2003). Embryo development can be monitored by cytochemical and biochemical methods for proteins, carbohydrate, lipids and enzymes such as glucose-6-phosphate, esterases, acid phosphatases, peroxidases and polyphenoloxidases (Viana and Mantell, 1999).

3.1. Staining for carbohydrates, proteins and lipids

Protocol 1

Material and equipment required: 2.5% paraformaldehyde in 0.2 M phosphate buffer pH 7.3 (1:1), phosphate buffer, EtOH (30, 50, 70, 90 and 100% v/v), hystoresin, Coomassie R250, Schiff Periodic Acid, Toluidine Blue, Alcian Blue, Anilin Blue, Sudan Black B, light microscope.

1. Fix somatic embryos at globular, early cotyledonary, cotyledonary and mature stages grown on medium M5 with 2.5% paraformaldehyde in 0.2 M phosphate buffer pH 7.3 (1:1) for 24 h at 4°C (Bouzon, 1993).
2. Wash the tissue samples twice in phosphate buffer for 15 minutes.
3. Dehydrate in a series of EtOH (30, 50, 70, 90 and 100% v/v) (two changes of 15 minutes in each concentration) at 25°C.
4. Pre-infiltrate tissue samples with (1:1) 100% EtOH and hystoresin for 12 h and infiltrate with 100% hystoresin for 24 h.
5. Embed tissue samples in at 25°C.
6. Prepare thin sections (4 μm) using a microtome.
7. Stain sections with either:
    - Toluidine Blue (for acid polysaccharides): 0.5% solution at pH 1.0 for 30-60 seconds (O'Brien et al., 1965).
    - Alcian Blue (for sulfated acid polysaccharides): 1% (w/v) at pH 0.5 for 24 h (La Claire & Dawes, 1976).
    - Schiff Periodic Acid (for neutral polysaccharides): 1% aquous periodic acid solution for 20 minutes, wash for 10 minutes and stain with Schiff reagent for 30 minutes (Bouzon, 1993).
    - Coomassie Brilliant Blue R250 (for total proteins): 0.02% (w/v) Clarke's acidified ethanolic solution for 24 h (Gahan, 1984).
    - Aniline Blue (for observations on cell morphology and somatic embryo structure): 0.1% aquous solution acidified with 1 N HCl (Bouzon, 1993).
8. Examine sections under a light microscope.

For lipid observation:

1. Fix somatic embryos at globular, early cotyledonary, cotyledonary and mature stages with 2.5% paraformaldehyde in 0.2 M phosphate buffer pH 7.3 (1:1) for 24 h at 4°C.
2. Wash the tissue samples twice in (1:1) solution of distilled water and phosphate buffer for 15 minutes.
3. Pre-infiltrate tissue samples with (1:1) solution of distilled water and hystoresin for 12 h and 100% hystoresin for 24 h at 4°C (Soares, 2000).
4. Embed tissue samples in hystoresin at 25°C.
5. Prepare thin sections (4 μm) using a microtome.
6. Stain sections with Sudan Black B (for 15-30 minutes) (Gahan, 1984).

7. Examine sections under a light microscope.

Protocol 2

Material and equipment required: Napthol Yellow S, Oil Red O (Sudan Black B, cryostat, light microscope.

1. Prepare sections 20μM thick of fresh somatic embryos at the globular, early cotyledonary, cotyledonary and mature stages using a cryostat (-28°C).
2. Stain sections with Napthol Yellow S (for proteins), Oil Red O (for lipid droplets) and Sudan Black B (total lipids) according to the methods described by Gahan et al. (1967) and Gahan (1984).
3. Examine sections under a light microscope.

The cytochemical staining with aniline blue shows the mature somatic embryos have two well-formed cotyledons, radicle, vascular bundles and an apical meristem not completely developed (Figure 2A). Starch and lipids are the main storage compounds accumulated in the cotyledon cells of somatic embryos at early cotyledonary and mature stages (Figures 2BC). Somatic embryos at globular and early cotyledonary stages show higher levels of proteins. Secretory cells are present in the cotyledons of the somatic embryos at all stages of development and give positive staining for neutral and acid polysaccharides, lipids and proteins (Figure 2D) (Viana and Mantell, 1999; Catarina, 2001).

3.2. Staining for enzymes

Material and equipment required: cryostat, light microscope.

4. Prepare sections 20μM thick of fresh somatic embryos at the globular, early cotyledonary, cotyledonary and mature stages using a cryostat (-28°C).
5. Stain sections for glucose-6-phosphate dehydrogenases, esterases, acid phosphatases, peroxidases and polyphenoloxidases according to the methods described by Gahan et al. (1967) and Gahan (1984).
6. Examine sections under a light microscope.

Test for esterases indicates that the secretory cells contain phenolics. The secretory cells show intense activity for peroxidases and polyphenoloxidases. The activity of peroxidases is especially high in the dehydrated and germinated somatic embryos. The activity of glucose-6-phosphate

dehydrogenases is intense in the somatic embryos at globular and early cotyledonary stages. The epidermal and subepidermal cells of mature somatic embryo cotyledons show strong glucose-6-phosphate dehydrogenase activity (Viana and Mantell, 1999).

4. EMBRYO GERMINATION

1. Collect mature somatic embryos from embryogenic culture grown on medium M5.
2. Transfer the somatic embryos aseptically to Petri dishes (15 x 60 mm) containing two layers of sterilized filter paper.
3. Seal the Petri dishes with PVC film and keep at 25°C in darkness at 70% RH for 4 days.
4. Transfer the somatic embryos to baby food culture jars (72 x 55 mm) containing medium M6.
5. Seal the jars with polypropilene film.
6. Incubate the cultures at 25±2°C, under a 16-h photoperiod and photon flux of 20-23 μmol m^{-2} s^{-1} for 4 weeks.
7. Assess embryo germination (radicle protrusion) after 4 weeks.

A minimum decrease in fresh weight (12-15%) is necessary to trigger germination of the somatic embyos but moderate dehydration for 4 days in Petri dishes (Figure 1D) producing moisture losses of 25% stimulates the highest germination percentages (30-40%) (Figure 3A) (Viana, 1998).

5. *IN VITRO* CONSERVATION

5.1. Short term *in vitro* conservation

1. Collect mature somatic embryos from embryogenic culture grown on medium M5.
2. Transfer the somatic embryos aseptically to Petri dishes (15 x 60 mm) (twelve somatic embryos per dish) containing two layers of sterilized filter paper.
3. Seal the Petri dishes with PVC film and keep at 25°C in darkness at 70% RH for 4 days.
4. Transfer the somatic embryos aseptically to empty sterilized test tubes (25 x 100 mm) (twelve somatic embryos/tube) and close with polypropilene caps.
5. Seal the test tubes with PVC film and keep at 25°C in darkness at 70% RH for 12 weeks.

6. Transfer the somatic embryos to baby food culture jars containing medium M7.
7. Seal the jars with polypropilene film.
8. Incubate the cultures the cultures at 25±2°C, under a 16-h photoperiod and photon flux of 20-23 μmol m^{-2} s^{-1} for four weeks.
9. Assess embryo germination (radicle protrusion) and repetitive embryogenesis after 4 weeks.

The dehydrated somatic embryos can be stored for twelve weeks showing 25% germination and 40% of the mature somatic embryos show repetitive embryogenesis (Figure 3B). The site of repetitive embryogenesis from mature somatic embryos is the root pole as shown in Figure 2A (Viana, 1998; Viana and Mantell, 1999).

5.2. Cryoprotective air dehydration

1. Collect early cotyledonary and cotyledonary somatic embryos from embryogenic cultures grown on medium M5.
2. Transfer the somatic embryos aseptically to sterilized open Petri dishes, over two filter paper layers (24 somatic embryos per dish).
3. Dehydrate the somatic embryos in a running flow laminar cabinet at 25°C and 70% RH for periods varying from 0 to 8 hours, and in Petri dishes from 0 to 6 days.
4. Transfer the early cotyledonary and cotyledonary somatic embryos, respectively to medium M5 and M7.
5. Seal the culture tubes with polypropylene film and maintain the cultures at 25±2°C, under a 16-h photoperiod and photon flux of 20-23 μmol m^{-2} s^{-1} for 8 weeks.
6. Determine levels of survival assessing the cultures weekly during 8 weeks for the frequencies of somatic embryos showing repetitive embryogenesis.

Replicate samples should be enough to determine the tissue fresh and dry weights before and after the step 3. The early cotyledonary somatic embryos tolerate 95% fresh weight loss, after 6 days of slow dehydration in Petri dishes, without losing the repetitive embryogenesis potential (85%). The cotyledonary somatic embryos tolerate extreme fresh weight losses (86%), after 6 hours dehydration in the flow cabinet, without losing their repetitive embryogenesis potential (15%) (Figure 3C). The treatment, gives maximum dehydration and survival, is selected for pre-freeze dehydration.

5.3. Encapsulation dehydration

1. Collect cotyledonary somatic embryos from embryogenic cultures grown on medium M5.
2. Transfer 2 g fresh weight of somatic embryos aseptically to the conical flask containing 20 ml WPM liquid medium and 3 g l^{-1} sodium alginate, then swirl to ensure the complete submersion of the embryos.
3. Using a 100-1000 µl adjustable digital pippet collect aliquots of the alginate solution with several somatic embryos.
4. Dispense drops of the alginate solution into the conical flask containing WPM liquid medium and 100 mM $CaCl_2$.
5. Allow the beads to polymerize for 30 minutes.
6. Transfer the alginate encapsulated somatic embryos to the conical flasks containing medium M8 (twelve somatic embryos per flask).
7. Seal the conical flasks with polypropylene film and maintain the cultures in a rotatory shaker at 100 rpm, at 25±2°C, under a 16-h photoperiod and photon flux of 20-23µmol m^{-2} s^{-1} for 1, 4 and 8 days.
8. After 1, 4 and 8 days of incubation remove the somatic embryos and dehydrate them in a running laminar flow cabinet at 25°C and 70% RH for 1 and 2 hours, over filter paper, in open Petri dishes.
9. Transfer the encapsulated somatic embryos to culture tubes containing medium M7.
10. Seal the culture tubes with polypropylene film and incubate the cultures at 25±2°C, under a 16-h photoperiod and photon flux of 20-23µmol m^{-2} s^{-1} for 1, 4 and 8 days.
11. Determine levels of survival assessing the cultures during 8 weeks for the frequencies of somatic embryos showing repetitive embryogenesis.

Replicate samples should be enough to determine the beads fresh and dry weights after the steps 5 and 8. The alginate encapsulated dehydrated somatic embryos survival (ca. 47%) (Figure 3D) occurs at extreme exposures to 1.5 M sorbitol for 4 days combined with 1hour air dehydration. The lethal treatments are the exposures to 1.5 sorbitol for 8 days combined with 2 hours dehydration, and to either 1 or 1.5 M sorbitol for 4 days with 2 h dehydration. The treatment, which gives maximum survival and maximum water loss is selected for pre-freeze dehydration.

6. CONCLUDING REMARKS

The somatic embryogenesis system developed for *O. catharinensis* has two main important features which focused our attention: 1- the ability of the somatic embryos, at any developmental stage to undergo repetitive embryogenesis and produce either globular or cotyledonary somatic embryos *de novo* in the absence of plant growth regulators, 2- the potential of the system to be used for plant production and secondary metabolite studies. In view of the difficulties of obtaining new seeds to initiate new cultures our main goal was to optimize protocols to ensure the continuous availability of embryogenic cultures for the subsequent studies. At the same time efforts were devoted to overcome the difficulties encountered to optimize somatic embryo germination and plant conversion, which directed our research to understand morphological and physiological aspects of this system, concerning the cytochemical and morphological characterization of the somatic embryos at different developmental stages and to the development of desiccation protocols. The tolerance of the somatic embryos to desiccation and their ability to undergo repetitive embryogenesis opened up the windows to investigate a possible approach to use this feature to develop *in vitro* conservation systems either using solely desiccation or combining it with cryopreservation strategies. Research in our laboratory is presently also oriented toward this goal. Parallel to these studies the preliminary studies on essential oils and on neolignans carried out by Lordello (1996) leaded us presently to optimize the development of cell suspension at cell aggregate and cotyledonary stages, which will assist the needs not only for the studies on biosynthesis, identification and quantification of essential oils but also the large scale production of somatic embryos. Preliminary studies on producing cell suspension in bioreactors were conducted by Moura-Costa (1992). Our current goal is to improve the system. The research carried out on the *O. catharinensis* somatic embryogenesis system was profitable as it helped to develop very complete protocols for induction, maintenance of embryogenic tissues, development of cell suspension, cytochemical characterization, germination, short term conservation and desiccation which are directly applicable to other *Ocotea* species.

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REFERENCES

Benson EB (1994) Cryopreservation. In: Dixon RA, Gonzales RA (eds) Plant Cell Culture A Pratical Approach (pp 147-168). IRL Press, Oxford.

Carvalho PER (1994) Espécies Florestais Brasileiras. Recomendações Silviculturais, Potencialidades e Uso da Madeira. Embrapa-CNPF/SPI, Brasília.

Catarina SC (2001) *In vitro* growth, biochemical, morphological and hystochemical characterization of embryogenic cultures of *Ocotea catharinensis* Mez. (Lauraceae). MSc. Thesis, Federal University of Santa Catarina, Florianópolis, SC, Brazil (in Brazilian Portuguese with English summary)

Catarina SC, Randi AM & Viana AM (2003) Growth and accumulation of storage reserves by somatic embryos of *Ocotea catharinensis* Mez. (Lauraceae). Plant Cell Tiss. Org. Cult. 74: 67-71

Bouzon, ZL (1993) Hystochemical and ultrastructure of *Hypnea musciformis* (Gigartinales - Rodophyta). MsC. Thesis, Federal University of Paraná, Curitiba, PR, Brazil (in Brazilian Portuguese with English summary)

Gahan PB (1984) Plant Histochemistry and Cytochemistry. New York: Academic Press.

Gahan PB, McLean J, Kalina M & Sharma W (1967) Freeze sectioning of plant tissues: the technique and its use in plant histochemistry. J. Exp. Bot. 18: 151-159

Gamborg OL, Miller RA & Ogima K (1968) Plant Cell Cultures. I. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158

La Claire JW & Dawes CJ (1976) An autoradiographic and histochemical localization of sulphated polysaccharides in *Eucheuma nudum* (Rodophyta). J. Phycology 12: 368-375

Lloyd G & McCown B (1981) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture. Intl. Plant Prop. Soc. Proc. 30: 421-427

Lordello ALL (1996) Chemical constituents from leaves, tissue and cell cultures of *Ocotea catharinensis* Mez. (Lauraceae). PhD. Thesis, University of São Paulo, São Paulo, SP, Brazil (in Brazilian Portuguese with English summary)

Maruyama E, Kinoshita I, Ishii K and Ohba K (1997a) Germplasm conservation of the tropical forest trees *Cedrela odorata* L., *Guazuma crinita* Mart. and *Jacaranda mimosaefolia* D. Don. by shoot tip encapsulation in calcium-alginate and storage at 12-25° C. Plant Cell Reports 16: 393-396

Moura-Costa PM (1992) Somatic embryogenesis and plant regeneration of *Ocotea catharinensis* Mez. (Lauraceae), an endangered forest tree of S. Brazil. PhD. Thesis, University of London (Wye College), UK

Moura-Costa PM, Viana AM & Mantell SH (1993) *In vitro* plantlet regeneration of *Ocotea catharinensis*, an endangered Brasilian hardwood forest tree. Plant Cell Tiss. Org. Cult. 35: 279-286

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473- 497

O'Brien TP, Feder N & McCully ME (1965) Polycromatic staining of plant cell walls by toluidine blue O. Protoplasma 59(2): 368-373

Sakita MN & Yatagai M (1992) Essential oil of bark of *Ocotea catharinensis* Mez. (Lauraceae). Rev. Inst. Flor. (4): 684-687

Viana AM. 1998. Somatic embryogenesis in *Ocotea catharinensis* Mez. (Lauraceae). In: Bruns S, Mantell SH, Tragardh C, Viana AM (eds) Recent Advances in

Biotechnology For Tree Conservation and Management (pp 244-253). International Foundation for Science, Stockholm

Viana AM & Mantell SH (1999) Somatic embryogenesis of *Ocotea catharinensis*: an endangered tree of the Mata Atlantica (S. Brasil). In: Jain SM, Gupta PK, Newton RJ (eds) Somatic Embryogenesis in Woody Plants, Vol 5 (pp 3-30). Kluwer Academic Publishers, Dordrecht

Table 1. Composition of culture media used for different purposes in the somatic embryogenic system of *Ocotea catharinensis*.

Purpose	**Medium**	**Sucrose ($g.l^{-1}$)**	**PGRs type**	**PGRs ($mg.l^{-1}$)**	**AC ($g.l^{-1}$)**	**Glutamine ($mg.l^{-1}$)**	**Sorbitol ($g.l^{-1}$)**	**Phytagel ($g.l^{-1}$)**
Induction (M1)	MS	20	-	-	3	-	-	2
Induction (M2)	MS	20	2,4-D	4	-	-	-	2
Cell aggregate Multiplicatio n (M3)	MS	20	2,4-D	40	3	-	-	2
Cell suspension (M4)	½ MS	20	2,4-D	0.5	-	-	-	-
RE from early cotyledonary SEs and maturation (M5)	WPM	20	-	-	-	400	22.7	2
Germination (M6)	½ B5	20	GA+ NAA	40	1.5	-	-	2
RE from cotyledonary SEs (M7)	½ WPM	20	-	-	1.5	400	-	2
Chemical dehydration (M8)	WPM	20	-	-	-	-	0 – 364	-

RE = repetitive embryogenesis

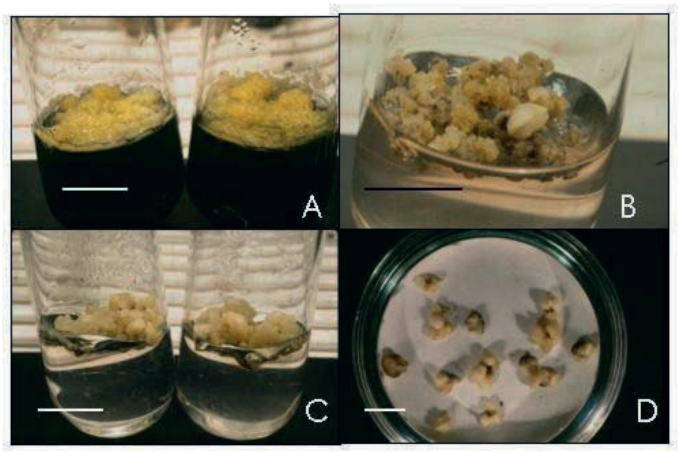

Figure1A-D. Embryogenic friable callus of *Ocotea catharinensis* obtained from mature zygotic embryos grown on M1 medium (A). Proliferation of embryogenic tissue at globular, early cotyledonary, cotyledonary and mature stages on medium M5 (B, C). Mature somatic embryos desicccated in Petri dish (D). (Bar 1 cm).

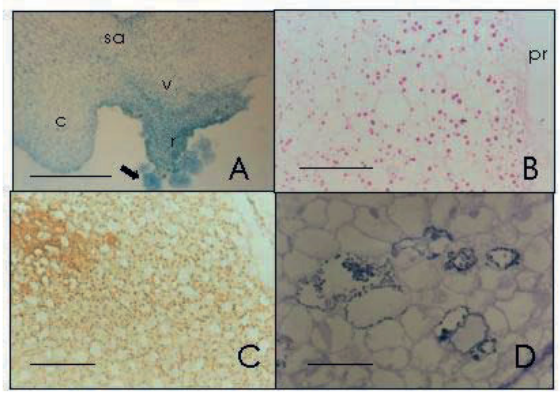

Figure 2A-D. Longitudinal sections of somatic embryos of *Ocotea catharinensis* after staining with Aniline Blue (A, note repetitive embryogenesis from mature somatic embryos associated with the root cap), PAS (B, note the distribution of starch grains in epidermal and cotyledon cells), Sudan Black B (C, note lipid droplets in the cotyledon cells) and Coumassie Brilliant Blue (D, note the accumulation of protein granules in the secretory cells and positive reaction in the cytoplasm of cotyledon cells). (c, cotyledon; v vascular tissue; r, radicle; sa, apical meristem; p, epidermis). Bar 500μm (A). Bar 100μm (B,C,D).

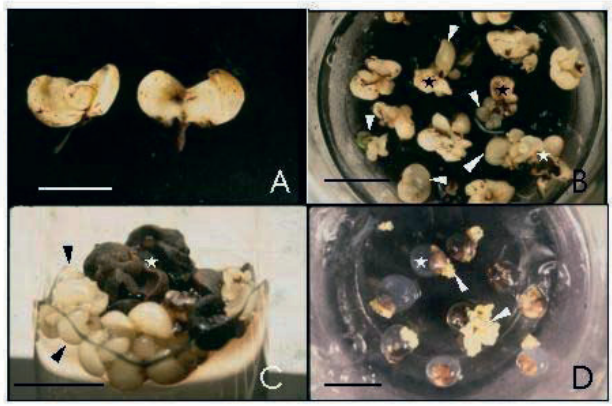

Figure 3A-D. Geminated somatic embryos of *Ocotea catharinensis* on M6 medium (A). Repetitive somatic embryogenesis (arrows) from desiccated mature somatic embryos (*) stored for 12 weeks (B). Repetitive somatic embryogenesis (arrows) from cotyledonary somatic embryos (*) dehydrated for 6 hours in the flow cabinet (C). Repetitive somatic embryogenesis (arrows) from alginate encapsulated cotyledonary somatic embryos (*) after chemical dehydration and air dehydration in the flow cabinet (D). (Bar 1 cm).

CORK OAK, *QUERCUS SUBER* L.

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1. INTRODUCTION

The cork oak species belongs to the Section *Suber* of the subgenus *Cerris*, genus *Quercus*, which is one of the most important in forestry. This genus is also a member of the family *Fagaceae* and includes other genus, *Castanea* and *Fagus*, equally important for the Northern Hemisphere (Ceballos and Ruiz de la Torre, 1979). This species is extremely polymorphic, endemic to the Western Mediterranean region, growing in different European and African countries and territories: Portugal, Spain, South France, Morocco, Algeria, Tunisia, Corsica, Sardinia, Italy, and Slovenia. It stretches in latitude between South Mogador (Morocco) at 31ºN and South France at 44ºN; in longitude between Portugal at 9º10'W to Slovenia at 15ºE (Ceballos and Ruiz de la Torre, 1979). At present, cork oak covers about 2.35 million ha, most of them in Portugal (676000 ha), Spain (500000 ha), Algeria (480000 ha) and Morocco (400000 ha) (Montoya, 1988).

The cork oak is a medium size, evergreen tree with a broad round crown that reaches up to 20 m tall, rarely up to 25 m. Its most distinctive characteristic is a secondary meristem, the phellem, produces cork in trunk and branches. The bark of the cork oak is composed of death cells with trapped air, whose walls contain high amounts of suberin that confer cork very good gas and water insulating properties. The tree continuously generates abundant cork that can be stripped without severe damage at regular intervals, usually each nine years, to provide commercial cork. Therefore, apart of it great ecological value for the Mediterranean ecosystem, the cork oak is the source of cork, a natural and renewable product of economic interest for diverse applications. Entire cork pieces are used in wine bottling: high quality wines that have to be aged in the bottle for many years require this high-quality closure. Heated ground cork can be manufactured to form tiles and carpets used in floor covering due to their pleasant

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 445–457.

elastic feel and sound absorbing properties (Anonymous, 2000). Novel applications of cork include the use of by-products generated during the processing of corkboards: some of them have been reported to act as inhibitors of human lymphocyte proliferation and of the growth of a human cancer cell line (Moiteiro et al., 2001).
Portugal is the leading producer of cork with 55 % of the world production, followed by Spain with 26 % (Anonymous, 2000). The annual cork production in Spain is quite steady: during the period 1985-1999, was of 77204 ± 4115 (mean ± sem) metric tons, with a peak of 122257 tons in 1998 to the value of 132.7 million euros (MAPA, 2001).

The cork oak also forms part of the Mediterranean open woodlands called "montados" in Portugal and "dehesas" in Spain. They are managed as silvopastororal systems that are very important for the rural development of the zone. This value is threatened in the last years due to several causes: forest fires, tree decay, difficult natural regeneration and the commercial competition of cork substitutes (Montero et al., 1998; Varela, 1999). Therefore, conservation and improvement of cork oak genetic resources are of paramount importance. Vegetative propagation plays a key role to meet these objectives (Savill and Kanowski, 1993).

The cork oak is considered a recalcitrant species, not only for seed conservation purposes, but also for morphogenic capability. Vegetative propagation by cuttings has been tried, even with material from adult trees after rejuvenation or reinvigoration by grafting. Although some success has been reported, rooting percentages decline with the age of grafting (Freitas, 2002). Tissue culture micropropagation via organogenesis has been successful in this species (Manzanera and Pardos, 1990; Romano et al., 1992). Plant regeneration via somatic embryogenesis, that presents many advantages over organogenesis, has recently been reviewed for oaks (Wilhelm, 2000). Briefly, cork oak somatic embryos have been obtained from stem fragments (El Mâataoui et al., 1990), zygotic embryos (Bueno et al., 1992), leaves of seedlings (Fernández-Guijarro et al., 1995) and adult trees (Hernández et al. 2001). This chapter describes protocols for complete plant regeneration of cork oak via somatic embryogenesis.

2. MATERIALS

1. Fungicides: Benoagrex® (50% benomyl) and CaptosanR® (8% carbendazim plus 40% captan); 70 % ethanol; commercial bleach (sodium hypochlorite: 35 % active chlorine); Tween 20; sterile water.

2. Water treatment equipment; balances; autoclave; laminar-flow hood; scissors, forceps and scalpels; Petri dishes (60 mm diameter), baby food jars (55 mm diameter, 72 mm height), plastic film and Parafilm®; growth chambers.
3. Plant tissue culture media (see Tables 1 and 2).
4. Plant growth regulators (PGRs): BA, 6-benzyladenine; NAA, 1-naphtaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.
5. Plant material: immature acorns; leaves from seedlings; pieces of branches (15 cm in length and between 1 to 4 cm diameter) from adult trees.

Table 1: Medium composition for the induction of somatic embryogenesis in cork oak zygotic embryos [(1)]

Mineral salts	**Organic additives**	**Concentration**
Macronutrients: Sommer et al., 1975	Ascorbic acid	11.35 µM
	Nicotinic acid	8.12 µM
	Glutamine	3.42 mM
	Calcium pantothenate	4.19 µM
Micronutrients and Fe-EDTA: Murashige & Skoog, 1962	Pyridoxine-HCl	4.86 µM
	Thiamine	2.96 µM
	Sucrose	87.64 mM
	Agar	8 g/L

[(1)] From Bueno et al., 1992

Table 2: Protocol of media formulations and culture conditions for the induction of somatic embryos in leaves from seedlings or mature cork oak trees, and maintenance of embryogenic lines [(1)]

Stage / **Culture media**	Stage 1: Pre-conditioning	Stage 2: Primary stage for induction	Stage 3: Secondary stage for induction	Stage 4: Expression - Proliferation
Macros [(2)]	½ G	SH	SH	SH
Sucrose (g/L)	10	30	30	30
PGRs (µM)	Without	10 BA / 50 NAA	0.5 BA / 0.5 NAA	Without
Culture conditions				
Lighting [(3)]	Darkness	Darkness	120-180	120-180
Length	7 days	30 days	30 days	Montly subculture

[(1)] From Hernández et al., 2003
[(2)] Macros (macronutrients): ½ G, half-strength Gamborg (1966, PRL-4-C); SH, Schenk and Hildebrandt (1972). Micronutrients, vitamins and Fe-EDTA are from MS medium (Murashige and Skoog, 1962) in all stages. The pH of all media are adjusted to 5.7
[(3)] In µmol m^{-2} sec^{-1}. Photoperiod: 16 hours. Temperature: 25 ºC in all the stages

3. METHODS

Plant regeneration by somatic embryogenesis in cork oak can be achieved following four main phases: induction of somatic embryogenesis in the initial explants, that in turn need to be subdivided in at least three stages depending on the explant, recurrent embryo cloning by secondary embryogenesis, maturation of somatic embryos, and conversion of somatic embryos into somatic seedlings.

3.1 Initiation of Embryogenic Cultures

Somatic embryogenesis can be obtained in different explants from cork oak, such as isolated somatic embryos or leaves from seedlings and from mature trees.

3.1.1 Initiation of embryogenic cultures from zygotic embryos

To induce somatic embryogenesis in zygotic embryos, developing acorns are collected between August and September. Developmental stage, genotype and their interaction significantly influence the embryogenic ability of zygotic embryos. As development depends on genotype and also on climatic conditions, to decide when to collect on the basis of the date is not reliable. Because there is a good correlation between the fresh weight of the acorns and their dry weight, both with and without covers, and also between fresh weight and volume, all these traits are adequate indicators of developmental stage.

Steps

1. Take developing acorns and wash them thoroughly with soap and tap water, put them in a vessel with water and discard those float to the surface and that show insect stings.
2. Wipe the acorns, weigh them and select those that are less than 0.3 g fresh weight.
3. Treat the acorns with a 20% solution of commercial bleach (v/v) plus two drops of Tween 20 for 20 min. Shake from time to time.
4. Rinse the acorns three times with sterile distilled water, in the laminar-flow hood.
5. Remove the seed coat with forceps and scalpel.
6. Place three embryos in Petri dishes filled with 10 ml semisolid culture medium (Table 1) containing 3 μM 2,4-D, and seal them with Parafilm.
7. Put the Petri dishes in a growth chamber at 25 ºC and a 16-h photoperiod of 50μmol m^{-2} sec^{-1} photon flux density for 30 days.

8. Subculture the proliferating embryos onto fresh medium lacking growth regulators, and place the Petri dishes again in the growth chamber for additional 30 days.
9. Transfer the induced somatic embryo clusters onto proliferation medium (Table 2, stage 4), for maintenance of embryogenic lines.

Description of the embryogenic response in zygotic embryos

After the third week in culture, globular structures arise mainly on the hypocotyl of zygotic embryos. They soon develop into clusters of somatic embryos, generally when cultures are transferred onto medium without regulators. The developmental stage of zygotic embryos is the main factor influencing the response: in a study with nine half-sib families and three weight classes in a factorial design, lees than 0.3 g fresh weight acorns showed a percentage of induction of 30.2 % in average, that dropped to 7.1 % when 0.5-0.8 g fresh weight acorns were used, and to 1.4 % when the acorns were more than 1 g fresh weight (Fernández-Guijarro, 1997) (Fig. 1). Other interesting feature of the embryogenic response in cork oak zygotic embryos is that it can be obtained without PGRs, providing that the embryos are at the proper developmental stage (Fernández-Guijarro, 1997).

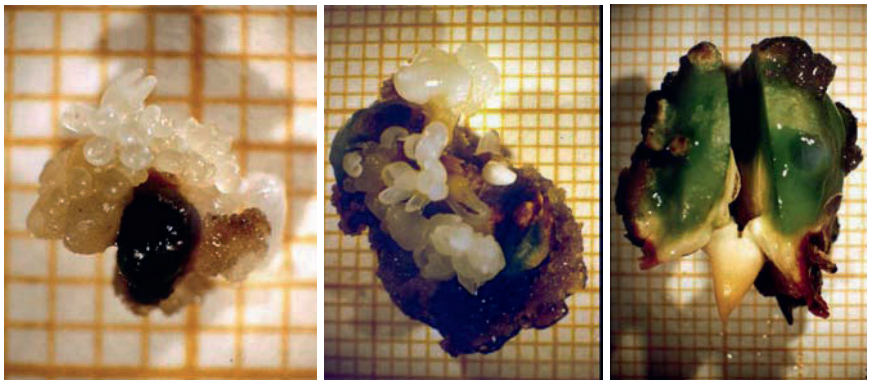

Figure 1. Induction of somatic embryogenesis in cork oak zygotic embryos that are less than 0.3 g (left), between 0.5-0.8 g (centre) and more than 1 g (right) fresh weight.

3.1.2 Initiation of embryogenic cultures in leaves from seedlings

To initiate embryogenic cultures in leaves from 2-4-month-old seedlings, collect expanding leaves from the apex or from lateral sprouts of plants weekly sprayed with a solution, which is a mixture of 1g/l each Benoagrex and CaptosanR.

Steps

1. Treat the leaves with 70% ethanol for 30 seconds, shaking vigorously.
2. Immerse the leaves in a 10% solution of commercial bleach plus two drops of Tween 20 for 10 min. Shake from time to time.
3. Rinse the leaves three times with sterile distilled water, in the laminar-flow hood.
4. Place two leaves with the abaxial surface down on semisolid culture medium, in Petri dishes filled with 10 ml of medium (Table 2) and seal them with Parafilm.
5. Follow the cultural scheme described in Table 2.
6. Transplant the induced somatic embryos and embryo clusters onto proliferation medium (Table 2, stage 4), for maintenance of embryogenic lines.

3.1.3 Initiation of embryogenic cultures in leaves from mature trees

The easiest way to collect leaves for inducing somatic embryogenesis in leaves from mature 100-year-old trees, is by forcing epicormic shoots to sprout in pieces of branches taken from the crown of trees.

Steps

1. Cut branches from trees in the field and make pieces of about 15 cm in length and 1-4 cm diameter without lateral shoots and leaves, put them in a plastic bag, and close it tightly.
2. Wash thoroughly the pieces of branches with a strong brush under tap water. Immerse these pieces in a mixture of 1g/l each of Benoagrex and CaptosanR for 10 minutes.
3. Insert the pieces upright into wet perlite in trays, and place them in a greenhouse or growth chamber with a 16-h photoperiod, at 25 ºC and 80-95% relative humidity. Spray them weekly with the fungicide mixture of 1 g/l of each Benoagrex and CaptosanR.
4. Take expanding leaves (0.5 to 1.5 cm from the base to the apex) from the growing epicormic shoots that start to appear after one or two weeks under high humidity conditions, and remain healthy for at least one month before wilting takes place.
5. Follow the same steps 1-6 described to induce embryogenesis in leaves from seedlings.

Description of the embryogenic response in leaves

Contamination is not usually a problem in leaves from seedlings, but this is not the case with explants from adult trees, in spite of the fact that they are originated in controlled conditions (Fig. 2). Putative endogenous contamination is influenced by genotype, collection date and, mainly, by the time of harvesting: leaves harvested from recently sprouted shoots become less contaminated than leaves collected from older epicormic shoots. Both leaves from seedlings and leaves from adult trees, during the second half of culture on the secondary medium for induction show different degrees of proliferation and necrosis, and some of them begin to show early-stage somatic embryos after about 60 days in culture. Somatic embryos arise almost without callus formation and secondary embryogenesis begin very soon (Fig. 3). The full expression of embryogenesis happen when leaves are transferred to medium lacking plant growth regulators.

Figure 2. Sprouting of epicormic shoots in pieces of branches from mature cork oak trees.

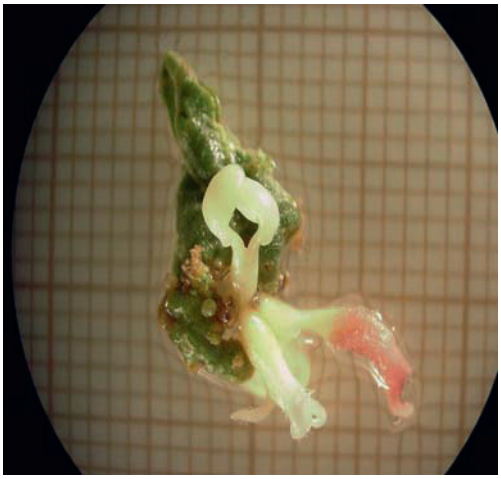

Figure 3. Induction of somatic embryogenesis in an expanding leaf from epicormic shoot

3.2. Maintenance and multiplication of embryogenic tissue

Either induced in zygotic embryos or in leaves, cork oak somatic embryos show the same developmental pattern. Secondary embryogenesis takes place continuously on media without plant growth regulators giving a recurrent process that lasts for years without apparent decline of multiplication ability, merely by monthly subculture to the fresh medium. The other relevant feature of this process

is that embryos reach their full cotyledonary stage without any specific treatment of differentiation, allowing the easy isolation of somatic embryos. A lot of new embryos are formed from previous somatic embryos mostly following a multicellular budding pattern (Fig. 4), hence they usually show a necrotic zone at the base that does not hamper germination.

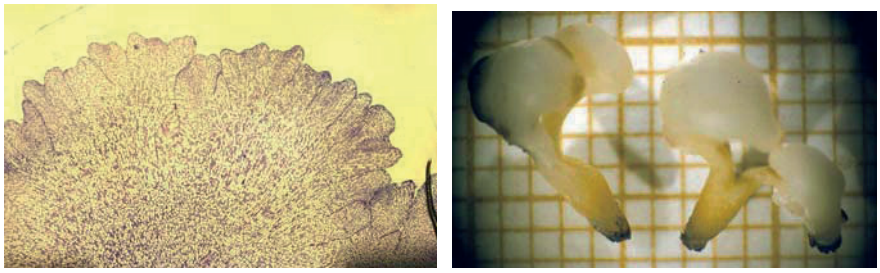

Figure 4. Secondary embryo formation: Transversal section of a cork oak somatic embryo (left) and newly formed somatic embryos (right).

Steps

1. Isolate embryos or embryo clusters from the initial explants and transplant them into baby food jars filled with 30 ml of the same medium used to accomplish Stage 4 of induction in leaves (Table 2), and seal them with plastic film.
2. Place the jars in a growth chamber with the Stage 4 culture conditions (Table 2) for one month.
3. Subculture monthly onto the same fresh medium to maintain the recurrent process. Cold storage or cryopreservation can be performed at this phase in order to prolong subcultures or to avoid them.

3.3 Arrest of secondary embryogenesis and maturation of somatic embryos

The recurrent cycle based on continuous secondary embryogenesis is the basis of the great potential of this technique for mass clonal somatic embryo production. However, it needs to be arrested in individual embryos when the production of individual plants is desired. Experiments aimed at controlling the recurrent cycle in cork oak, by adding abscisic acid (ABA) to culture medium that was successful in other species, have been inconclusive.

Either for *ex situ* conservation purposes or for genetic testing of selected genotypes a relatively low number of plants is required, and then it is possible to

take advantage of spontaneous maturation. By the end of the subculture time on proliferation medium, when embryo clusters have covered all the medium surface (Fig. 5) embryos that are not in contact with the medium mature spontaneously. It means that secondary embryogenesis ceases and these somatic embryos enlarge and become white opaque. Then, they can be picked for the next germination and conversion phase. However, to use somatic embryogenesis as a technique for mass vegetative propagation of high value trees for forest planting, maturation needs to be controlled. Two external stimuli that have rendered good results are the partial desiccation under high humidity conditions treatment, and the starvation treatment (Fernández-Guijarro et al., 1994)

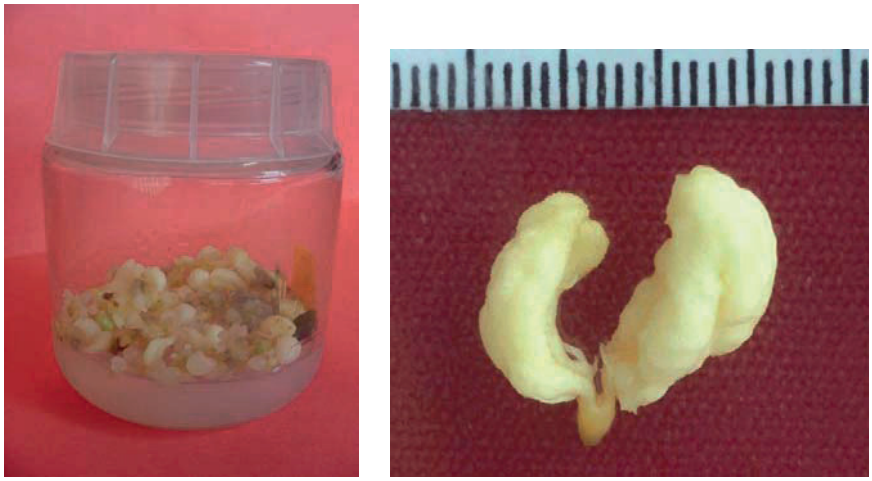

Figure 5. Proliferation culture of somatic embryos and embryo clusters (left) and spontaneously matured somatic embryo (right).

Steps

1. Pick easily detachable immature embryos out of the proliferation cultures. These embryos have to be small (3-5 mm length) soft and translucent, but clearly cotyledonary.
2. Transfer these embryos onto fresh proliferation medium in baby food jars, under the same standard growth chamber conditions for proliferation (Table 2, stage 4) for 30 days.
3. Place the embryos in small empty wells that are surrounded by sterile distilled water, inside the culture vessels tightly closed, at 25 ºC in darkness for seven days. This “desiccation under high humidity conditions” treatment can be substituted by a 7-day culture period on medium containing only 6g/L agar and water.

4. Subculture the embryos again in baby food jars filled with 30 ml fresh proliferation medium and seal them with plastic film to start the first step of the next phase, germination and conversion.

Alternatively, in Step 2 the transfer of embryos to medium with the same components but with half-strength Gamborg's PRL-4-C medium macronutrients (Gamborg, 1966), can give as good results as the previous protocol. In this case, even Step 3 can be avoided without significant decrease in the further germination percentages.

3.4. Germination and conversion to plantlets

This last phase of plant regeneration in cork oak can be accomplished by using the same medium and culture conditions employed to maintain recurrent cultures. However, to break the putative dormancy imposed by the arrest induced spontaneously, or as a consequence of the culture conditions during the previous maturation phase, a cold or stratification treatment is necessary before germination takes place.

Steps

1. Isolate embryos that matured spontaneously (white opaque, 15-20 mm length, average fresh weight 225 mg, without signs of secondary embryogenesis, Fig. 5) from proliferation cultures, and transplant them into baby food jars filled with 30 ml of the same medium used to accomplish Stage 4 of induction in leaves (Table 2).
2. Place these embryos or, alternatively those from Step 4 of the previous phase of maturation, in cold storage at 4 ºC in darkness for 60 days.
3. Without sub culturing, return the baby food jars to the growth chamber with the same light ant temperature conditions used for proliferation (Table 2, stage 4).
4. After 30 days, 40-70 % embryos germinate (root and shoot growth), depending on maturation treatment and genotype (Fernández-Guijarro et al., 1994; Hernández et al., 2003b).
5. Transplant the somatic seedlings (Fig. 6) into 180 ml forest containers filled with substrate (3 pine bark: 1 peat: 1 sand, v:v:v). Place them in the growth chamber under the lighting and temperature conditions described before, covered with glass beakers turned upside down. Water once a week with a solution containig 1/5 of SH macronutrients and 1/10 of MS micronutrients and Fe-EDTA.
6. After two months, remove the beakers one hour per day for one month. After acclimatisation (Fig. 6), move the plants to the nursery under shade.

3.5 Concluding remarks

Up to date, plant regeneration in cork oak has been obtained from different initial explants, in a process highly conditioned by the individual genotype. Some of the phases of this process show a significant additive genetic control, particularly the induction of somatic embryogenesis in zygotic embryos (Fernández-Guijarro, 1997), and therefore they are amenable to genetic improvement. Although present percentages of germination are acceptable, percentages of conversion are still low, likely due to the scarce reserves stored in the small cotyledons (Fig. 6).

Figure 6. Cork oak somatic seedling recently germinated (left) and acclimatised (right).

4. STEPS FOR FURTHER MODIFICATION

Cork oak is one of the few forest species in which complete plant regeneration from adult trees has been obtained. This response is quite reproducible, and embryogenic lines were obtained from all the selected genotypes that were studied (Hernández et al., 2003b). Therefore one of the main bottlenecks facing other forest species has overcome in cork oak. The multiplication ability of this technique is also guaranteed by the high multiplication rates obtained in the recurrent process that continuously performs, and this is very important, without the external addition of plant growth regulators. In fact, almost whole plant regeneration process can be achieved without plant growth regulators. Currently, plant regeneration in cork oak is well developed for objectives in which a limited number of plants is required, i.e. conservation, genetic testing purposes and for basic studies on plant development such as the expression of Heat Shock Proteins during the development of somatic embryos (Puigderrajols et al., 2002). However, the main bottleneck in this species is to control the recurrent process that needs to

be improved. Synchronisation may be achieved by promoting the unicellular pathway of regeneration in opposition to the multicellular pathway (Puigderrajols et al., 1996, 2001). Proper maturation may be achieved in liquid medium, using the temporary immersion technique to switch embryos from the embryogenic program to the germination potential, based on the role showed by starvation in controlling secondary embryogenesis and enhancing germination. Proper maturation, that includes adequate nutrient deposition in cotyledons, will also help to solve current problems with the acclimatisation of cork oak somatic seedlings.

5. ACKNOWLEDGEMENTS

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6. REFERENCES

Anonymous (2000) O sobreiro e a cortiça – The cork oak and cork. Direcção-Geral de Desenvolvimento Rural. Lisboa, Portugal.

Bueno MA, Astorga R, Manzanera JA (1992) Plant regeneration through somatic embryogenesis in *Quercus suber*. Physiologia Plantarum, 85: 30-34.

Ceballos L, Ruiz de la Torre J (1979) Árboles y arbustos de la España peninsular. E.T.S. Ingenieros de Montes. Madrid, Spain.

El Mâataoui M, Espagnac H, Michaux-Ferriere M (1990) Histology of callogenesis and somatic embryogenesis induced in stem fragments of cork oak (*Quercus suber*) cultured in vitro. Annals of Botany, 66: 183-190.

Fernández-Guijarro B (1997) Embriogénesis somática en alcornoque (*Quercus suber* L.). Doctoral Thesis. Polytechnic University of Madrid, Spain.

Fernández-Guijarro B, Celestino C, Toribio M (1994) Somatic embryogenesis in *Quercus suber* L. In: Pardos JA, Ahuja MR, Elena-Rossello R (eds.) Biotecnology of Trees. Investigación Agraria. Sistemas y Recursos Forestales. Fuera de Serie nº 4: 105-110.

Fernández-Guijarro B, Celestino C, Toribio M (1995) Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber* L. Plant Cell, Tissue and Organ Culture, 41: 99-106.

Freitas MIC de (2002) Propagação vegetativa de sobreiros seleccionados. Silva Lusitana, 10: 17-52.

Gamborg OL (1966) Aromatic metabolism in plants. II. Enzymes of the shikimate pathway in suspension cultures of plant cells. Canadian Journal of Biochemistry, 44: 791-799.

Hernández I, Celestino C, Martínez I, Manjón JL, Díez J, Fernández-Guijarro B, Toribio M (2001) Cloning mature cork oak (*Quercus suber* L.) trees by somatic embryogenesis. Melhoramento, 37: 50-57.

Hernández I, Celestino C, Toribio M (2003a) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis: I. Factors affecting the induction in leaves from mature cork oak trees. Plant Cell Reports, 21: 759-764.

Hernández I, Celestino C, Alegre J, Toribio M (2003b) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis: II. Plant regeneration from selected cork oak trees. Plant Cell Reports, 21: 765-770.

Manzanera JA, Pardos JA (1990) Micropropagation of juvenile and adult *Quercus suber* L. Plant Cell, Tissue and Organ Culture, 21: 1-8.

MAPA (2001) Otras producciones forestales. Capítulo 28. Anuario de Estadística Agroalimentaria. Ministerio de Agricultura, Pesca y Alimentación. Madrid, Spain.

Moiteiro C, Justino F, Tavares R, Marcelo-Curto MJ, Florencio MH, Nascimento MSJ, Pedro M, Cerqueira F, Pinto MMM (2001) Synthetic secofriedelane and friedelane derivatives as inhibitors of human lymphocyte proliferation and growth of human cancer cell lines *in vitro*. Journal of Natural Products, 64: 1273-1277.

Montero G, San Miguel A, Cañellas I (1998) Systems of Mediterranean silviculture "La Dehesa". In: Jiménez R, Lamo de Espinosa J (eds.) Agricultura sostenible. Agrofuturo, Life and Mundi-Prensa, Madrid. English transcription Chapter 24, pp 519-554.

Montoya JM (1988) Los alcornocales. Ministerio de Agricultura, Pesca y Alimentación. Madrid, Spain.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

Puigderrajols P, Fernández-Guijarro B, Toribio M, Molinas M (1996). Origin and early development of secondary embryos in *Quercus suber* L. International Journal of Plant Sciences, 157: 674-684.

Puigderrajols P, Mir G, Molinas M (2001) Ultrastructure of early secondary embryogenesis by multicellular and unicellular pathways in cork oak (*Quercus suber* L.). Annals of Botany, 87: 179-189.

Puigderrajols P, Jofre A, Mir G, Pla M, Verdaguer D, Huguet G, Molinas M (2002) Developmentally and stress-induced small heat shock proteins in cork oak somatic embryos. Journal of Experimental Botany, 53: 1445-1452.

Romano A, Noronha C, Martins-Loução MA (1992) Influence of growth regulators on shoot proliferation in *Quercus suber* L. Annals of Botany, 70: 531-536.

Savill PS, Kanowski PJ (1993) Tree improvement programs for European oaks: goals and strategies. Annals des Sciences Forestières, 50: 368s-383s.

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Canadian Journal of Botany, 50:199-204

Toribio M, Celestino C, Gallego J, Martínez I (2000) Induction of somatic embryogenesis in tissues from mature oak trees. In: F Ó Ríordáin (ed) Development of integrated systems for large-scale propagation of elite plants using *in vitro* techniques. EUR 19237 – COST Action 822, Report of activities 1998. Office for Official Publications of the European Communities, Luxembourg, pp 236-237.

Varela MC (1999) Cork and the cork oak system. Special issue: Mediterranean forests. Unasylva (English ed.) 50(2) issue 197: 42-44.

Wilhelm E (2000) Somatic embryogenesis in oak (*Quercus* spp.) In Vitro Cellular & Developmental Biology – Plant, 36: 349-357.

SAWARA CYPRESS *Chamaecyparis pisifera* Sieb. *et* Zucc.

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1. INTRODUCTION

There are six species in genus *Chamaecyparis* worldwide, and Sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.) is one of the domestic species in Japan (Maruyama et al., 2002). Trees are 30 m tall and 1 m diameter at breast height and as its lumber is resistant to moisture, it is mainly used for wooden tubs or boxes. It is also suitable as an ornamental plant, e.g. as garden hedges (Maruyama et al., 2003). This species is also used in hybridization with the related valuable timber tree, Hinoki cypress (*Chamaecyparis obtusa*) (Fukuhara, 1989). The wood quality of Sawara cypress is considered inferior to Hinoki cypress; however, it grows faster and is considered much cold hardier than Hinoki cypress (Fukuhara, 1978). In addition, Sawara cypress also has high adaptability to humid and unproductive soil and resistance against termite injury (Maeta, 1982). Plant regeneration through somatic embryogenesis of Sawara cypress is important for genetic engineering and somatic hybridization breeding to develop disease resistant hybrids. A stable and efficient plant regeneration system is important for genetic engineering. Somatic embryogenesis is an ideal procedure for effective propagation of not only plus trees but also target tissue for genetic transformation. In this chapter we describe a stable and an efficient plant regeneration system for propagation of Sawara cypress by somatic embryogenesis from immature seeds.

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2. PROTOCOL OF SOMATIC EMBRYOGENESIS IN SAWARA CYPRESS

2.1 Culture Media

1. The culture media used for Sawara cypress somatic embryogenesis are described in Table 1.
2. Note that this protocol consists of eight *in vitro* culture stages differing in medium, plant growth regulators, and additives contents 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. Petri dishes and culture plates are sealed with Novix-II film (Iwaki Glass Co., Ltd., Chiba, Japan). Flasks are capped with autoclavable transparent film (Tetoron, Toray Ind., Tokyo, Japan).

2.2 Explant Preparation

1. Collect immature cones (Fig. 1A) from mother trees during late-June to early July (Tsukuba, Japan).
2. Disinfect cones by 15 min immersion in 70% ethanol containing few drops of neutral detergent and then wash in tap water before dissection.
3. Disinfect excised seeds (Fig. 1B) with 3% (w/v available chlorine) sodium hypochlorite solution for 30 min and then rinse five times with sterile distilled water.

2.3 Induction of Embryogenic Cells

1. For induction of embryogenic cells, prepare whole seed explants as described above and culture in 24-well tissue culture plates (one per well) containing induction medium as specified in Table 1 and 2.
2. Seal culture plates with Novix-II film and incubate under conditions described in Table 2.
3. The presence (Fig. 1C) 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.
4. Using a transferpipette, transfer induced cells to proliferation medium and culture as described in Table 2. Induction of embryogenic cells is recorded if distinct early stages of somatic embryos proliferated after the first subculture.

2.4 Maintenance of Embryogenic Cells

1. Collect embryogenic cells from proliferation medium on 100µm nylon screen.

2. Using a tweezers, transfer five embryonal masses (10-20 mg FW each) to maintenance medium and culture as described in Table 2. Seal petri dishes with Novix-II film.
3. For subsequently maintenance routines, subculture embryonal masses on maintenance medium at 4 to 6-wk intervals.

2.5 Proliferation of Embryogenic Cells

1. Transfer 10-20 mg FW embryogenic cells from maintenance medium to proliferation medium and culture as described in Table 2.
2. For continuously proliferation routines, subculture embryogenic cells from proliferation medium (Fig. 1D) to same fresh medium using a transferpipette (about 0.5 ml suspension culture in 30-40 ml fresh medium) and incubate as specified in Table 2.
3. Subculture at intervals as specified in Table 2.

2.6 Development of Somatic Embryos

1. Collect embryogenic cells from proliferation medium on 100µm nylon screen.
2. Rinse embryogenic cells on nylon screen with 50-100 ml development medium.
3. Transfer about 500 mg FW embryogenic cells from nylon screen to 100 ml flask containing 30-40 ml development medium and culture as describe in Table 2.

2.7 Maturation of Somatic Embryos

1. Collect embryogenic cells from development medium (Fig. 1E) on 100µm nylon screen.
2. Resuspend embryogenic cells in fresh development medium (about 500 mg FW per 10 ml cell suspension medium).
3. Dispense as 2 ml aliquots on filter paper disk over each petri dish containing maturation medium as specified in Table 1 and 2.
4. Seal petri dishes and culture as described in Table 2.
5. Initial formation of cotyledonary embryos are observed about 4 wk after transfer of embryogenic cells to the maturation medium, and is evident at 6 wk of culture (Fig. 1F-J). After 8 wk of culture, 500-1000 mature cotyledonary somatic embryos per petri dish can be obtained (Fig. 1K-L). Normally, maturation process is recorded up to 12 wk of culture, however in some cases this might be continuous up to more than 28 wk *via* repetitive embryogenesis.

2.8 Germination of Somatic Embryos

1. Collect somatic embyos from maturation medium and transfer to filter paper disk over each petri dish containing germination medium as described in Table 2.
2. Seal petri dishes and culture at 25±1°C under 16-h photoperiod (65 µmol.m^{-2}.s^{-1}) provided by cool-fluorescent lamps.
3. Root emergence is observed about 1 wk after transfer to germination medium (Fig. 1M), and more than 95% germination is recorded at 2 wk of culture.

2.9 Conversion of Somatic Embryos

1. Collect germinated somatic embryos from germination medium and transfer to flasks containing conversion medium (Table 2).
2. Flasks are capped with autoclavable transparent film and culture as described in Table 2.
3. Epicotyl development is observed about 2 wk after transfer to conversion medium, and more than 90% conversion is recorded at 4 wk of culture.
4. Emblings showing faster growth (Fig. 1N) can be transferred to *ex vitro* conditions for acclimatization, omitting the next step for shoot and root development in flasks kept under culture room conditions.

2.10 *In Vitro* Growth of Emblings

1. Collect emblings from conversion medium and wash roots with sterile distilled water to free any adhering agar.
2. Transfer emblings carefully to flasks containing vermiculite fertilized with plant food solution as specified in Table 2.
3. Flasks are capped with autoclavable transparent film and culture as described in Table 2.
4. About 4-6 wk after transfer to vermiculite medium emblings can be transferred to *ex vitro* conditions for acclimatization.

2.11 *Ex Vitro* Acclimatization

1. Remove emblings from culture flasks and transplant into plastic pots filled with vermiculite and perlite (volume ratio 1:1).
2. For the first 2 wk emblings are kept under high relative humidity by covering pots with transparent plastic films and irrigate with tap water and subsequently as needed.
3. Best acclimatization and growth of emblings are recorded keeping the pots inside the Phytotron (under natural light, 75% relative humidity, and alternating temperature of 20°C for 16-h and 15°C for 8-h).
4. After the first 2 wk, open the covers gradually and irrigate the pots with Nm nutrient solution as described in Table 1.

5. Remove covers completely 4 wk after transplanting.
6. Keep acclimatizated emblings at same conditions for another 4 wk before field transfer. At this time emblings are normally on an average 5 cm long (Fig. 1O).

2.12 Field Transfer

1. Transplantation is recommended in spring season. If necessary, keep emblings in a greenhouse until the spring arrival.
2. Remove emblings from pots and transfer to the nursery soil.
3. Nurture emblings in the nursery for one year.
4. Transplant emblings to a permanent field location (Fig.2).

3. CONCLUSION

An efficient plant regeneration system *via* somatic embryogenesis has been achieved for Sawara cypress with protocol described. In addition to high somatic embryo maturation efficiency, the subsequently high germination and plant conversion frequencies attained demonstrated the "high quality" of somatic embryos produced. Somatic embryos readily germinate after transfer to plant growth regulator-free medium without any kind of treatments, e.g. partial drying treatment which is necessary to promote germination of somatic embryos of some other species.

4. REFERENCES

Aitken-Christie, J. and Thorpe, T, A. (1984) Clonal Propagation: Gymnosperms. *In* Cell Culture and Somatic Cell Genetics of Plants, Vol. 1., Vasil, I.K. (Ed.), 480pp, Academic Press Inc., San Diego, 82-95.

Fukuhara, N. (1978) Meiotic observation in the pollen mother cell of interspecific hybrid between *Chamaecyparis obtusa* and *C. pisifera*. J. Jpn. For. Soc. 60: 437-441.

Fukuhara, N. (1989) Fertility in interspecific-crossing between hinoki (*Chamaecyparis obtusa* Endl.) and Sawara (*C. pisifera* Endl.) and identification of the hybrids. Bulletin of the Forestry and Forest Products Research Institute **354:** 1-38.

Maeta, T. (1982) Effects of gamma-rays irradiation on interspecific hybridization between *Chamaecyparis obtusa* S. et Z. and *C. pisifera* S. et Z. Hoshasen Ikusyujo Kenkyu Hokoku 5: 1-87. (in Japanese)

Maruyama, E., Tanaka, T., Hosoi, Y., Ishii, K., and Morohoshi, N. (2000) Embryogenic cell culture, protoplast regeneration, cryopreservation, biolistic gene transfer and plant regeneration in Japanese cedar (*Cryptomeria japonica* D. Don). Plant Biotechnology, **17:** 281-296.

Maruyama, E., Hosoi, Y., and Ishii, K. (2002) Somatic embryogenesis in Sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.) for stable and efficient plant regeneration, propagation and protoplast culture. J. For. Res. 7: 23-34.

Maruyama, E., Hosoi, Y., and Ishii, K. (2003) Somatic embryo culture for propagation, artificial seed production, and conservation of Sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.). J For Res 8: 1-8.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant., **15:** 473-497.

Smith, D.R. (1996) Growth medium. United States Patent # 5,565,355.

Table 1. Constituents of culture media for Sawara cypress plant regeneration system *via* somatic embryogenesis.

Constituents	MS[1] (mg/L)	LPm[2] (mg/L)	SMm[3] (mg/L)	Nm[4] (mg/L)	
Basal salts					
NH_4NO_3	1650	200		143	
KNO_3	1900	900	1431		
$NaNO_3$			310		
$NaH_2PO_4{\cdot}2H_2O$					55.1
KH_2PO_4	170	135			
$NH_4H_2PO_4$			225		
$MgSO_4{\cdot}7H_2O$		370	180	400	61
$CaCl_2{\cdot}2H_2O$	440		25	52.5	
$Ca(NO_3)_2{\cdot}4\ H_2O$			600		
KCl					47.1
$MnSO_4{\cdot}4\ H_2O$		22.3	0.5	3.6	
H_3BO_3	6.2	3.1	8	1.5	
$ZnSO_4{\cdot}7\ H_2O$		10.58	4.3	25	
KI	0.83	0.04	1	0.01	
$CuSO_4{\cdot}5\ H_2O$		0.025	0.0125	2.4	
$Na_2MoO_4{\cdot}2\ H_2O$		0.25	0.125	0.2	
$CoCl_2{\cdot}6\ H_2O$	0.025	0.0125	0.2	0.005	
MoO_3				0.005	
$FeSO_4{\cdot}7\ H_2O$	27.8	13.9	30		
Na_2-EDTA	37.3	18.65	40		
Fe(III)-EDTA					25
$CuNa_2$-EDTA					0.1
$MnNa_2$-EDTA					0.1
$ZnNa_2$-EDTA					0.1
Vitamins					
Myo-Inositol	100	1000	1000		
Thiamine hydrochloride		0.1	0.4	5	
Pyridoxine hydrochloride		0.5		0.5	
Nicotinic acid		0.5		5	
Glycine	2.0				
Other additives					
Glutamine		Table2	550		
Asparagine			510		
Arginine			175		
Citrulline			19.75		
Ornithine			19		
Lysine			13.75		
Alanine			10		
Proline			8.75		
Sucrose	30000	Table2			
Maltose			50000		
Polyethylene glycol 4000				100000	
Activated charcoal (AC)			Table 2	2000	
Plant growth regulators	Table 2	Table 2	Table 2		
Gelrite			5000		
Agar			Table 2		
pH	5.8	5.8	5.8		

[1] MS medium (Murashige and Skoog, 1962). [2] Modified from Quorin and Lepoivre's medium (Aitken-Christie and Thorpe, 1984). [3] Modified from Embryo Development medium (Smith, 1996). [4] Modified from Nagao's nutrient solution (Maruyama et al., 2000).

Table 2. Medium, additives, culture conditions, and culture durations for each stage of somatic embryogenesis in Sawara cypress.

Stage	Medium[1]	Additives (mg/L)	Culture conditions[2]	Duration (wk)
1. Induction	MS	2,4-D (0.66), BA (0.07)	Dark, 24-well plate (1 ml medium/well)	4-12
2. Maintenance	LPm	2,4-D (0.66), BA (0.07), Sucrose (10000), Glutamine (500), Gelrite (3000)	Dark, 90 x 20 mm petri dish (30-40 ml medium/petri dish)	4-6
3. Proliferation	LPm	2,4-D (2.21), Sucrose (10000), Glutamine (500)	Dark, 100 ml flask (30-40 ml medium/ flask), on rotatory shaker at 100 rpm	2-4
4. Development	LPm	Sucrose (30000), Glutamine (500)	Dark, 100 ml flask, (30-40 ml medium/ flask), on rotatory shaker at 100 rpm	1-2
5. Maturation	SM	ABA (26.43)	Dark, 90 x 20 mm petri dish (30-40 ml medium/petri dish)	6-12
6. Germination	LPm	Sucrose (20000), AC (2000), Agar (12000)	Light, 90 x 20 mm petri dish (30-40 ml medium/petri dish)	2-4
7. Conversion	LPm	Sucrose (30000), AC (5000), Agar (12500)	Light, 300 ml flask (100 ml medium/ flask)	2-4
8. Growth	Vermiculite	0.1% (v/v) Hyponex 5-10-5 plant food solution[3]	Light, 300 ml flask (100 ml vermiculite/ flask)	4-6

[1] See Table 1.
[2] Culture at 16-h photoperiod (65 $\mu mol.m^{-2}.s^{-1}$) or darkness at 25±1°C.
[3] The Hyponex Co., Inc., Hyponex Japan, Osaka, Japan; containing in w/v: 5.00% N, 4.36% P, and 4.15% K.

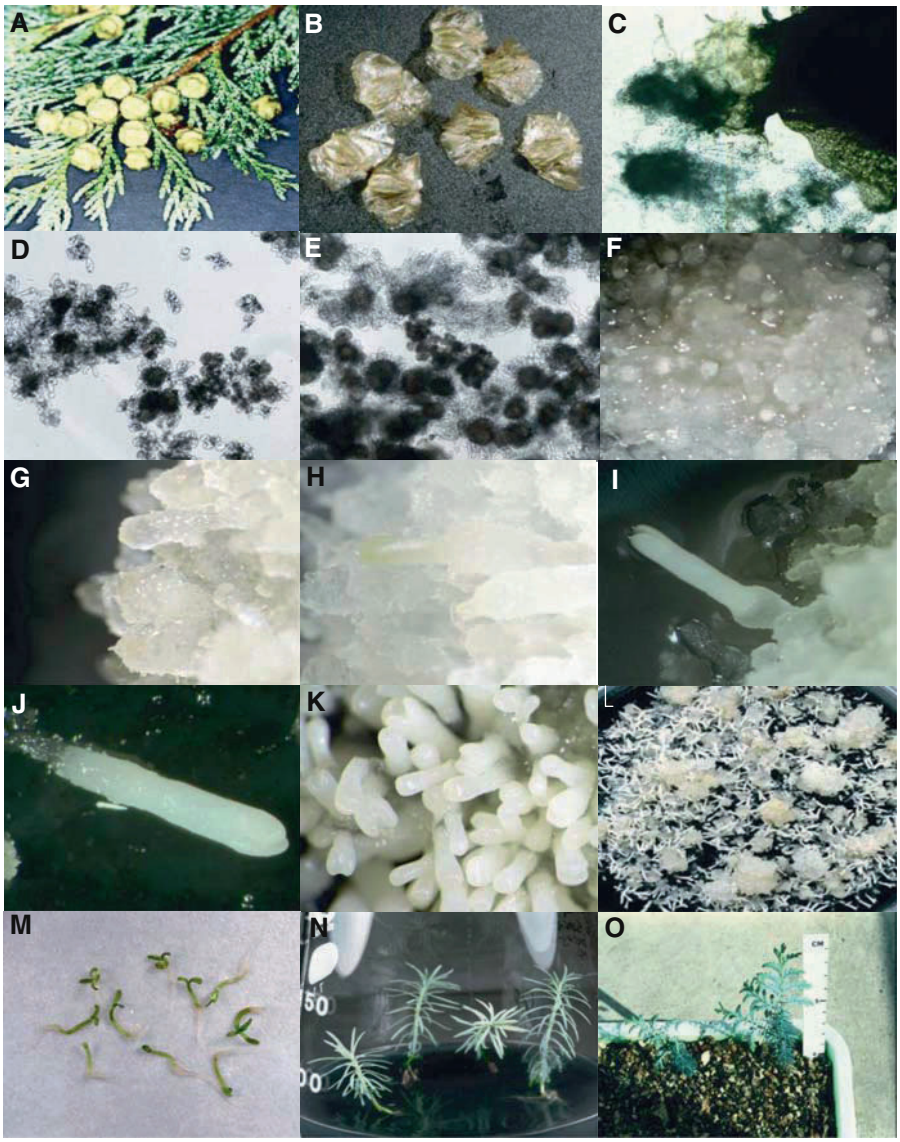

Figure 1. Somatic embryogenesis in Sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.). A: Collected cones. B: Excised immature seeds. C: Embryogenic cell initiation from whole seed in liquid medium. D: Embryogenic cells proliferating in suspension culture. E: Development of somatic embryos in plant growth regulator-free liquid medium. F-J: Different developmental maturation stages of somatic embryos on solid medium containing maltose, ABA, PEG and AC. K-L: Production of somatic embryos after 8 wk of culture on maturation medium. M: Germination of somatic embryos. N: Emblings growing in vitro. O: Acclimatized emblings.

Figure 2. Embling of Sawara cypress (*Chamaecyparis pisifera* Sieb. et Zucc.) growing in the field.

PROTOCOL OF SOMATIC EMBRYOGENESIS: HOLM OAK (*QUERCUS ILEX* L.).

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1. INTRODUCTION

Holm oak (*Quercus ilex*) is a characteristic evergreen woody species present in most countries surrounding the Mediterranean. The most important features of this hardwood tree are hardiness, resistance to drought, high ecological value as climacic, soil improving tree, game nourishment and refuge, etc. (figure 1a). Formerly, the wood was appreciated for its density and resistance in ship and house building and other traditional manufactures. Genetic variability is considerable, with a range of ecotypes with different morphologies, growth patterns and fruit (acorn) taste. The use of holm oak trees for symbiotic culture of truffle has a high economic importance and would contribute to rural development and to the stabilisation of the population in depressed areas. Nevertheless, basic research on this species has been scarce. Some problems include the variability of acorn production, the difficulty of conserving the acorns and the lack of conventional vegetative propagation, especially for adult elite trees (Cornu *et al*., 1977; L'Helgoual'ch and Espagnac, 1987).

Micropropagation of oaks from apical and axillary bud cultures has been tried in *Q. ilex* juvenile cultures (Bellarosa, 1989), and shoot regeneration has been obtained in related species (Vieitez *et al*., 1993). Micropropagation from adult oak trees is in general more difficult, with moderate rates of plant regeneration (e.g., San José *et al*., 1985; Manzanera and Pardos, 1990; Manzanera *et al.,* 1996; Sánchez *et al*., 1996).

Somatic embryogenesis is preferred to bud micropropagation for potential in culture automation and artificial seed production. Much research effort

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has been devoted to oak somatic embryogenesis leading to plant production. Somatic embryos have been induced from culturing *Q. rubra* immature zygotic embryos (Gingas and Lineberger, 1989), *Q. robur* immature zygotic embryos (Chalupa, 1990), *Q. robur* stems and leaves (Cuenca et al., 1999), *Q. suber* immature embryos (Bueno *et al*., 1992), *Q. suber* leaves (Fernández-Guijarro *et al*., 1995) and *Q. suber* anthers (Bueno *et al*., 1997), *Q. acutissima* immature zygotic embryos (Kim *et al*., 1994), *Q. variabilis* immature zygotic embryos (Kim *et al*., 1995) and *Q. canariensis* immature zygotic embryos (Bueno *et al*., 1996). In the past, there was hardly any work done on somatic embryogenesis of *Q. ilex*, except the first work done by Feraud-Keller and Espagnac (1989) and now recently by Mauri and Manzanera (2003).

The bottle-neck of somatic embryogenesis in *Q. ilex* is the maturation phase. Maturation has been hampered, in many woody species, by precocious germination and spontaneous repetitive embryogenesis, which may reach a 70% frequency. To prevent these unwanted side effects, osmotic treatments and the addition of abscisic acid (ABA) in the early stages of embryo development have been used in related species (Garcia-Martin *et al*., 2001). The role of ABA in the induction of maturation and dormancy of zygotic embryos and seeds has been studied in many other species as well its inhibitory effect on germination. Nevertheless, there are no references on the effects of exogenous application of ABA on somatic embryos of holm oak.

2. INDUCTION OF EMBRYOGENIC TISSUE

Induction of somatic embryogenesis was obtained from immature zygotic embryos (Mauri and Manzanera, 2003), collected in August 10th only (4.3 % frequency), approximately four months post anthesis. No positive results were recorded in immature embryos collected in July or September, or in nodal segments or leaves from seedlings or adult trees. Féraud-Keller and Espagnac (1989) reported somatic embryogenesis induction from the leaves of seedlings.

Acorns collected from the selected trees (figure 1b) are first rinsed in 70% (v/v) ethanol for 3 min, followed by immersion in 0.2% (w/v) Benlate® (Du Pont, 50% benomyl) with a few drops of Tween 20, then they are surface-sterilized for 20 min in a solution containing 0.5% (w/v) sodium hypochlorite prepared from commercial bleach to which 1-2 drops of Tween 20 are added. After rinsing three times in sterile distilled water,

they are left to imbibe for 10 min before dissection of the zygotic embryos. Remove the acorn coats with scalpel and forceps and transfer the embryos. Establish the excised zygotic embryos on G macronutrients (Gamborg *et al.*, 1968), with the micronutrients and cofactors of Murashige and Skoog (1962) (G*: Table 1), with 10 μM benzylaminopurine (BA) and 10 μM □-naphthaleneacetic acid (NAA) and 0.6 % (w/v) agar (Sigma, Type E) for one month. This plant growth regulator (PGR) combination was the best treatment for somatic embryo induction of all tested (Table 2). Adjust the pH of the medium to 5.8 with 1 N NaOH before autoclaving at 121 °C. We found that ten zygotic embryos per Petri dish are optimal for induction, which is realized at 25 °C in the dark. Wrap plates with a double layer of parafilm. Transfer the sterile embryos in petri plates containing G* medium with 0.5 μM BA and 0.5 μM NAA for 30 days. Transfer the embryos to basal medium free of PGR for 30 days. All through the induction period and even before embryogenic tissue can be observed, embryo cultures are transferred onto the fresh medium at every 4-week interval since this increases the frequency of induction as compared to cultures without subculture.

A white-translucent callus was induced on the zygotic embryo surface, which showed early embryo-like structures 3 to 8 weeks after the beginning of the induction treatment (figure 1c). Somatic embryos grow further and are eventually transferred to basal G* medium without PGR's when they reach 0.5-1.0 cm in diameter.

3. MAINTENANCE OF EMBRYOGENIC TISSUE

After 4-6 months, a proliferating, yellowish, friable callus is induced in the embryogenic cultures, from which somatic embryos develop after two weeks. On medium supplemented with 3 μM 2,4-dichlorophenoxyacetic acid (2,4-D) alone, non embryogenic callus is formed (Table 2). This non-embryogenic callus is normally characterised by a whitish appearance. Maintenance conditions are set at 25 °C with a 16-h photoperiod under light (50 μmol. $m^{-2}.s^{-1}$). Maintain embryogenic cultures by subculture every four weeks onto fresh basal medium without PGR and incubate at 25 ºC. Cultures are maintained on the same medium as for induction, i.e, G* medium without PGR.

Like other oak species, holm oak embryogenic tissue can be maintained either on solid (0.6 % agar) or in liquid medium (figure 1d-g) by serial subcultures to fresh medium every 28 or 14 days, respectively. Somatic

embryos have higher fresh weight (FW) increments in liquid medium (figure 2).

4. MATURATION

Holm oak somatic embryo development and maturation are carried out according to Mauri (1999). Maturation medium contains Schenk and Hildebrant's macronutrients (SH; 1972), with the micronutrients and cofactors of Murashige and Skoog (1962) supplemented with 0.1 or 1 μM ABA. Sucrose (90 mM, unless stated otherwise) was used as the carbon source. The medium was solidified with agar (Sigma, Type E) (6 g l^{-1}). Dispense the medium in Magenta™ glass flasks (59 x 66 mm, 30 ml medium) or in Magenta™ polycarbonate flasks (95 x 67 mm, 45 ml medium) for somatic embryo maturation. Adjust the pH of the medium to 5.8 with 0.1 M NaOH or 0.1 M HCl, and autoclave the medium at 1 atmosphere at 120 ºC for 20 min. Incubate the cultures in a climate-controlled chamber under a 16 h photoperiod, and a photon flux density of 50 μmol m^{-2} s^{-1} provided by cool-white fluorescent lamps.

Culture immature somatic embryos on SH medium supplemented with 0.1 or 1 μM ABA for 11 monthly subcultures. It will be observed that recurrent embryogenesis is significantly reduced and maturation increased with continuous culture on medium with ABA when compared with a medium without it (figure 3). Spontaneous germination is lower than 5%. Higher concentrations of ABA (10 μM) are not recommended becuase growth is arrested, while somatic embryos cultured on a medium supplemented with 0.1 and 1 μM ABA reach the highest values of dry weight (figure 4).

The addition of ABA to the culture medium significantly reduces unwanted recurrent embryogenesis in mature somatic embryos without affecting the germination of embryos subjected to stratification at 4 ºC. ABA is important for cotyledonary embryo development. It inhibits the polyembryony and allows embryo separation and further embryo development. ABA plays a role in embryo development and meristem dormancy and prepares tissues to tolerate physiological stages previous to germination.

5. GERMINATION AND TRANSFER TO SOIL

Transfer the matured somatic embryos (1 cm-size white embryos) to flasks containing basal SH medium without ABA and store them in a refrigerator

for a stratification treatment at 4ºC. The optimal period of stratification is normally 2-months (figure 5). After the stratification period, transfer the mature embryos to SH* germination medium (table 1). Incubate the cultures in light (50 μmol. $m^{-2}.s^{-1}$) with a 16-hour photoperiod and a continuos temperature of 25 ºC both during the day and at night. After 6-8 weeks, somatic embryos germinate and develop somatic seedlings (figure 1h-i). Transplant somatic seedlings, germinated *in vitro,* to pots filled with soil substrate. Grow the somatic seedlings in pots for 6 months in the greenhouse at 25 ºC.

Somatic embryos previously cultured on 0.1 and 1 μM ABA for long periods of time and stratified for two months reached germination rates as high as that of the control without ABA, i.e. 73%, while the 10 μM ABA treatment significantly reduces germination rate (data not shown). Stratification can neutralise the effect of ABA as a germination inhibitor. But stratification also promotes germination of somatic embryos in different species, such as *Q. suber* (Bueno *et al*., 1992; Manzanera *et al*., 1993; González-Benito *et al*., 2002). In holm oak, the stratification of somatic embryos at 4ºC has been significantly positive on germination for treatments longer than one month, two months being the optimum. Another positive effect of stratification was the shortening of the mean germination time, which was reduced to 3 to 7 days for the two month treatment at 4 ºC.

Sucrose concentration in the medium significantly affected germination. The best rates were obtained between 90 and 450 mM sucrose, which had no statistical differences between them (figure 6). No significant difference was observed between embryos cultured in light and those cultured in darkness. When comparing temperature regimes, i.e., continuous temperature at 25 ºC or a thermoperiod of 25 ºC/15 ºC, no statistical differences were recorded in embryo germination.

The best germination rates were obtained with the addition of 90 to 450 mM sucrose in the culture medium, as in *Q. suber* (Garcia-Martin *et al*., 2001). Higher concentrations (630 mM) of sucrose significantly inhibited germination rate (figure 6). Temperature was not a critical factor. In *Q. robur* acorns, light negatively affected germination (Finch-Savage and Clay, 1994) but in our case, light and darkness treatments provided similar germination rates, in agreement with observations of somatic embryos from other species, such as *Q. suber* (Fernández-Guijarro et al., 1995) or *Q. robur* (Chalupa, 1990).

7. CONCLUDING REMARKS

Somatic embryogenesis has been induced in *Q. ilex* by culturing immature zygotic embryos (Mauri and Manzanera, 2003) and leaves (Feraud-Keller and Espagnac, 1989).

To overcome the difficulties encountered in plant production, efforts were devoted during the last 10 years to understand different physiological aspects of this species. This approach was beneficial as it helped to develop a complete protocol of somatic embryogenesis, that is from induction of embryogenic tissues to plants in the field passing through short-term preservation of embryos, *in vitro* acclimatization, etc. Furthermore, most of these conditions are directly applicable to other species.

In conclusion, somatic embryogenesis can be induced in holm oak immature zygotic embryos during a narrow developmental window in August. A possible explanation is that at the end of the maturation period in September, the tissues of late zygotic embryos become more specialised and lose embryogenic capacity. The exogenous application of 0.1 to 1 μM ABA to immature somatic embryos induces maturation and lowers secondary embryogenesis, improving synchronisation of the cultures. The main factor for breaking dormancy and promoting germination in holm oak somatic embryos is a stratification treatment at 4ºC for two months.

Further research work should be devoted to the optimisation of the maturation rates and to the improvement of plant acclimation to greenhouse and nursery conditions. This would secure the large-scale production of holm oak somatic seedlings.

REFERENCES

Bellarosa R (1989) Oak (*Quercus* spp.). In: Bajaj YPS (ed) Biotechnology in Agriculture and Forestry, Vol. 5 Trees II. (387-401) Springer-Verlag, Berlin, Heidelberg.

Bueno MA, Astorga R & Manzanera JA (1992) Plant regeneration through somatic embryogenesis in *Quercus suber*. Physiol Plant 85: 30-34.

Bueno MA, Gómez A, Vicente O & Manzanera JA (1996) Stability in ploidy level during somatic embryogenesis in *Quercus canariensis*. In: MR Ahuja, W Boerjan & DB Neale (eds). Somatic Cell Genetics and Molecular Genetics of Trees. (23-28). Kluwer Academic Publishers ISBN 0-7923-4179-1.

Bueno MA, Gómez A, Boscaiu M, Manzanera JA & Vicente O (1997) Stress induced haploid plant production from anther cultures of *Quercus suber*. Physiol Plant 99: 335-341.

Chalupa V (1990) Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). Plant Cell Reports 9: 398-401.

Cornu D, Delran S, Garbaye J & Le Tacon F (1977) Research of the best rooting conditions for green shoots of oak (*Q. petraea* (M) Liebl.) and beech (*Fagus sylvatica* L.) (in French). Ann Sci For 34: 1-16.

Cuenca B, San José MC, Martínez MT, Ballester A & Vieitez AM (1999) Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Reports 18: 538-543.

Féraud-Keller C & Espagnac H (1989) Conditions for the appearance of somatic embryogenesis on callus from leaf tissue cultures of holm oak (*Quercus ilex*) (in French). Can. J. Bot. 67: 1066-1070.

Fernández-Guijarro B, Celestino C and Toribio M (1995) Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber* L. Plant Cell Tiss. Org. Cult. 41: 99-106.

Finch-Savage, W.E.; Clay, H.A. (1994) Evidence that ethylene, light and abscisic acid interact to inhibit germination in the recalcitrant seeds of *Quercus robur* L. Journal of Experimental Botany. 45: 1295-1299.

Gamborg OL, Miller RA & Ojima K (1968) Nutrient requirement of suspensions cultures of soybean root cells. Exp. Cell. Res. 50:151.

Garcia-Martin G, Gonzalez-Benito ME & Manzanera JA (2001) *Quercus suber* L. somatic embryo germination and plant conversion: pretreatments and germination conditions. In Vitro Cellular and Developmental Biology-Plant 37: 190-198.

Gingas VM & Lineberger RD (1989) Asexual embryogenesis and plant regeneration in *Quercus*. Plant Cell Tiss. Org. Cult. 17: 191-203.

González-Benito, M.E.; García-Martín. G.; Manzanera, J.A. (2002) Shoot development in *Quercus suber* L. somatic embryos. In Vitro Cellular and Developmental Biology-Plant 38: 477-480.

Kim YW, Lee BC, Lee SK & Jang SS (1994) Somatic embryogenesis and plant regeneration in *Quercus acutissima*. Plant Cell Reports 13: 315-318.

Kim YW, Youn Y & Noh ER. (1995) Somatic embryogenesis and germination from immature embryos of *Quercus variabilis*. Research Report of the Forest Genetics Research Institute Kyonggido 31: 147-152.

L'Helgoual´ch M & Espagnac H (1987) First observations on the adventitious rhizogenic capacity of holm oak (*Quercus ilex* L.) (in French). Annal Sci For 44: 325-334.

Manzanera, J.A.; Astorga, R.; Bueno, M.A. (1993) Somatic embryo induction and germination in *Quercus suber* L. Silvae Genetica. 42: 90-93.

Manzanera JA & Pardos JA (1990) Micropropagation of juvenile and adult *Quercus suber* L. Plant Cell Tissue Organ Cult 21: 1-8.

Manzanera JA, Bueno MA and Pardos JA (1996) *Quercus robur* L. (Pedunculate Oak). In: Bajaj YPS (ed). Biotechnology in agriculture and forestry 35. Trees IV.

(321-341) Springer Verlag, Berlin
Mauri PV (1999) Inducción de embryogenesis somática en encina y estudios sobre los procesos de desarrollo y maduración. Tesis doctoral: 1-235. UPM. Madrid.
Mauri PV & Manzanera JA (2003) Induction, development and maturation of holm oak (*Quercus ilex* L.) somatic embryos. Plant Cell Tissue Organ Cult 74: 229-235.
Murashige T & Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
San José MC, Vieitez AM & Vieitez E (1985) Establishment and in vitro multiplication of shoots from genus *Quercus* (in Spanish). Fiton 45: 31-40.
Sánchez MC, San Jose MC, Ballester A & Vieitez AM (1996) Requirements for in vitro rooting of *Quercus robur* and *Quercus rubra* shoots derived from mature trees. Tree Physiology 16: 673-680.
Schenk RU & Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can. J. Bot. 50: 199-204.
Vieitez AM, Pintos F, San Jose MC & Ballester A (1993) In vitro shoot proliferation determined by explant orientation of juvenile and mature *Quercus rubra* L. Tree Physiology 12: 107-117.

Figure 1. Somatic embryogenesis in Holm oak: (a) Holm oak trees (b) Holm oak acorns (c) Somatic embryos on induction medium. (d) Zygotic embryo in solid medium. (e; f) Somatic embryos matured according to solid maturation medium and (g) liquid medium. (h) Plantlet from a somatic embryo on germination medium and (i) Somatic embryos on germination medium.

Table 1. Composition of the different media used for the different stages of somatic embryogenesis in Holm oak.

	Induction medium (G*) (mg/L)	Maturation medium (SH*) (mg/L)	Germination medium (SH*) (mg/L)
KNO_3	2500	2500	2500
$MgSO_4.7H_2O$	250	400	400
$CaCl_2.2H_2O$	150	200	200
$(NH_4)_2 SO_4$	134	-	-
$NaH_2 PO_4.H_2O$	150	300	300
KI	0.83	0.83	0.83
H_3BO_3	6.2	6.2	6.2
$MnSO_4.H_2O$	16.9	16.9	16.9
$ZnSO_4.7H_2O$	8.6	8.6	8.6
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.025	0.025
$CoCl.6H_2O$	0.025	0.025	0.025
$FeSO_4 7H_2O$	278.5	278.5	278.5
Na_2-EDTA	372.5	372.5	372.5
Nicotinic Acid	0.5	0.5	0.5
Pyridoxine-HCL	0.5	0.5	0.5
Thiamine-HCl	0.1	0.1	0.1
Myo-inositol	100	100	100
Glycine	2.0	2.0	2.0
Sucrose	3 %	3 %	3 %
BA	0.5-10* μM	-	-
NAA	0.5-10* μM	-	-
ABA	-	0.1-1μM	-
Sigma, Type E-agar	0.6	0.6	0.6
pH	5.8	5.8	5.8

* 10.0 μM = for the first month.

* 0.5 μM = for the second month.

Table 2. Effect of plant growth regulator composition on the percentage of somatic embryo induction. in Holm oak. BA: benzyl-adenine; NAA: naphthalene acetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid. Percentages followed by the same letter were not significantly different at the 0.05 level.

Plant growth regulators	**Induction percentage (%)**
10 μM BA + 10 μM NAA	40 a
10 μM BA + 3 μM 2,4-D	30 a
5 μM 2,4-D	20 ab
3 μM 2,4-D	0 b
Control	33 a

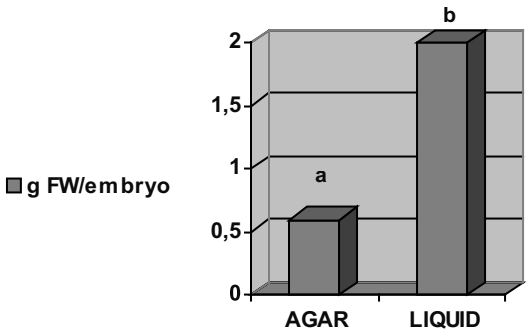

Figure 2. Holm oak somatic embryo mean fresh weight (FW, g) after 30 days in culture, either in agar-solidified (AGAR) or in liquid medium (LIQUID). Treatments with the same letter are not significantly different at the 0.05 level.

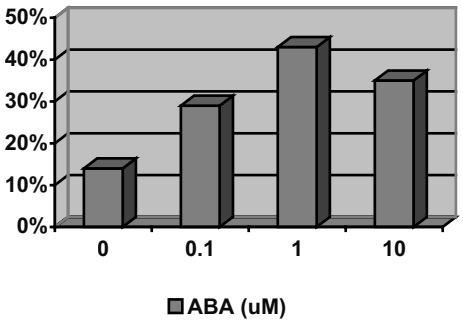

Figure 3. Maturation percentage (%) of Holm oak immature somatic embryos cultured in SH medium with the addition of ABA for 11 subcultures. Treatments with the same letter are not significantly different at the 0.05 level.

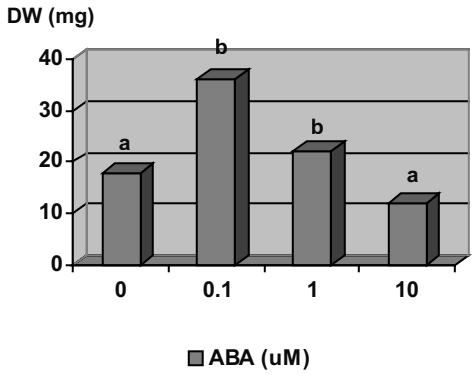

Figure 4. Dry weight (DW, mg) of Holm oak somatic embryos matured in SH medium with the addition of ABA for 11 subcultures. Treatments with the same letter are not significantly different at the 0.05 level.

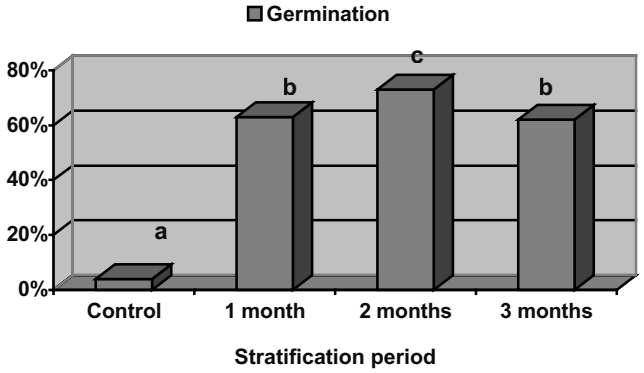

Figure 5. Percentage of germination of Holm oak mature somatic embryos previously cultured on SH medium with the addition of 0.1 μM ABA for 11 subcultures, and then stratified at 4 ºC for different periods. Treatments with the same letter are not significantly different at the 0.05 level.

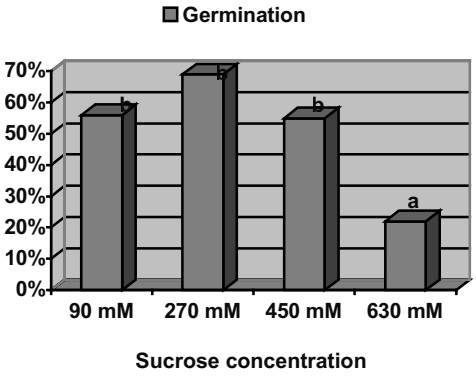

Figure 6. Germination percentage of Holm oak mature somatic embryos subjected to different sucrose concentrations in the germination medium, after two months stratification at 4 ºC. Treatments with the same letter are not significantly different at the 0.05 level.

PROTOCOLS FOR SOMATIC EMBRYOGENESIS OF HYBRID FIRS

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1. INTRODUCTION

Silver fir (*Abies alba* Mill.) is an important forest tree species in Slovakian dendroflora. In recent decades a significant silver fir dieback has been observed, resulting decrease of silver fir proportion in the forest of Slovakia from 9% in 1953 to less than 5% at present (Jásik et al., 1999). Views concerning the nature of this phenomenon are in principle reducible to two theories, one of which emphasizes the ecological aspect of fir forest death, while the other interprets this fact genetically (Kormu ák, 1986). Unfavorable ecological conditions (acid rains, accumulation of toxic metals in plant body) cause physiological weakening of organism which is accompanied by attack of parasitic or saprophytic bacteria and as a consequence desiccation of crowns may appear in trees. The genetic background of silver fir decline is caused by high degree of genetic uniformity has been suggested by Larsen (1989). Artificial hybridization of silver fir with allochthonous representatives of genus Abies is one of the promising and effective ways of enriching fir gene pool and hence increasing of its resistance and growth potential (Kormu ák, 1986; Greguss, 1984; Greguss and Paule, 1989). The hybridisation programme for increasing the resistance and growth potential of *Abies alba* is preferentially oriented at *A. nordmanniana, A. cephalonica, A. numidica* (Kormu ák, 1985; Kormu ák, 1986)

In recent years the hybridization experiments on Slovakian *Abies alba* with different introduced species resulted in obtaining of hybrid embryos. We have attempted to multiply this hybrid material through somatic embryogenesis (Gajdošová et al., 1995; Salajová et al., 1996; Vooková et al., 1998; Salajová and Salaj, 2001; Vooková and Kormu ák, 2003). The aim of this chapter is to give protocols on initiation, maturation and regeneration of hybrid fir embryos obtained in hybridization experiments.

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 483–496.

2. EMBRYOGENIC CULTURE INITIATION

2.1. Initiation from immature embryo explants

To obtain hybrid seeds, the female strobili of *Abies alba* Mill. trees are pollinated with the pollen of *A.cephalonica* Lond. and *A.numidica* deLann at the beginning of May. Artificial pollination of *Abies cilicica* female flowers is also performed at the beginning of May, using freshly collected pollen of *Abies nordmanniana.* The green cones with immature seeds are collected during the maturation period of zygotic embryos (July and August).

The immature seeds are isolated from cones and surface sterilized with 10% H_2O_2 followed by three washing in sterile distilled water. Excised megagametophytes (or developing embryos in one case, August 30) are aseptically dissected and culture on the culture medium.

Table 1 Media for embryogenic callus initiation ($mg.l^{-1}$)

Basal salts	DCR[1]	1/2 LM[2]	SH[3]
pyridoxine	0.5	0.1	0.5
thiamine	1.0	0.1	5.0
nicotinic acid	0.5	0.5	0.5
casein hydrolysate	500.0	1000.0	1000.0
glutamine	50.0	500.0	500.0
glycine	2.0	-	-
myo-inositol	200.0	100.0	1000.0
sucrose	2000.0	2000.0	2000.0
2,4-D	2.0	-	-
NAA	-	2.0	-
BA	0.5	2.0	1.0

[1]Gupta and Durzan (1985), [2]half concentration of salt according to Litvay et al. (1981), [3]Schenk and Hildebrandt (1972)
(From Salajová et al., 1996 - with permission of Springer-Verlag)

For the initiation (hybrids *Abies alba x A. cephalonica* and *Abies alba x A. numidica*) three different media have been used (Table 1). Embryogenic tissue of *Abies cilicica* x *A. nordmanniana* is induced on SH medium supplemented with 4.4μM BA. The explants are cultured in the dark at 25°C. After initiation the embryogenic tissues are separated from original explants and cultured as individual cell lines. For long-term maintenance we use the most successful initiation medium (SH medium with 4.4μM BA). The embryogenic tissues are transferred to new media regularly at 3-4 week interval.

The first proliferation is observed within 12-14 days after placing of explants on the culture media. The tissues appear on the suspensor extrude from megagametophyte (Fig. 1A) and proliferate relatively quickly. When zygotic embryos are isolated from megagametophytes and use them as explants (collection date August 30) the tissue starts proliferate at radicula end. Some embryos produce adventitious buds, mainly from those fail to develop embryogenic tissue. Non-embryogenic callus proliferation, swelling and necrosis of explants are also observable.

The embryogenic tissues apparently differ from hard non-embryogenic callus. The white color and mucilaginous consistence are the main characteristics observed under dissected microscope and transfer procedure. Microscopic examinations after aceto-carmine staining reveal the presence of somatic embryos with embryonal part compose of meristematic cells and long suspensors form by elongated highly vacuolised cells (Fig. 1B).

Table 2 Induction (%) of embryogenic tissue from megagametophytes with immature embryos or from excised cotyledonary zygotic embryos (*) of Abies alba x A. cephalonica (1) and A. alba x A. numidica (2). At least 40 explants for 1 and 45 explants for two in four Petri dishes were used.

Collection date in 1991	SH medium 1 Mean	SE	SH medium 2 Mean	SE	DCR medium 1 Mean	SE	DCR medium 2 Mean	SE
July, 10	38.1	6.9	34.9	9.0	5.3	3.1	0.0	0.0
July, 23	14.4	2.6	44.6	7.8	0.0	0.0	0.0	0.0
August 5	8.3	4.6	0.0	0.0	0.0	0.0	0.0	0.0
August 13	14.1	5.9	0.0	0.0	0.0	0.0	0.0	0.0
August 30*	4.4	2.5	15.8	3.5	0.0	0.0	0.0	0.0

(From Salajova et al., 1996 - with permission of Springer-Verlag)

The initiation is influenced by the developmental stage of zygotic embryo and the composition of culture media. Immature zygotic embryos collected in July give the highest initiation frequencies (Table 2). In August collected embryos, these values go down. In *Abies numidica,* explants collected during August 5 and 13, don't produce embryogenic tissue. We presume that the megagametophyte explants in this developmental stage have limitation of initiation to somatic embryogenesis and therefore, excise zygotic embryos and culture them directly on to the medium.

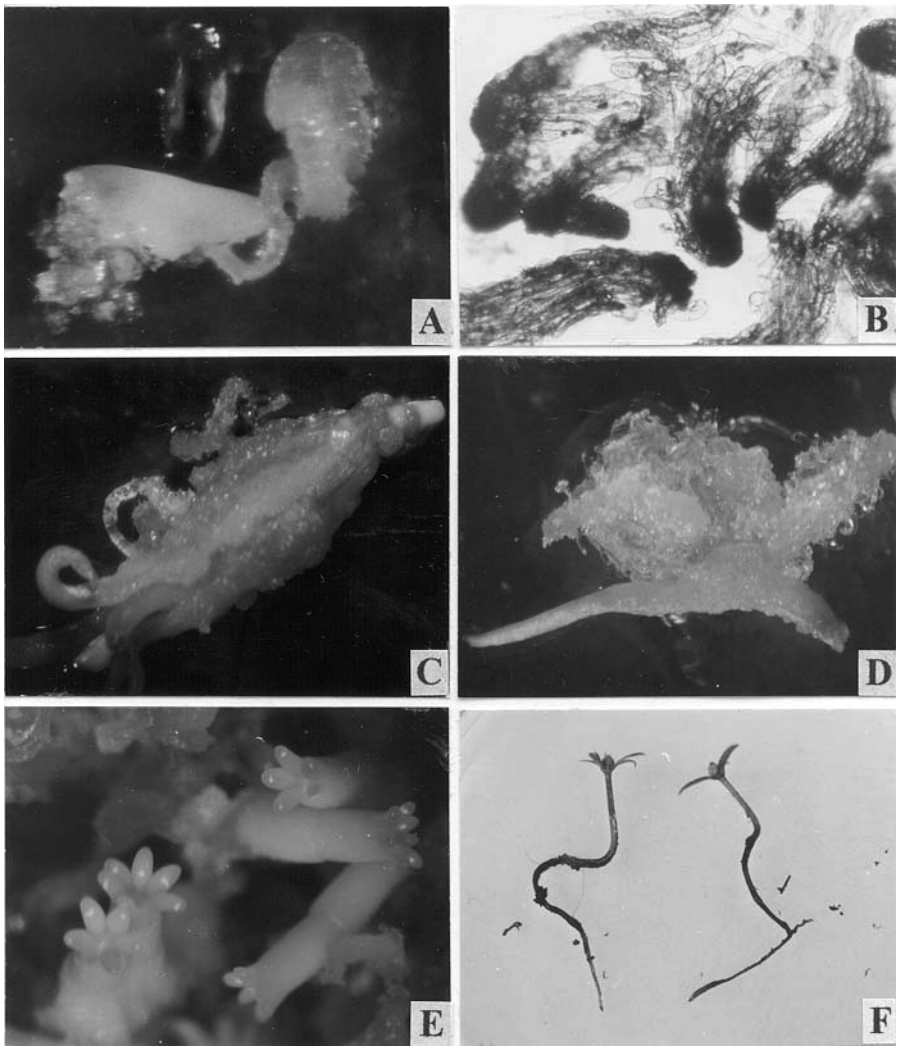

Figure 1 Induction, development and regeneration of hybrid firs somatic embryos. A -Initiation of embryogenic tissue on megagameophyte containing immature zygotic embryo. B - Extrusion of single somatic embryos from hypocotyl of mature zygotic embryo. C -Proliferation of embryogenic tissue initiated on cotyledon dissected from embling (reprinted from Salaj and Salaj, 2003 - with permission of Kluwer Academic Publishers). D - Immature somatic embryos occuring in embryogenic tissue. E - Cotyledonary stage somatic embryos (matured in the presence of 3% sucrose, 7.5% PEG-4000 and 10 $mg.l^{-1}$ ABA). F - Plantlets regenerated through somatic embryogenesis and growing for 4-5 months in soil.

The initiation frequency rate increases again reaching up to 15.8 %. Among media, SH supplemented with 1mg/l gives the best results. Limited success is achieved on DCR medium supplemented with 2,4-D and BA. Medium LM gives no positive results. In our later experiments (1994) explants isolated from unripe seeds collected in July were used. Both media SH and DCR

with different contents yielded embryogenic tissue formation although comparing with results from 1991 the initiation frequencies were lower (Salajová and Salaj, 1996).

Immature zygotic embyos of *Abies cilicica* x *A. nordmanniana* show initiation frequency rate from 3 (July 24) to 27.6% (August 5). Results are presented in Table 3. In July 5 the mucilaginous megagametophytes are unsuitable for experiments. In August 26, the cotyledonary zygotic embryos don't produce embryogenic tissue.

Table 3 Initiation (%) of embryogenic tissue from megagametophytes with immature embryos or from excised cotyledonary zygotic embryos (*) of *Abies cilicica x A. nordmanniana* on SH medium supplemented with 4.4 μM BA .

Collection date in 1997	%	number of explants
July 8	-	-
July 15	6.5	108
July 24	3.0	108
August 5 *	27.6	105
August 26 *	0.0	101

2.2. Initiation from mature zygotic embryo explants

Immature zygotic embryos are excellent explants for the initiation of somatic embryogenesis and as a result the initiation frequencies reach high values. The disadvantage of using these explants is restriction of their availability to very short period during the maturation of zygotic embryos. Mature zygotic embryos are available during the whole year and the seeds can be stored for long period giving explants even after several years of storage.

We have used mature embryos dissected from *Abies alba x A. cephalonica* hybrid seeds stored for different period: 6 months, 12 and 48 months. The seeds are collected in September (the female strobili of *Abies alba* trees are pollinated with the pollen of *Abies cephalonica* at the beginning of May.) The seeds are surface sterilized with 0.1% $HgCl_2$ for 15-30 min. and rinse four times with sterile distilled water. After imbibition for 48 hours in last wash the mature embryos are carefully dissected from seeds and place on the surface of culture medium. Altogether 264 mature zygotic embryos are used as explants. The embryos are dissected from seeds, stored for 6,12, and 48

months. The nutrient medium Schenk and Hildebrandt (1972) containing BA (1 $mg.l^{-1}$) as sole PGR is used in the initiation experiments. In addition, the medium is supplemented with myo-inositol (1000 $mg.l^{-1}$), enzymatic caseinhydrolysate (1000 $mg.l^{-1}$), glutamine (500 $mg.l^{-1}$) and sucrose (2%). The explants are cultured in petri plates and each plate contains 6-7 isolated zygotic embryos.

The initiation of somatic embryos also occurs on the hypocotyl of mature zygotic embryos. White single somatic embryos (Fig. 1C) protrude from hypocotyls around the 20^{th} day of culture and in contact with medium proliferate embryogenic tissue. The embryogenic tissue is separated from explants and culture as an individual cell line. The induction of somatic embryos and non-embryogenic callus occurs on the same explants at the same time.

To trace the origin of somatic embryos histological study, histological examinations show the somatic embryos arise directly on hypocotyls and we conclude that the induction of somatic embryos and non-embryogenic calli initiation are two distinct processes (Salaj and Salaj, 2003).

The initiation frequency rates are 27.2% (embryos from seeds stored for 6 month) and 29% (seeds stored for 12 months), respectively. Embryos isolated from seeds stored for 48 months do not respond. For long-term maintenance the tissues are cultured on the same medium similar to initiation medium.

2.3. Initiation from cotyledon explants of zygotic seedlings

Hybrid seeds of *Abies alba x A. cephalonica*, stored for one year, are surface sterilized; embryos are dissected and cultured on DCR (Gupta and Durzan, 1985) medium without PGR content. The embryos are cultured in the dark, and approximately after two weeks, small seedlings develop. Their hypocotyls grow up to 2.0-2.5 cm long and the length of cotyledons is 6-10 mm. Excise cotyledons and culture on DCR medium containing BA (1 $mg.l^{-1}$), enzymatic casein hydrolysate (1000 $mg.l^{-1}$), glutamine (500 $mg.l^{-1}$), myo-inositol (1000 $mg.l^{-1}$) and sucrose (20 $g.l^{-1}$). The medium is solidified with Phytagel (3 $g.l^{-1}$). The explants are cultured at $23^{o}C$ in the dark. Altogether 103 cotyledons dissected from 21 seedlings have been cultured.

The first embryogenic structures initiate on the cotyledon explants after five months of culture. Although the explants are transferred at 5-6 week interval to new media, some of them turn brown and necrotic. The single structures of somatic embryos soon proliferate white, mucilaginous embryogenic tissue with presence of somatic embryos. Transfer to fresh medium is

helpful for additional initiation on the cotyledon tip. The initiation frequency is very low in comparison with immature or mature zygotic embryo explants. Out of 103 explants only two form embryogenic structures (1.94%). The embryogenic tissues are separated from cotyledon explants and culture them as cell lines AC13 and AC14 on medium DCR containing only BA. The cell line AC14 does not last longer than one year,whereas cell line AC13 is being cultured even after four years of initiation. In both cell lines somatic embryos have been identified using squash preparations and microscopic examinations.

2.4. Initiation from cotyledon explants of emblings

Small emblings have been regenerated from different embryogenic cell lines of hybrid firs. Two of *Abies alba x A. cephalonica* cell lines are derived from immature zygotic embryos (AC78, AC79) and the remaining three (AC1, AC2, AC5) from mature zygotic embryos. The cell line *Abies alba x A. numidica* was derived from immature zygotic embryos (Salajová et al., 1996; Jásik et al., 1999). The embling cotyledons are dissected and place on culture medium of the same composition as used for seedling cotyledons (containing BA as sole PGR). For each genotype, use minimum 78 explants. (Culture conditions see below for seedling cotyledons).

Somatic embryo initiation on embling cotyledons occur earlier as on seedling cotyledons. The cotyledons slightly elongate and swell after 2 weeks in culture. Following, protuberances appear on the cotyledon surface, subsequently giving rise to somatic embryos. The somatic embryos protrude as single structures and soon develop white, soft mucilaginous embryogenic tissue (Fig. 1D). Except the cell line AC5, all genotypes show “secondary” somatic embryogenesis. Initiation frequencies values reach up to 1.25 (AC1) to 24.28% (AC78). The “secondary” embryogenic tissues can be maintained as long-term cultures on DCR medium containing BA (1 $mg.l^{-1}$).

The origin of somatic embryos on cotyledon explant can be followed using histological study. Cell division in epidermal and subepidermal cell layers form meristematic cell clusters, their size increase and gradually develop nodular structures on the surface of cotyledons. The nodular structures subsequently emerge from epidermis. These structures are considered as early developmental stages of somatic embryos owing to the presence of protoderm-like layer. The embryonal “head” is connected with highly vacuolised cells, forming subsequently the suspensor by elongation (Salajová and Salaj, 2001).

3. SOMATIC EMBRYO MATURATION

The embryogenic tissues of *Abies alba x A. cephalonica* and *Abies alba x A. numidica* derived from different primary explants can be used for maturation. Start with the use of ABA (1 and 10 mg.l^{-1}) to stimulate somatic embryo development in embryogenic tissue initiated from immature zygotic embryos. Early stage bipolar somatic embryos develop (in both hybrids) at substantially high frequency rate, and the number goes down when they reach at the cotyledonary stage (Salajová et al., 1996). PEG as a high molecular weight osmoticum improves somatic embryo maturation causing nondestructive plasmolysis (Attree and Fowke, 1993). When PEG-4000 is used (combined with ABA) to improve somatic embryo maturation in five cell lines initiated in 1994 from immature zygotic embryos. The promontory effect of PEG-4000 is apparently positive, although somatic embryo maturation is cell line dependent (Jásik et al., 1999).

When PEG-4000 is used together with different carbohydrates (sucrose, maltose, glucose in conc. 3, 6, 9%) or carbohydrates alone, there is no positive influence of carbohydrates on somatic embryo maturation. PEG-4000 in combination with mentioned carbohydrates gives positive response in developing well-formed cotyledonary somatic embryos (Fig. 1E).

Maturation of somatic embryogenic cultures derived from mature zygotic embryos is done in the presence of ABA (10 mg/l) and PEG-4000 (7.5%). All the five tested cell lines developed cotyledonary somatic embryos capable of germination and plantlet regeneration (Salaj and Salaj, 2003). Similar results have been achieved in cell lines derived from embling ("secondary" tissues) or seedling cotyledons using medium of similar composition (Salajová and Salaj, 2001).

To determine whether *A. cilicica* x *A. nordmanniana* embryogenic tissue respond to maturation treatment 23 cell lines are subjected to maturation treatment. SH medium supplemented with 3% maltose and 10% PEG-4000 is used. Somatic embryos at the cotyledonary developmental stage are seen in 34,8% cell lines. To assess the most beneficial medium for somatic embryos maturation, three cell lines are cultured on SH, GD (Gresshoff and Doy, 1972) and modified MS (Vooková et al., 1998). All media are supplemented with 40μM ABA. The cell lines differed in their response to the three maturation media but, in general, the tendency for the best maturation is on MS medium. GD medium is not suitable because slow maturation rate and embryos reach only at the globular developmental stage. The production of cotyledonary embryos is influenced by the ABA concentration. The addition of 80μM ABA to MS maturation medium (in

comparison to 40μM ABA) has a very significant ($P < 0.01$) positive influence on embryo maturation (Vooková and Kormu ák, 2003).

4. GERMINATION OF SOMATIC EMBRYOS

Select well-developed cotyledonary somatic embryos, characterized by the presence of at least 4 cotyledons for germination. Our first experiments resulted in very week germination frequencies and for both hybrids only 12% cotyledonary embryos formed emblings (Salajová et al., 1996). Relatively higher but still low germination frequencies have been obtained for somatic embryos initiated from mature zygotic embryos (18.75 to 39.28%). In both cases the germination occur without desiccation treatment. Partial desiccation improves germination rate of somatic embryos. Somatic embryos mature in the presence of PEG-4000 combined with maltose or sucrose give germination frequencies 71.35 and 78.62%. Somatic embryos develop in "secondary" cell lines germinate in frequencies 20.26 to 61.48 %. Similar germination frequency (58.86%) has been obtained in AC13 cell line derived from seedling cotyledon (Salajová and Salaj, 2001).

Somatic embryos of *Abies cilicica* x *A. nordmanniana* germinate at different frequencies ranging from 83.61 to 99.60 %. Before germination, selected somatic embryos with four to six cotyledons are desiccated partially for three weeks. The germination SH medium contains ½ strength of SH basal salts, 1% sucrose and 1% charcoal.

Small emblings are transferred to soil and after 4-5 months in the soil they develop a root system (Fig. 1F).

MATERIAL AND METHODS

A. Material

1. Immature female cones, mature seeds
2. Culture media (see Tables 5-6)
3. Sterile water, 10 % H_2O_2
4. Flow hood, petri plates, Erlenmayer flasks, pipettes, forceps, scalpels

Induction

Initiation of embryogenic cultures occurredon SH medium (Table 4).

Proliferation

For proliferation and maintenance, the same medium as for initiation can be used (Table 4). The embryonal suspensor masses (ESM) are sub-cultured at 3-week intervals.

Table 4. Induction SH medium (Schenk and Hildebrandt, 1972)

Constituent	$mg.l^{-1}$
Basal Salts	
KNO_3	2500.0
$MgSO_4.7H_2O$	400.0
$NH_4H_2PO_4$	300.0
$CaCl_2.2H_2O$	200.0
$MnSO_4.H_2O$	10.0
H_3BO_3	5.0
$ZnSO_4.7H_2O$	1.0
KJ	1.0
$CuSO_4.5H_2O$	0.2
$NaMoO_4.2H_2O$	0.1
$CoCl_2.6H_2O$	0.1
$FeSO_4.7H_2O$	15.0
$Na_2EDTA.2H_2O$	20.0
Organic Additives	
Myo-inositol	1000.0
Casein hydrolysate	1000.0
L-glutamine	500.0
Thiamine-HCl	5.0
Nicotinic acid	5.0
Pyridoxine-HCl	0.5
Sucrose	20 000.0
Growth regulator	
6-benzylaminopurine	1.0
Phytagel	3 000.0
pH	5.6-5.8

Maturation

MS medium contains ½-strength MS macro nutrients, except for ¼-strength of potassium nitrate, original micro nutrients and FeEDTA, and modified vitamins (Table 5). All media contain 10 or 20 $mg.l^{-1}$ (40 or 80 µM) ABA, 4% maltose, 10% PEG-4000, casein hydrolysate and L-glutamine each of 500 $mg.l^{-1}$.

Germination and plant regeneration

Prior to germination, isolated mature SE with 4-6 cotyledons are subjected to

Table 5. Modified MS (Murashige and Skoog, 1962) maturation medium

Constituent	$mg.l^{-1}$
Basal salts	
$CaCl_2 .2H_2O$	220.0
KH_2PO_4	85.0
KNO_3	475.0
$MgSO_4.7H_2O$	185.0
NH_4NO_3	824.0
$FeSO_4.7H_2O$	27.85
$Na_2EDTA.2H_2O$	37.25
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
KJ	0.83
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
$Na_2MoO_4.2H_2O$	0.25
Organic additives	
Myo-inositol	100.0
Glycine	2.0
Nicotic acid	1.0
Thiamine-HCl	1.0
Pyridoxine-HCl	1.0
L-glutamine	500.0
Casein hydrolysate	1000.0
Maltose	40 000.0
PEG-4000	100 000.0
Growth regulator	
Abscisic acid	10.0 or 20.0
Phytagel	3 000.0
pH	5.6-5.8

partial drying (Vooková et al., 1998) at 21-23°C for 3 weeks in the darkness. Embryos were then transferred to germination medium containing ½-SH medium salts, 100 $mg.l^{-1}$ myo-inositol, 1% sucrose, 1% charcoal and 0.3% Phytagel. Rooted plantlets are transferred to small pots containing autoclaved peat / vermiculite / perlite mixture.

B. Methods

The plantlet regeneration through somatic embryogenesis includes several steps: initiation of embryogenic tissues, maintenance of embryogenic cultures, maturation of somatic embryos, germination and plantlet regeneration.

Initiation of embryogenic cultures from immature zygotic embryos

1. Remove the immature seeds from green cone.
2. Sterilize the seeds by soaking them 10% H_2O_2 for 10 min. Remove the H_2O_2 solution and rinse the seeds 3-4 times with sterile distilled water.
3. Isolate the megagametophytes containing immature zygotic embryos with forceps and scalpel in sterile conditions and put them on the surface of initiation medium (Table 5). Make sure the explants are in contact with medium.
4. Seal the plates with strips of parafilm and incubate them in culture room in dark at 25˚C.
5. Under stereo-microscope check the cultures regularly for the initiation of embryogenic tissue.

Maintenance of embryogenic cultures

When embryogenic tissue reaches size 5-10 mm, remove it from initial explant and transfer to the fresh medium (the same as for initiation, see Table 5). The embryogenic tissue is white and mucilaginous. Under stereomicroscope the somatic embryos are distinguisable on the surface of the white embryogenic mass (ESM, embryo-suspensor mass). Staining with acetocarmine reveals the morphology of somatic embryos (Fig. 1B). Maintain the cultures by subculturing onto fresh media every three weeks and incubate in dark.

Somatic embryo maturation

For somatic embryo maturation in hybrid firs abscisic acid and polyethylene-glycol (PEG-4000) as well as sucrose or maltose as carbon source were effective. After treatments, cotyledonary somatic embryos developed (Fig. 1E in this chapter).

1. Transfer the embryogenic tissue to maturation medium (Table 6) in petri plates. Seal the plates with strips of parafilm and incubate in dark in culture room at 25 ˚C.
2. In regular intervals check the development of somatic embryos under stereomicroscope.
3. After appearing of the cotyledonary somatic embryos select them by forceps for desiccation. Somatic embryos containing at least four cotyledons should be selected. For selection use stereomicroscope and handle careful to avoid the damage of somatic embryos.

4. For desiccation put the somatic embryos into small petri dish (6 cm in diameter) and do not cover the dish with lid. Transfer the petri dish on moist filter paper placed in another petri dish with 9 cm diameter and seal the bigger petri plate. Incubate in dark at 23 ˚C for 3 weeks.

Germination of somatic embryos

1. Transfer carefully with forceps the partially desiccated somatic embryos on germination medium
2. Incubate the cultures in dark for 4-6 weeks.
3. The somatic embryos germinate and somatic seedlings develop.
4. Transfer the somatic seedlings into small pots containing peat perlite mixture (1:1).
5. Growth the seedlings in pots in greenhouse at temperature 23-25˚C.

5. CONCLUSION AND PERSPECTIVES

Somatic embryogenesis has been initiated from juvenile material of different hybrid firs. Immature and mature zygotic embryos proliferated embryogenic tissues, maintained by regular transfers in long-term cultures. The tissues retain their embryogenic and regeneration capacity even after prolonged period of cultivation (7 to 13 years). The initiation “window” has also been extended to more mature but still juvenile explants of cotyledons dissected from seedlings or emblings. Somatic embryo maturation occurred in embryogenic tissues of different origin (from immature and mature zygotic embryos as well as from cotyledon explants) and as a result emblings have been obtained. The regenerated plantlets have been transferred into soil and survived for several months. These results suggest the possibility of multiplication of hybrid firs material through somatic embryogenesis.

The present research is focused mainly on physiological markers of somatic embryo maturation, because the maturation is a key step in the whole process. More attention will be paid to the transfer of emblings into the soil and their survival in natural conditions. Transformation experiments have been started recently with the aim to introduce GUS-gene into somatic embryos as model plant developmental system, using *Agrobacterium tumefaciens* vector.

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7. REFERENCES

Attree S.M., Fowke L.C. Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. Plant Cell Tissue Org Cult 1993, 35: 1-35

Gajdošová A., Vooková B., Kormu ák A., Libiaková G., Doležel J. Induction, protein composition and DNA ploidy level of embryogenic calli of silver fir and its hybrids. Biol Plant 1995, 37: 169-176

Greguss L. The results of species hybridization within the genus *Abies.* Zprávy Lesníckeho Výskumu 1984, 1: 10-16

Greguss L., Paule L. Artificial hybridization in the genus *Abies*. In: Proc. 5th IUFRO-Tannensymposium. Hochschule für Forstwirtschaft und Holztechnologie Zvolen, VSLD Zvolen. 1989, pp.179-188

Gresshoff P.M., Doy C.H. Development and differentiation of haploid *Lycopersicum esculentum* (tomato). Planta 1972, 107: 161-170

Gupta P.K., Durzan D. Soot multiplication from mature trees of Douglas fir (*Pseudotsuga menzienzii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep 1985, 4: 177-179

Jásik J., Salajová T., Kormu ák A., Salaj J. Somatic embryogenesis in hybrid firs. In: Jain S.M., Gupta P.K., Newton R.J. (eds) Somatic embryogenesis in woody plants. Vol. 4 - Gymnosperms. Kluwer Academic Publishers, Dordrecht pp. 505-523, 1999

Kormu ák A. Study on species hybridization within the genus *Abies*. Acta Dendrologica, Veda Bratislava, pp. 127, 1985

Kormu ák A. Possibilities of extending the silver fir (*Abies alba* Mill.) genepool by means of interspecific hybridization. In: Genepool of forest woody species, its conservation and utilization. Kormu ák A., Užák D.(eds), Veda Bratislava, pp. 341-353, 1986

Murashige T., Skoog F.A. A revised medium for rapid growth and bioassays with tobaco tissue cultures. Physiol Plant 1962, 15: 473-497

Salaj T., Salaj J. Somatic embryo formation on mature *Abies alba x Abies cephalonica* zygotic embryo explants. Biol Plant 2003, 47: 7-11

Salajová T., Jásik J., Kormu ák A., Salaj J., Hakman I. Embryogenic culture initiation and somatic embryo development in hybrid firs (*Abies alba x A. cephalonica* and *Abies alba x A. numidica*). Plant Cell Rep 1996, 15: 527-530

Salajová T., Salaj J. Somatic embryogenesis and plantlet regeneration from cotyledon explants isolated from emblings and seedlings of hybrid firs. J Plant Physiol 2001, 158: 747-755

Schenk R.U., Hildebrandt A.C. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J Bot 1972, 50: 199-204

Vooková B., Gajdošová A, Matúšová R. Somatic embryogenesis in *Abies alba* and *Abies alba x Abies nordmanniana* hybrids. Biol Plant 1998, 40: 523-530

Vooková B., Kormu ák A. Plantlet regeneration in *Abies cilicica* Carr. and *Abies cilicica* x *Abies nordmanniana* hybrid via somatic embryogenesis. Turkish J Bot 2003, 27: 71-76

SOMATIC EMBRYOGENESIS IN SANDALWOOD

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1. INTRODUCTION

Santalum album L., family Santalaceae, is known as Indian sandalwood or chandan. Sandal is a small evergreen tree, partial root parasite attaining a height of 12-13 m and girth of 1-2.4 m. It requires primary host at nursery stage and secondary host or long-term host in the field.

Indian sandalwood is closely tied to the culture and economies of many Asian countries. It has been highly valued for its fragrant heartwood, consist sandal oil used in world-class perfumes, cosmetics and medicine (Ananthapadmanabha, 2000a). Its wood is second only to ivory for use in intricate carvings. Indian sandal consists of highest oil (upto 6 percent) as well as santalol content in oil as compared to other species of genus *Santalum*. The compositional difference in oil and santalol varies with age (Shankaranarayana and Parthasarathi, 1984).

Sandalwood oil has important fixative properties, which is critical in the blending process for perfume manufacture. These fixative properties are due to the chemical composition of two key compounds, □-santalol and β-santalol (Shankaranarayana and Theagarajan, 2000). The predominant markets for Indian sandalwood oil are France, the USA, the UK, and Middle East. It is estimated that 30,000 kg of oil is exported to the US, Europe, Asia and Middle East each year. Both domestic and international demand is increasing, especially in new markets, such as in aromatherapy and pharmaceutical application and cosmetics. Today India is the largest exporter of sandalwood oil supplying around 75% of the world demand. Currently the sandalwood is sold in the international market at US $20,000 or more per tonne. This represents a premium of 40% (Ananthapadmanabha, 2000b).

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Seed is the major way for natural regeneration of sandal. Although germination of seed is profuse, mortality is equally high, due to excessive heat of the sun, drought, fire, grazing, trampling, attack of insects and pests, excessive weed growth (Chadha, 1972). Vegetative propagation through cleft grafting is common method with limited success due to graft incompatibility. Low success has been reported through air layering (Rao and Srimathi, 1977).

Natural sandalwood forests in India have declined dramatically over the past few decades due to unsustainable harvesting. However, spike disease and wide spread smuggling have left India's sandalwood strands dangerously depleted. Some sap sucking insects like *Inglisia bivalvata* cause die back, lessening of flowering and fruits in sandalwood. Lack of understanding of dynamics of parasitism has been the cause of failure of pure plantations in the past (Shobha Rai, 1990). The gap between the supply and demand is widening over the time. Cultivation undertaken by government and the private sectors in India are not adequate to meet future demand. An effort is now needed to increase the area of cultivation and to improve productivity with the aim of sustainable supply (Srinivasan et al., 1998).

Early attempts to develop somatic embryogenesis for sandalwood propagation focussed on indirect embryogenesis using hypocotyl, nodal and endosperm explants (Rao and Rangaswamy, 1971; Bapat and Rao, 1979, Lakshmi Sita et al., 1979; 1980). Rai (1996) and Rai and McComb (2002) reported the regeneration of sandalwood plantlets from somatic embryos developed from mature zygotic embryos of sandalwood through direct somatic embryogenesis. The procedures employed in our laboratory for the induction of somatic embryos and for the conversion of embryos into plantlets will be described in the remains of this chapter.

2. MATERIALS

1. Sodium hypochlorite solution, Tween-20 detergents and sterile water
2. Laminar flow hood, 200-ml screw capped glass jars and 25x120mm culture tubes
3. Mature fruits of sandalwood
4. Dissecting microscope
5. Media

The induction medium is MS (Murashige and Skoog, 1962) basal medium supplemented with 3% (w/v) sucrose (Table-1). The medium is solidified with 0.8% agar and the pH is adjusted to 5.8 with 1N NaoH or 1N Hcl before autoclaving at 121^{o}C for 20 min. Growth regulators are filter sterilized through 0.22μM Millipore filters and added to media after autoclaving.

Table 1: Composition of induction medium (Murashige and Skoog basal medium)

Constituents	Amount in mg/l
In organic	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
KI	0.83
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
$FeSO_4.7H_2O$	27.8
Na_2.EDTA. $2H_2O$	37.3
Organic	
Inositol	100
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Glycine	2.0
Sucrose	3%

3. METHOD

3.1. Explant–type and sterilization

Use mature embryos at cotyledonary stage for initiation of embryogenic culture. Mature fruits are collected from elite trees of *S. album*. The fruits are purplish drupe when fully matured and single seeded

1. Select a fresh stock of sandalwood fruits.
2. The pericarps is cracked open and remove the seed.
3. Seeds are soaked in deionised water overnight at room temperature then disinfected in 2% sodium hypochlorite solution and 0.005% Tween-20 for 10-15 min.
4. Then seeds are washed three times with sterile distilled water.
5. Seeds are air dried in a laminar flow hood for 10 min. prior to dissection and embryo excision.
6. Each sandalwood seed consists of a mature embryo with two cotyledons, a short plumule and a thick radical. Polyembryony seeds are avoided for culture.

3.2. Initiation of embryos

1. Mature zygotic embryos are removed from sterilized seeds with surgical scalpel and are placed horizontally on MS medium with TDZ (4.5μM).
2. Cultures are incubated at 25^{o}C±2^{o}C under a 16-h photoperiod with cool white fluorescent light (40 μmol m^{-2} s^{-1}).
3. After a week in culture on MS containing TDZ (4.5μM), sandalwood embryos commence swelling and hypocotyls and cotyledons turn green.
4. After 20-25 days in culture, small globular structures are conspicuous on the excised embryos (*Figure*1 A). Generally these structures appear on the plumule, hypocotyl as well as cotyledonary part of embryos. This embryo formation occurs without an intervening callus phase on medium with TDZ (4.5μM).

3.3. Embryo development

After 8-10 weeks, globular embryos continue to develop through heart and torpedo stages on the induction medium, resulting in cotyledonary stage embryos (*Figure*1 D-F). Sandalwood somatic embryos are bipolar structures flanked by cotyledons and opaque-white in appearance. The development of somatic embryos is asynchronous. As a result, various stages of embryo development can be observed in the same cluster of embryos originating from the explant. Some of embryos are abnormal. Aberrant embryos have single or multiple cotyledons and few are fasciated. When somatic embryos are maintained on the induction medium with TDZ over a period of 6 months, the occurrence of fasciated embryos is noticed.

3.4. Maintenance of embryogenic competence or secondary embryogenesis

The production of secondary somatic embryos from primary embryos frequently occurs when somatic embryos are maintained for an extended period of 4 months on induction medium with TDZ (4.5μM). Secondary embryos are also produced by isolating primary somatic embryos by using fine forceps from the original mature zygotic embryos and subculture separately (*Figure*1 B). Single primary embryos are cultured horizontally in test tubes (25x100mm) containing 15 ml of MS basal medium. The primary embryos are transferred to fresh medium at 4-week intervals.

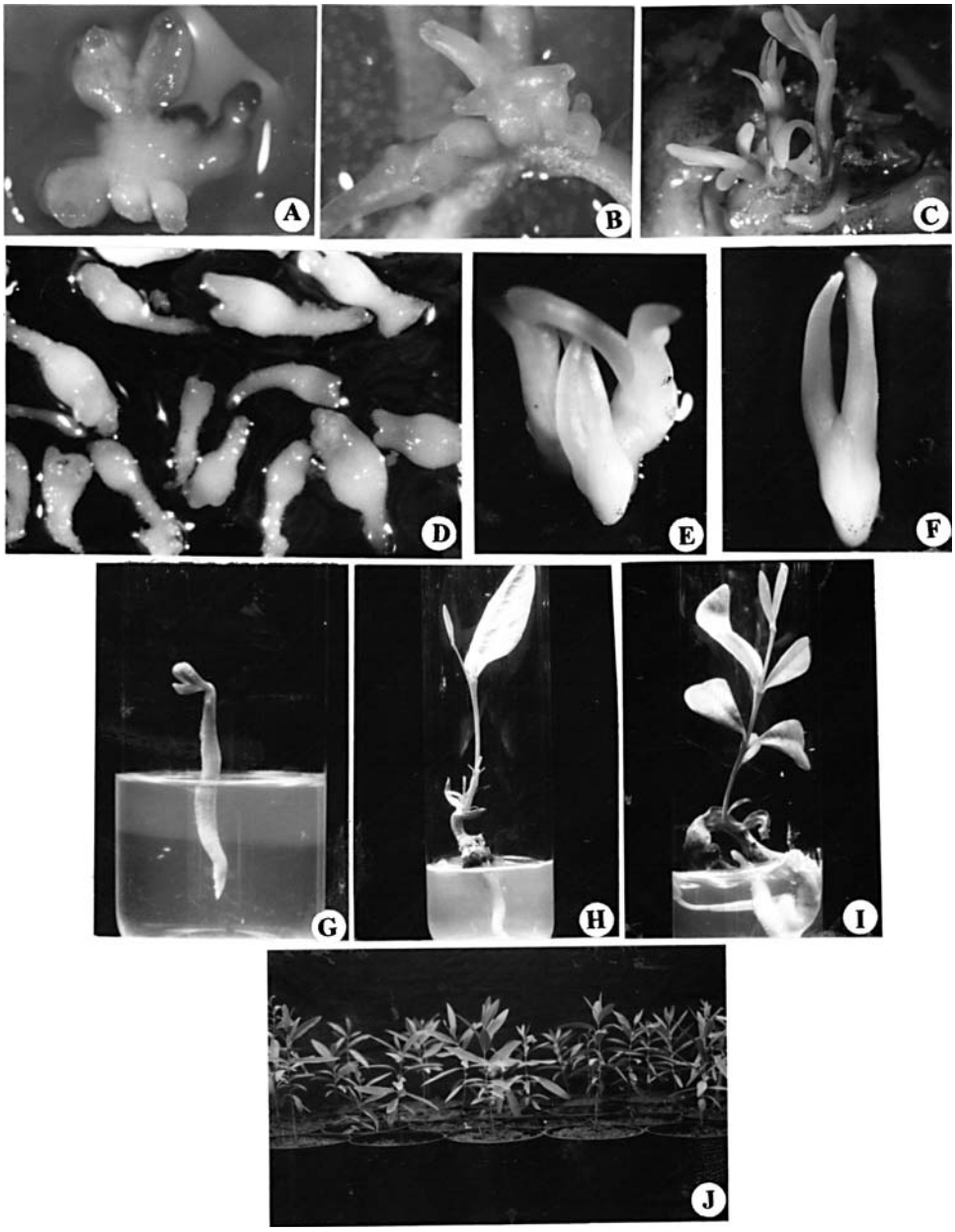

Figure 1. Somatic embryogenesis in *Santalum album*. (*A*) Clusters of globular-stages somatic embryos developing directly from mature zygotic embryos (x6). (*B*) Secondary embryos growing from lower surface of primary somatic embryos (x6). (*C*) Germination of somatic embryos on the explant (zygotic embryo) (x6). (*D*) Isolated somatic embryos (x6). (*E*) Cotyledonary stage somatic embryos with different cotyledon configuration (x12). (*F*) Individual somatic embryo (x12). (*G-I*) Stages of germination of somatic embryos on half-strength basal MS medium with 2.8 μ M GA_3 (*J*) Plants obtained from somatic embryos.

3.5. Staining to confirm embryogenic nature

Histological observation clearly revel that development of embryoids in sandalwood follows a normal embryogical morphogenetic sequence and somatic embryos (primary and secondary) are formed on the explant surface without any vascular connection with the maternal tissue. The dicotyledonary embryos consist two well-developed cotyledons with a prominent shoot and root meristem.

1. For histological examination, samples are fixed in formalin/acetic acid/alcohol (FAA), dehydrated gradually in an ethanol series, then embedded in paraffin wax.
2. Sections are cut at 10-12μm thickness and stained with alcoholic haematoxylin.

3.6. Embryo maturation and embryo germination

1. Using a stereomicroscope, select cotyledonary stage somatic embryos using forceps. Transfer the selected embryos to 25x100mm culture tubes containing 15ml of medium.
2. Embryos are inserted vertically into half-strength MS medium supplemented with GA_3 (2.8μM) and 0.15% Phytagel.
3. Cultures are incubated under same light and temperature regime as used for culture initiation.
4. After 5-6 weeks, embryos germinate and develop into somatic seedlings (Figure 1G-I).
5. When somatic embryos are not excised from the original zygotic embryos before they are transferred to germination media, the hypocotyl part elongate further without forming a well-defined radical (*Figure*1 C).

3.7. Acclimatization and field transfer

1. Regenerated plantlets are transferred to plastic pots (9.0 cm in diameter) containing 1:1 soil and vermiculate and kept in a growth chamber.
2. After a month the surviving plants with three to four green leaves and healthy roots are placed in the greenhouse where they develop further.
3. Then plantlets are transplanted at four to six leaf stage to plastic pots (11.5cm in diameter) containing sand, soil and farm manure with ratio 2:1:1 along with one or two seeds of red gram (*Cajanus cajan*). The seedlings of *Cajanus* serve as primary host.
4. After transplanting the regenerated plants are kept in the greenhouse and watered regularly with care to avoid excess moisture.
5. Host plants are pruned periodically to check their growth.

6. In early stage of development the plants derived from somatic embryos appear morphologically indistinguishable from similarly aged sandalwood plants derived from seed (*Figure*1 J).

4. CONCLUSION AND PROSPECTS

In conclusion, we developed a direct somatic embryogenesis and plantlet regeneration system from zygotic embryos of sandalwood. Direct somatic embryogenesis from mature embryos would seem to offer the possibility for avoiding passage through callus and provides a useful tool for rapid and uniform clonal propagation of selected forest trees. The maintenance of a repetitive secondary embryogenesis process makes available permanent sources of embryogenic material that can be used for the genetic modification of this species. Spike disease caused by mycoplasm is a major threat to sandal populations. Application of biotechnology to improvement in sandalwood mainly aims at evolving trees that can yield more heartwood and are resistant to spike disease and other pests. The protocol developed here for regeneration of sandalwood through direct somatic embryogenesis can possibly be used in introducing genes resistant to spike diseases and pests in sandalwood.

Although high yield of cotyledonary somatic embryos are obtained from mature embryos in sandalwood, embryos development is asynchronous. In order to realize the potential of this micropropagation technique, further research is needed to improve the conversion rate of somatic embryos to plantlets and also to achieve synchronized embryo development. Micropropagated plants regenerated through somatic embryos must be field tested to evaluate the occurrence of morphological and/or genetic changes.

5. REFERENCES

Ananthapadmanabha H.S. The present status of sandalwood in India and Australia. Fafai Journal. 2000a; 3: 33-36

Ananthapadmanabha H.S. Sandalwood and its marketing trend. My Forest 2000b; 36: 147-152

Bapat V.A.. Rao P.S. Somatic embryogenesis and plantlet formation in tissue cultures of sandalwood (*Santalum album* L.) Ann. Bot. 1979; 44:629-630

Chadha Y.R. *The Wealth of India*. Raw materials. Vol 9, Publications and informations, Directorate CSIR, New Delhi, India, 1972; 208-224

Lakshmi Sita G., Raghava Ram N.V., Vaidyanathan C.S. Differentiation of embryoids and plantlets from shoot callus of sandalwood. Plant Sci. Lett. 1979; 15:265-270

Lakshmi Sita G., Raghava Ram N.V., Vaidyanathan C.S. Triploid plantlets from endosperm culture of sandalwood by experimental embryogenesis. Plant Sci. Lett. 1980; 20: 63-69

Murashige T., Skoog F. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 1962; 15: 473-497

Rai V.R. Direct somatic embryogenesis from mature embryos of sandalwood. Sandalwood Research Newsletter 1996; 5: 4

Rai V.R., Jen McComb. Direct somatic embryogesis from mature embryos of sandalwood Plant Cell Tissue and Organ Culture 2002; 69: 65-70

Rao P.S., Rangaswamy N.S. Morphogenetic studies in tissue culture of parasite *Santalum album* L. Biologia Plantarum 1971; 13:200-206

Rao P.S., Srimathi R. A. Vegetative propagation of sandal (*Santalum album* L.). Current Science 1977; 46: 276-277

Shankaranarayana K.H., Parthasarathi K. Compositional differences in sandal oil undergoing color change on standing. Indian Perfumer 1984; 28: 138-141

Shankaranarayana K.H., Theagarajan K.S. Recent developments in chemistry and utilization of sandalwood. Wood News 2000; 5: 17-20

Shobha N. Rai. Status and cultivation of sandalwood in India. USDA Forest Service Gen. Tech. Rep. 1990; 122: 66-71

Srinivasan V.V., Ananthapadmanabha H.S., Rangaswamy C.R. A strategy for sustainable supply of sandal. In: Radomiljac A.M., Ananthapadmanabha H.S., Welbourn R.M., Satyanarayana Rao K. (eds.) Sandal and Its Products (p.22). ACIAR Proceedings, Canberra, 1998

ECHINACEA PURPUREA L.: SOMATIC EMBRYOGENESIS FROM LEAF EXPLANT

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1. INTRODUCTION

Somatic embryogenesis is defined as a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell without vascular connection with the original tissue (von Arnold *et al.*, 2002). Production of somatic embryos from cell, tissue and organ cultures may occur directly which involves the formation of an asexual embryo from a single cell or a group of cells on a part of the explant tissue with or without an intervening callus phase. These embryos usually germinate and grow as a normal plant, however, they may functionally behave as a clone of the parent (same genotype). Starting with the first accounts of somatic embryogenesis in carrot, there have been a steadily increasing number of reports of embryogenic induction from somatic cells of a variety of plants, initially confined to members of the carrot family (Umbelliferae) and later extending to members spread among a number of angiosperm and gymnosperm families.

The genus *Echinacea* (purple coneflower) is represented by 11 taxa found in the United States and in south central Canada (Koroch *et al.,* 2002). Among the genus, *Echinacea purpurea* is the most widespread (McGregor, 1968) and widely cultivated medicinal plant species (McKeown, 1999). *Echinacea* phytopharmaceuticals represent the most popular group of herbal immunostimulants in Europe and USA (Bauer, 1999) and are ranked among the top ten herbal products (Clarke, 1999).

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Regeneration of *E. purpurea* has been accomplished by both somatic embryogenesis and organogenesis (Choffe *et al.,* 2000; Harbage, 2001; Koroch *et al.,* 2002). However, these regeneration systems for *E. purpurea* produce relatively few propagules. Therefore, the current study is designed to investigate the efficiency of several physiochemical parameters with an objective to develop an optimized protocol for somatic embryogenesis of *E. purpurea*.

2. MATERIAL AND METHODS

2.1 Seed sterilization

Seeds of *Echinacea purpurea* L. (Richters, The Herb Specialists, Goodwood, ON) are surface sterilized by immersing in 70% ethanol for 30 s and soaking in a 5.4% sodium hypochlorite solution (Colgate-Palmolive Inc., Toronto, ON) containing one drop of Tween 20 per 500 ml for 20 min, followed by three rinses in sterile deionized water.

2.2 Establishment of sterile seedlings

Twenty disinfected seeds are cultured in 100 x 50 mm disposable Petri dishes containing 25 ml of culture medium comprised of half-strength MS salts (Murashige and Skoog, 1962), half-strength B5 vitamins (Gamborg *et al.,* 1968), 20 g l^{-1} sucrose and 1.0 ml l^{-1} of PPM (Preservative for Plant Tissue Culture Media; Plant Cell Technology Inc., Washington DC). The pH is adjusted to 5.7 and the medium is solidified with 8.0 gl^{-1} agar (Fisher Scientific, Nepean, ON) prior to autoclaving at 1.4 kg cm^{-1} for 20 min. Sterile seeds are germinated in a growth cabinet in darkness at 24 ^{o}C. After 14 d, four seedlings are subcultured per Magenta vessel (Magenta Corporation, Chicago, IL) onto a basal medium (MSO) containing full strength MS salts and B5 vitamins, 30 g l^{-1} sucrose and 3.0 g l^{-1} Gelrite (Sigma Chemical Co, St. Louis, MO) with the pH adjusted to 5.7. Cultures are incubated in a growth cabinet with 16 h photoperiod (30-35 µmol m^{-2} s^{-1}) at 24^{o}C for 2 months.

2.3 Somatic embryogenesis

Explants of leaves (20 x 10 mm) of 2-month-old seedlings with 4-5 unfolded leaves of *Echinacea purpurea* are excised and sub-cultured onto MSO supplemented with benzylaminopurine (BA; 0 – 50µM), indolebutyric acid

(IBA; 0 – 2.5μM), thidiazuron (TDZ; 0 – 10μM) and indoleacetic acid (IAA; 0 – 0.25μM) (for exact concentrations see Table 1). Cultures are incubated in a growth cabinet at 24°C in the dark for the first 0, 7, 14 or 28 d followed by 16 h photoperiod under cool white fluorescence lamps (PPF 30 μmol m^{-2} s^{-1}). Regeneration is quantified after 28 d as per cent explant response and number of embryos per explant. Each treatment consists of 10 replicate dishes each containing 6 explants and all experiments are repeated twice.

2.4 Germination of somatic embryos

After 35 d of culture, regenerated somatic embryos from optimal treatments are transferred into Magenta vessels containing MSO. The percentage germination of embryos is quantified after 1 week (day 42).

2.5 Rooting and greenhouse transplantation

To accelerate *in vitro* rooting, germinated embryos are transferred onto MSO (60 ml glass tubes; Fisher Scientific, Nepean, ON) with various auxins, naphthaleneacetic acid (NAA, 0–20μM), IAA (0–20μM) and IBA (0–20 μM) (Table 2). The percentage of embryos that formed roots, the number of roots per plantlet and root lengths are quantified after 21 d of culture on auxin-supplemented media. Twenty embryos are cultured, one embryo per tube, for each treatment and the experiment is repeated twice. Plantlets are transplanted in greenhouse and are assessed for viability and survival.

2.6 Statistical analysis

Statistical significance is determined by one-way analysis of variance (ANOVA) using the General Linear Model Protocol of SAS (SAS Ver. 6.12, SAS Institute Inc., Cary, NC). Differences between means are assessed with a Student Newman-Keulls means separation test.

3. RESULTS

Somatic embryogenesis is observed in leaf explant initiated mainly on the cut-edge of the leaves within 3 weeks of culture. An initial 14-d of continuous dark treatment followed by transfer to 16h photoperiod resulted in the development of red pigment on the cells of the cut-edge of the leaves within 2-3 d of exposure to light (day 16-17 of the treatment). Globular stage somatic embryos are observed by day 19-20 of the treatment (Fig. 1). Within

a few days these globular somatic embryos are observed to develop into cotyledonary stage embryos (Fig. 1) and spontaneously germinated within 34 d of culture with root development from the base of the embryo.

Incubation of cultures in the dark for an initial period of 14-d significantly increased the number of somatic embryos formed in leaf explant cultures with an 8-fold increase (from 10 to 83) with 5.0μM BA + 2.5μM IBA and a 6-fold increase (from 11 to 65) with 10μM BA + 2.5μM IBA compared to those without any dark incubation (Fig. 2).

Exposure of explants to the medium with cytokinin (BA) as the sole plant growth regulator resulted in embryo development although the frequency is low (2-7 embryos per explant) (Fig. 3). The presence of exogenous auxin (2.5μM IBA) significantly increased this cytokinin induced somatic embryogenesis. The optimal medium for induction of somatic embryogenesis is 5μM BA + 2.5μM IBA with an average of 83 embryos per leaf explant after 28 d (Fig. 3). Exposure of leaf explants to 10μM BA + 2.5μM IBA and 20μM BA + 2.5μM IBA resulted in approximately 60 embryos per explant (Fig. 3a). The higher concentrations of cytokinin (10 or 20μM BA) delayed somatic embryo development by 10-15 d (Table 1).

Other cytokinins are also compared for their efficacy for the induction of somatic embryogenesis in *E. purpurea.* When TDZ is used either alone or in combination with IAA, significantly fewer (<20) somatic embryos are observed from leaf explants and embryo development is delayed by 7-10 d (Table 1) compared to the 5μM BA + 2.5μM IBA treatment. The number of somatic embryos per leaf explant increases with time and reach a maximum of *ca.* 35 per explant on day 42 in the TDZ + IAA treatments. Callus is observed on leaf explants cultured with 1.0μM TDZ and after 45 d of treatment and an average of 20 somatic embryos are developed per explant either directly from the leaf surface or from the callus.

Orientation of the explants onto the medium is an important factor for the induction of somatic embryogenesis from leaf explant (Fig. 3). Direct contact of the abaxial surface of leaves to the medium significantly increases the number of embryos as compared to direct contact of the adaxial surface with the medium. Explants cultured on the medium containing 5μMBA + 2.5μM IBA, develop somatic embryos both on cut edges and on the surface of the leaves when the abaxial surface of the leaf discs is in contact with the medium. In contrast, cultures with the adaxial surface exposed to the medium, the average number of somatic embryos significantly decreased and

somatic embryo development is limited mostly to the cut-edge of the leaves (Fig. 3). Similarly, in the 10µM BA + 2.5µM IBA treatment, the number of somatic embryos on abaxial surface of leaf explants is double that observed with adaxial surface in contact with the medium (Fig. 3).

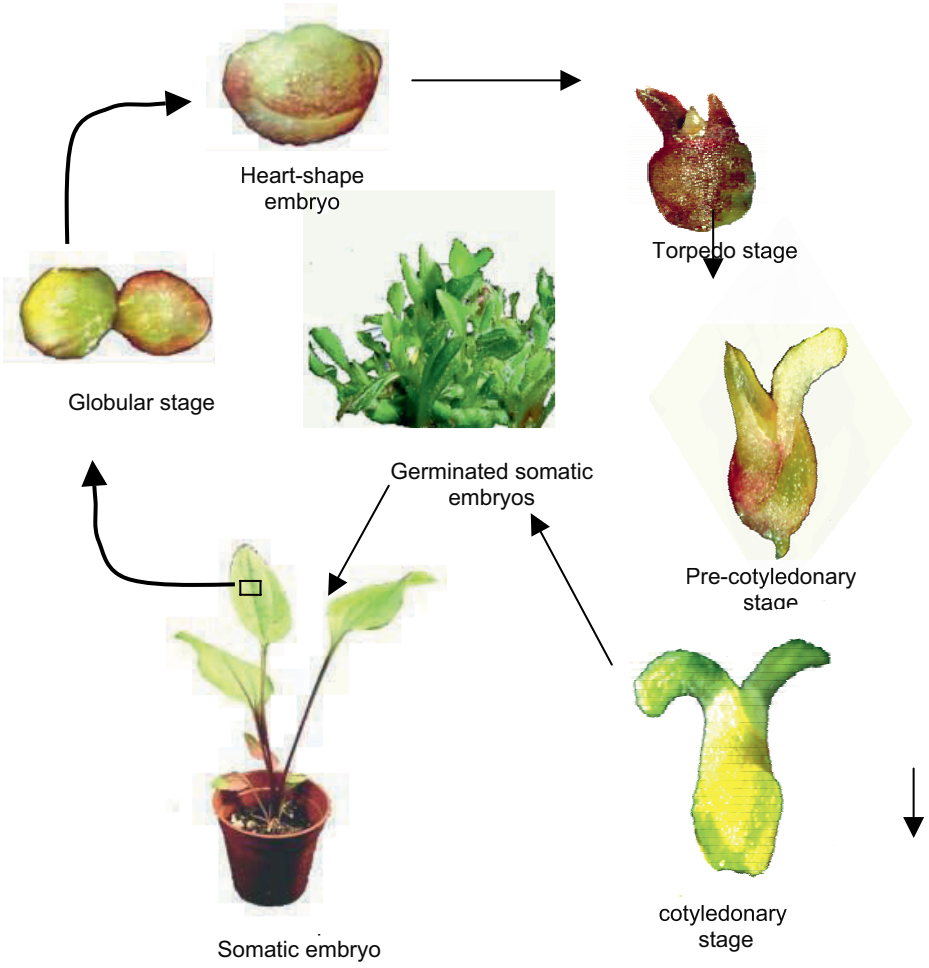

Fig. 1. Different stages of somatic embryo developed from leaf disc cultures.

Table 1. Percentage of explants forming embryos and number of days until embryo initiation is observed on leaf explant as affected by different concentrations of growth regulators added in the MS medium. Cultures are incubated in dark for initial 14-d. Data are collected after 4 weeks of culture; each value represents a mean±SE of 60 replicates.

Growth regulators	Leaf	
	Percentage of explants producing embryos	Days to embryo initiation
BA 1.0 µM	34±6g	19-24
BA 5.0 µM	43±5f	19-24
BA 10 µM	41±3.3fg	19-24
BA 20 µM	23±2.7i	19-24
BA 30 µM	29±2.1h	19-24
BA 5.0 µM + IBA 2.5 µM	95.1±4.6a	14-21
BA 10 µM + IBA 2.5 µM	97.8±3.6a	14-21
BA 20 µM + IBA 2.5 µM	90.5±4.1b	14-21
BA 50 µM + IBA 2.5 µM	68.8±7.2de	14-21
TDZ 0.1 µM	76±14d	21-28
TDZ 0.25 µM	56±23ef	21-28
TDZ 0.5 µM	84±6c	21-28
TDZ 1.0 µM	79.8±7.4c	21-28
TDZ 2.5 µM	47.8±1.4f	21-28
TDZ 5.0 µM	72.3±12.5d	21-28
TDZ 10 µM	63.4±15e	21-28
TDZ 0.1 µM + IAA 0.1 µM	96±5.9a	21-28
TDZ 0.25 µM + IAA 0.1 µM	66±13de	21-28
TDZ 0.25 µM + IAA 0.25 µM	82±12c	21-28
TDZ 0.5 µM + IAA 0.25 µM	88±5.9c	21-28
TDZ 1.0 µM + IAA 0.25 µM	72±10.3d	21-28

Significant differences between the treatments at $P \leq 0.05$ determined by Student-Newman-Keuls test within columns.

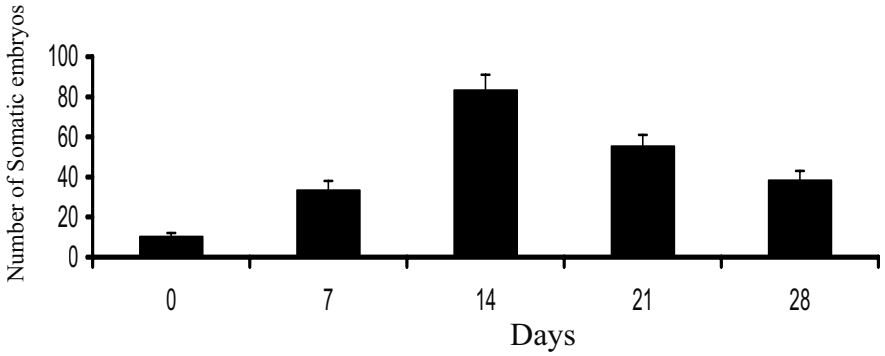

Fig. 2. Effect of dark incubation on the number of somatic embryo formation from *Echinacea purpurea* leaf explant

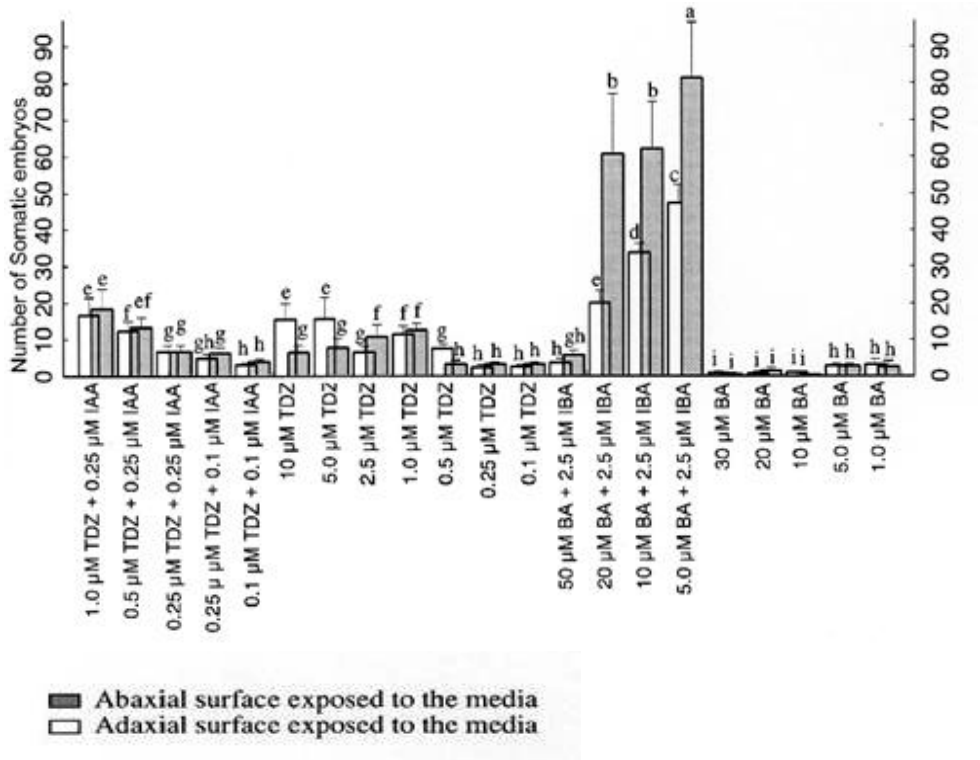

Fig. 3. Total number of somatic embryos developed from leaf disc explant of *Echinacea purpurea* after 28 d of culture on medium supplemented with various growth regulators. Cultures are incubated in the dark for 14-d and later transferred to light (16h photoperiod). Each bar represents mean±SE of 60 replicates (after Zobayed and Saxena, 2003).

About 38% of the somatic embryos induced by the 5μMBA + 2.5μM IBA treatment germinated spontaneously by day 34 and without transfer onto the basal medium. In total, about 80% somatic embryos induced with 5μMBA + 2.5μM IBA germinated within 7 d (day 42) of transferring onto a medium devoid of plant growth regulators. Similarly, somatic embryos induced by exposure to 10μM BA + 2.5μM IBA spontaneously germinated with a frequency of 36%.

Table 2. Development of roots from germinated somatic embryos of *Echinacea purpurea* as affected by different concentrations of an auxin added to the MS culture medium. Data are collected after 4 weeks of culture; each value represents a mean±SE of 10 replicates.

Growth regulators	Concentration (μM)	Percentage of explants producing roots	Length of main root (mm)	Number of roots per explant
IBA	1.0	0	-	-
IBA	2.5	0	-	-
IBA	5.0	23	11.4±0.7b	5.2±1c
IBA	10	20	10.0±0.4b	3.5±1cd
IBA	20	12	5.0±0.4d	1.2±0.3d
NAA	1.0	0	-	-
NAA	2.5	40	8.3±1bc	6.5±0.6bc
NAA	5.0	49	8.0±1c	5.6±1c
NAA	10	73	13.1±1.1a	9.8±1.1a
NAA	20	69	7.2±1c	4.7±0.4c
IAA	1.0	0	-	-
IAA	2.5	10	7.5±0.5c	6.5±0.7bc
IAA	5.0	25	5.5±0.3d	5.5±0.3c
IAA	10	60	6.9±0.5c	7.3±0.4b
IAA	20	41	5.3±0.5d	4.1±0.7c
No added growth regulator		27	3.2±0.1e	2.1±0.2d

Significant differences between the treatments at $P \leq 0.05$ determined by Student-Newman-Keuls test within columns.

Germinated somatic embryos are cultured on a medium containing different concentrations of auxins in order to accelerate root formation. The optimal medium for rooting contained 10μM NAA producing 9.8 roots per plantlet and 73% of the somatic embryos developed roots within 28 d of culture

(Table 2). The length of roots is also significantly higher in the 10µM NAA treatment (average 13 mm) compared to the growth regulator free MS medium (3.2 mm).

Plantlets are assessed for viability and survival after transplanting. The plantlets transplanted *ex vitro,* showed leaf wilting within an hour but these affected leaves recovered within 3 d of acclimatization period. More than 90% of the transplants established within 6 weeks of transplanting *ex vitro* and the plants are successfully grown to maturity in the greenhouse.

Fig. 4. Somatic embryo derived *E. purpurea* plants established in greenhouse (3 months after transplanting).

Result indicates that the somatic embryogenesis is observed when the cytokinin BA is combined with an auxin, IBA. The best response for the development of somatic embryogenesis is observed on a medium with both a cytokinin (BA 5µM) and an auxin (IBA 2.5µM). Other auxins are less effective in this regard as well other cytokinins are not as effective as BA for induction of somatic embryogenesis. BA-induced somatic embryogenesis in *E. purpurea* is also reported by Choffe *et al.* (2000) with a limited number of embryos developing. The Callus induction is noted in the presence of TDZ

and somatic embryos are developed from these calluses although the frequency of TDZ-induced somatic embryogenesis is lower. Induction of embryogenic callus by TDZ may be useful in development of callus suspensions and recovery of genetic variability in *E. purpurea.* In the present experiment a high percentage (80%) of somatic embryos are germinated and rooted. Plantlets developed from these embryos are successfully established in the greenhouse (Fig. 4).

Therefore, the somatic embryogenesis protocol developed in this study can be summarized as follows:

Step 1: Collect leaf from *in vitro* grown plantlet or 2-month-old seedlings and excise the explants (20 x 10 mm).
Step 2: Subculture the excised leaf explants into plastic Petri dishes containing MSO medium (MS medium with B5 vitamins) supplemented with benzylaminopurine (BA; 5.0μM), indolebutyric acid (IBA; 2.5μM).
Step 3: Incubate the cultures at 24^{o}C in the dark for the first 14 d followed by 16 h photoperiod under cool white fluorescence lamps (PPF 30 μmol m^{-2} s^{-1}).
Step 4: After 35 d of culture, transfer the regenerated somatic into Magenta vessels containing growth regulator free MSO medium.
Step 5: To accelerate *in vitro* rooting, transfer the germinated embryos onto MSO with 10μM naphthaleneacetic acid (NAA).
Step 6: Transplant the rooted plantlets after 28 d in the greenhouse.

4. CONCLUSION

Echinacea phytopharmaceuticals represent the most popular group of herbal immunostimulants in Europe and USA (Bauer, 1999) and are ranked among the top ten herbal products (Clarke, 1999). *In vitro* techniques can facilitate genetic manipulation of secondary metabolites (Koroch *et al.,* 2002). Regeneration protocol developed in the present study provides evidence of the induction of an endogenous, light sensitive mediator of plant morphogenesis and an efficient, high frequency propagation system that may be adapted for commercial production of *Echinacea*. The regeneration efficiency of this protocol is significantly higher than those reported earlier of inducing somatic embryos (Choffe *et al.,* 2000) or shoot organogenesis (Harbage, 2001; Koroch *et al.,* 2002). In addition, the regeneration system

may be useful in broadening the pool of genetic variability in *Echinacea purpurea* through genetic engineering using *Agrobacterium* or other methods of gene transfer.

5. REFERENCES

Bauer, R. 1999. Chemistry, analysis and immunological investigations of *Echinacea* phytopharmaceuticals. In: Wagner, H., ed. Immunomodulatory agents from plants. pp. 41-88, Birkhauser Verlag, Basel.

Clarke, A. 1999. Healthy and tasty. Too good to be true? J. Int. Food Ing. 5:54-56.

Choffe, K. I., Victor, J. M. R., Murch, S. J., Saxena, P. K. 2000. *In vitro* regeneration of *Echinacea purpurea* L.: Direct somatic embryogenesis and indirect shoot organogenesis in petiole culture. In Vitro Cell. Dev. Biol. – Plant. 36: 30-36.

Gamborg, O.L., Miller, R.A., Ojima, K. 1968. Nutrient requirement of suspention cultures of soybean root cells. Exp. Cell Res. 50:150-158.

Harbage, J. F. 2001. Micropropagation of *Echinacea angustifolia, E. pallida,* and *E. purpurea* from stem and seed explants. HortSci.. 36:360-364.

Koroch, A., Juliani, H. R., Kapteyn, J., Simon, J. E. 2002. *In vitro* regeneration of *Echinacea purpurea* from leaf explants. Plant Cell Tiss. Org. Cult. 69:79-83.

McGregor, R. L. 1968. The taxonomy of the Genus *Echinacea* (Compositae). The University of Kansas Science Bulletin. Vol XLVIII: 113-142.

McKeown, K. A. 1999. A Review of taxonomy of the Genus *Echinacea.* In: Janick, J. ed. Perspectives on New Crops and New Uses. pp. 482-490, Purdue University, West Lafayette, USA.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. 15: 473-497.

von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., Filonova L. 2002 Developmental pathways of somatic embryogenesis. Plant Cell Tissue and Org. Cult. 69: 233-249.

Zobayed, S.M.A., Saxena, P.K. 2003 Auxin and a dark incubation increase the frequency of somatic embryogenesis in *Echinacea purpurea* L. In Vitro Cell. Dev. Bio. - Plant. 39: 605-612.

HISTOLOGICAL TECHNIQUES

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1. INTRODUCTION

Manipulation of regeneration systems forms the basis of modern agriculture and horticulture. Mass propagation systems that allow for large scale multiplication of elite germplasm, development of virus-free stocks, and creation of genetic transformation systems rely on the capacity to regenerate plants (Cassells 2001). Each of these practical applications involves stimulation of organized development through differentiation of specific tissues (Thorpe 1994). Exposure of excised plant tissues to plant growth regulators results in de novo development of organs both *in vivo* and *in vitro*. This principle has been demonstrated innumerable times and the phenomenon forms the basis of modern asexual propagation industries wherein cuttings are exposed to commercial auxin preparations and induced to root in artificial media (Cameron et al. 2003). In addition, various experimental systems have been used to demonstrate that auxin, alone or in combination with other plant growth regulators, regulates cellular processes such as cell division, elongation and differentiation *in vitro*. There are literally tens of thousands of reports of the regeneration of tissues, organs, and plants in a wide variety of wild and cultivated species. *In vitro* regeneration is achieved by organogenesis (shoot and root development) or somatic embryogenesis that results in production of asexual embryos that are analogous to zygotic embryos (Raghavan 2000, Thorpe 1994, Thorpe and Stasolla 2001). Regeneration can occur directly on the explants or through an intermediary callus. Technologies for induction of somatic embryogenesis facilitates large-scale multiplication, selection of unique germplasm via mutation of single cells, in addition to the possibility of developing artificial seeds. The advantages of artificial seeds (somatic embryos desiccated to about 10% moisture) include genetic and physiological uniformity, bulk production, transportation, storage, as well as the potential for mass production of foreign proteins in transgenic embryos (Redenbaugh 1993). Documentation of events of cell division and differentiation at histological, physiological, and molecular levels is essential for advancement of fundamental and applied aspects of plant

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development. This chapter outlines basic histological techniques to study the process of morphogenesis with emphasis on somatic embryogenesis.

2. KNOW YOUR SYSTEM

A structural study is an important first step in the study of *in vitro* organized development. Different histological techniques have contributed significantly to our understanding of *in vitro* embryogenesis. However, before embarking on a histological study, it is important that a good understanding of the system is attained and culture conditions have been optimized. Careful observation of the changes of the explants during the course of the study will pinpoint the area in which events begin. This information is necessary because it identifies the area of activity and, thus, the focus for the study of structural changes during the course of somatic embryo development. Furthermore, optimization of a culture system is essential as this will provide a semi-quantitative evaluation of the developmental events. This information, such as the frequency and quality of somatic embryo formation *in vitro* will complement observations obtained from histological studies.

The careful examination of live cultures is the best way to understand a tissue culture system. For suspension cultures, cells and cell clusters can be examined either by using an inverted microscope or by using a stereomicroscope. If bulky explants are used as a source of embryogenic tissue, simple dissection, free hand sections and staining (Yeung 1998) can be carried out to take a closer look at the system. Photomacrography method (Bracegirdle 1995) is an efficient mean to document changes during the course of *in vitro* development (see next section).

3. MACROSCOPIC OBSERVATION TECHNIQUES

The use of photomacrographs is one of the best ways to document changes within a culture system. A good quality stereomicroscope or inverted microscope with a film or digital camera attached is often sufficient for recording macroscopic changes of the explants or cultures. Nowadays, the use of a good quality digital camera with a macro-lens can also be used to take pictures of the bulkier explants without the need of a microscope. Proper lighting during photography can be provided by a fiber optics lighting system or simple household halogen lamps. In photomacrography, it is possible to create a system that suits one's need without incurring major expenses.

For recording the information using a film camera system, different photographic films can be used. Since all print films are "daylight" film, an appropriate "daylight conversion filter" must be used if tungsten or halogen lamps are used as a light

source. The daylight conversion filter can be placed within the fiber optics lamp housing or put somewhere in front of the optical lens. For slide films balanced for tungsten light, a daylight conversion filter is not necessary. If a digital camera is used, a daylight conversion filter is not necessary. The images captured can be transferred to a computer and further manipulated using common software such as the Adobe Photoshop® or the software provided with the purchase of a digital camera. The selection of a camera system depends on one's resources. In our laboratories, the film camera system is preferred for normal bright field photography. We feel that the usual film camera provides a better depth of field and the cost is less than setting up a digital camera system and related hardware for printing.

For photomacrography, in order to obtain better contrast of the specimen, one simple procedure is to prepare "black" agar plates. Since a majority of somatic embryos are creamy yellow in colour, the black background provides an excellent contrast to the specimens. The black agar plates are prepared by adding a few grams of powdered charcoal to a 100 ml of 2% agar solution. A number of plates can be prepared ahead of time and stored in the refrigerator until needed. Certainly, other colour contrast backgrounds can also be used such as using different colored cardboard against the transparent agar plate.

4. PLANNING AND PREPARATION FOR A HISTOLOGICAL STUDY

Histological studies are useful in providing information concerning structural changes during the course of a developmental event such as somatic embryogenesis. A histological study, when properly performed, is an extremely labour intensive process. It involves the collection and fixation of a large number of explants/embryos over the entire course of their development. The quality of the structural study can easily be judged by the quality of micrographs presented.

Depending on the question of interest, once the system is optimized, one can begin to plan a histological study. In planning for this type of study, one has to determine the number of explants/embryos needed. The total number depends on the number of sampling points. Changes usually begin to occur immediately after the placement of explants on an induction medium. In our laboratories, we usually sample frequently during the first few days of culture and then gradually lengthen the sampling time until such time as the appearance of somatic embryos, etc. For each sampling point, a minimum of 10 explants or at least 50 somatic embryos should be processed. As a result, for each experiment, more than a hundred explants or somatic embryos are needed. Control treatments, e.g., explants cultured in

maintenance medium without growth substance, should also be fixed and processed at the same time. The entire procedure should be repeated at least once to ensure reproducibility of the observations.

5. THE PREPARATION OF TISSUES FOR PARAFFIN AND PLASTIC EMBEDDING

• General comments

The first step in the processing of a tissue sample is fixation. Fixation is the most important step in the entire process. The quality of the histological preparation depends on how the sample is fixed. The sample should be harvested and fixed at the same time (Berlyn and Miksche 1976). It is important that investigators have a good understanding of the principles of fixation and the chemical properties of the fixing agents used in the fixative. For a critical discussion on the properties of fixing agents used in plant tissues, readers are referred to the articles by O'Brien et al. (1973), Mersey and McCully (1978), Coetzee (1985), Jakstys (1988) and Dong et al. (1994). Many recipes for fixation are available in the literature. It is important to remember that recipes are not formulae. Recipes can be changed or modified to suit one's own need. The willingness to try and experiment using different recipes is the key to a good quality histological study.

In a tissue culture laboratory, one should never store fixatives and other histological and histochemical reagents with tissue culture chemicals. For example, aldehydes are common components of fixatives, their fumes can interact with and destroy many expensive chemicals used for tissue culture. It is important to designate a refrigerator to store chemicals, solutions related to histological preparations and fixed samples. Furthermore, many reagents used for histology can be highly toxic. It is essential that one has a proper understanding of properties of the chemicals used and takes proper precautions during the preparation and handling of such chemicals. It is also preferable not to mix tissue culture and histological glassware in order to avoid unnecessary contamination of histological chemicals.

Trimming of samples is an important step during fixation. Cuticular material is usually present on cells exposed to air. This could pose problems during fixation and subsequent processing and sectioning of these embryos. Trimming of samples removes some surface cells and this will also aid in the penetration of fixative into the tissue. Because of the small size, somatic embryos can be fixed whole without the need of trimming. However, if the quality of fixation is not satisfactory, one should consider careful trimming of somatic embryos at the time of fixation. Although small embryos appear to be soft and easy to process, cuticular material is

often present at the surface of the embryos. If trimming of embryo is required, it is advisable to cut the embryo into two unequal halves. Fix the larger half as it contains the undamaged shoot and root apical meristems. The other added advantage of trimming at the time of fixation is that this step also determines the orientation of the embryo for embedding and subsequent sectioning. Proper orientation is the key in obtaining a median section through the embryo with both apical meristems in the same section. This will reduce the number of slides needed for the project.

For adequate fixation and dehydration of samples, approximately 10 volumes of fixative/dehydrating solution should be used for 1 volume of tissue. The timing of fixation and subsequent processing steps depends on the size and the density of the tissues. Some somatic embryos may accumulate a large amount of storage materials. The dense storage products could pose problems for the infiltration of the embedding medium. In general, since somatic embryos are usually small in size, 4-6 hours is usually adequate for each step of the fixation and dehydration process. The infiltration time for the embedding medium should be lengthened in embryos with a large amount of storage products.

Paraffin wax and glycol methacrylate based formulations are common embedding media used for light microscopy. In general, it is less expensive to prepare specimens using the paraffin embedding procedure as more sophisticated equipment such as the Ralph knife maker is not required. However, because of the inherent problem of fixation and the requirement to infiltrate wax at about 60ºC, some cytological features are not well preserved, especially the distribution of vacuoles. If wax is the method of choice, it is imperative that different fixatives be tested, the processing time for dehydration and wax infiltration should be optimized to avoid unnecessary shrinkage of cells due to prolong extraction of macromolecules and exposure to high temperatures. Different embedding waxes with different melting points and sectioning properties are available commercially. It is useful to experiment with different waxes to achieve the best results. From the writers' point of view, the plastic embedding method is the preferred method for small somatic embryos as the quality of sections is always good. The improvement in the formulation of the plastic, together with the retraction-type microtome currently available, makes serial sectioning possible. Furthermore, 2-3µm thick sections are easily obtained. This allows for a greater resolution of the specimen. Fig. 1 provides examples of white spruce somatic embryos prepared using the plastic embedding technique. Cellular details are well preserved.

- **A protocol for the paraffin embedding method (see Jensen, 1962):**

a) Many fixatives are available in the literature for fixing specimens using the paraffin embedding method (see Jensen, 1962). The most common fixative for

botanical specimens is the FAA (formalin, acetic acid, and ethyl alcohol) formulation. The advantage of this fixative is that the fixing agents penetrate tissues rapidly, thereby stabilizing tissues allowing long term storage of tissue in the fixative until further processing. However, the quality of fixation is relatively poor when compared to fixative containing glutaraldehyde. Fixation using FAA often results in plasmolysis of cells. FAA is prepared by mixing 90 ml of 50% ethyl alcohol, 5 ml of glacial acetic acid, and 5 ml of commercial formalin (40% formaldehyde solution).

b) Embryos are placed directly into vials containing the fixative at 4ºC (on ice). The vials should be labeled by writing the information on a small piece of paper with pencil and included with the sample. During subsequent transfers, the paper label always stays with the sample so the information will not be misplaced. We prefer glass vials with a 10 ml capacity such as the scintillation vials for processing samples.

Since air can be present within tissue samples, it is important that air be removed as it will prevent the infiltration of fixative and embedding medium, resulting in poor quality samples. Therefore, after approximately one hour of fixation, place the vials into a vacuum chamber and evacuate the samples. Vacuum until no more air bubbles appear. Be sure not to allow the samples to "boil" too rapidly by regulating the rate of vacuuming. The fixative should also be replaced with a fresh one after the evacuation step as the fixative composition changes due to prolonged vacuuming. The samples should be stored in the refrigerator and can be processed after 12-24 h of fixation or stored in fixative until required. Before using the vacuum and the vacuum chamber, be sure to understand the operating procedures of such devices. Also confirm that the pump and chamber are compatible with one another. The chamber has to be able to withstand the negative pressure generated by the vacuum pump.

c) The tertiary butyl alcohol (TBA) series is a common dehydration scheme for tissue intended for paraffin embedding as TBA is compatible with wax. The TBA series is prepared as follows:

50	50 ml H_20, 40 ml 95% ethyl alcohol, 10 ml TBA
70	30 ml H_20, 50 ml 95% ethyl alcohol, 20 ml TBA
85	15 ml H_20, 50 ml 95% ethyl alcohol, 35 ml TBA
95	45 ml 95% ethyl alcohol, 55 ml TBA
100	25 ml absolute ethyl alcohol, 75 ml TBA
Absolute TBA	100% TBA

Since the fixative contains 50% ethyl alcohol, dehydration can start at the 50% step of the TBA series. Dehydration can be carried out at room temperature. It is useful

to dissolve a very small amount of stain (0.1%) such as safranin in the 100% TBA in order to pre-stain the samples. This will make embedding and subsequent specimen locating easier during the sectioning process. The stain will not interfere with the subsequent staining of specimens as it will be removed during the hydration step prior to staining.

For vials containing small somatic embryos, it is easier to decant the solution instead of trying to remove the solution completely using a pipette before introducing the next dehydrating solution. This will minimize sample loss. After several changes of absolute TBA, the samples are ready for wax infiltration. It is important to note that absolute TBA is a solid at room temperature. Since the melting point of TBA is 29.3ºC, a suitable amount of TBA should be kept at this temperature for dehydration purposes and the samples with absolute TBA should also be kept warm. A slide warmer can be used to maintain the absolute TBA at the proper melting temperature.

d) Prior to wax infiltration, a new set of vials half-full of wax should be prepared ahead of time. The samples are transferred into these wax vials with TBA covering the specimens. The vials are tightly capped and placed into an oven set at the melting point of the wax used. The wax will melt slowly within the oven allowing gradual mixing with TBA. This process also enables the gradual infiltration of wax into the samples. After 12-24 h, the cap should be removed allowing the TBA to evaporate. This process will also concentrate the wax within the vials. Fresh wax should be exchanged several times over a 2-3 day period to ensure that all of the TBA is no longer present in the specimen. It is important to note that different types of wax are available commercially. They have different additives and melting points. Be sure to understand the properties of the wax used. We routinely use the Paraplast Plus® wax which can be obtained from different commercial suppliers.

e) If a vacuum oven if available, it is advisable to carry out an additional vacuuming step at 60ºC using a vacuum oven. This ensures the complete extraction of air and residual solvent if it is still present within the samples.

f) Embedding is a process of immobilizing and properly placing samples for the subsequent sectioning process. There is no exact procedure for this step. Materials can be embedded using paper boats, weighing dishes, Peel-a-Way® molds, etc. For small somatic embryos, it is difficult to manipulate the embryos within the embedding container. We found the small Peel-A-Way moulds to be useful. A small amount of wax together with the somatic embryos can be poured into each mold. Embryos will settle down to the bottom of the mold. If not, simply place the moulds back into the wax oven for a short time to ensure all the embryos are rested at the bottom. Transfer the moulds to ice-cold water to solidify the wax. If Peel-A-Way molds are not available, small plastic or aluminum weighing boats are useful alternatives.

g) Prior to sectioning, the wax blocks containing the embryos need to be secured onto wooden or other suitable mounting chucks. This is best done using a hot spatula to melt the base of the wax block and "glue" onto the mounting chucks. The blocks should be carefully trimmed leaving about 1 mm of wax around the embryo/tissue prior to sectioning.

• A protocol for the plastic (glycol methacrylate–based) embedding method:

a) The tissue is usually fixed in a fixative containing a mixture of 1.6% paraformaldehyde and 2.5% glutaraldehyde in a 0.05M phosphate buffer at pH. 6.8. As an alternative, a 3% glutaraldehyde solution in a phosphate buffer can also be used. A stock solution of paraformaldehyde (16%) is prepared by adding the appropriate weight of paraformaldehyde into a beaker containing boiling distilled water in which a few drops of 1 N KOH have been added. The solution is stirred continuously with heat to dissolve the powder. After about 5 minutes, the solution should be clear with a few undissolved particles. The final volume of the solution is adjusted. The solution should then be filtered to get rid of a few undissolved particles. The stock solution is stored in a tightly capped glass bottle. Glutaraldehyde solution of different concentrations can be purchased commercially. Routinely, we use an "electron microscope grade", 25% glutaraldehyde solution to prepare our fixative. The phosphate buffer is prepared at a concentration of 0.1 M at pH 6.8. To prepare 100 ml of fixative, simply mix 50 ml of the 0.1 M phosphate buffer with 10 ml of each paraformaldehyde and glutaraldehyde stock solutions and 30 ml of distilled water. Using the above procedure, the final concentration of the fixative is a 1.6 % paraformaldeyde, 2.5% glutaraldehyde in 0.05 M phosphate buffer.

The preparation of the paraformaldehyde solution and fixative solution should always be carried out in the fume hood to avoid inhaling the toxic aldehyde fumes. Generally speaking, the phosphate buffer should be able to maintain the fixative pH at about 6.8. However, if too much hydroxide was used in preparing the paraformaldehyde solution, the pH of the final fixative may need to be adjusted. For adjusting the pH, 1 N sulfuric acid solution should be used instead of hydrochloric acid, as this can result in the production of a carcinogenic product (Goodbody and Lloyd 1994). Since the paraformaldehyde in the stock solution can repolymerize upon storage, a freshly prepared paraformaldehyde solution should be used in preparing a new batch of fixative solution.

b) The explants/embryos are collected from culture vessels. The appropriate part is carefully excised and trimmed to the desired orientation with a sharp double edge razor blade. This ensures proper fixation and infiltration of the embedding medium. This step should be done gently to avoid physical damage due to handling. It is

best to excise and trim the tissue in a pool of fixative. However, due to the toxicity of aldehyde fumes, the tissue can be excised and trimmed in its own medium and quickly transferred into vials containing the fixative. The vials can be labeled by directly writing on the outside of the vials using a permanent marker. In order to ensure that the ink is not washed off, a transparent adhesive tape is added to protect the writing.

c) The tissues are fixed at room temperature for 1-2 h prior to a vacuuming step. After vacuuming, replace the fixative and transfer the vials to a refrigerator and continue to fix the tissue overnight at 4ºC. The total fixation time should be no more than 24 h. Over-fixation can render the tissue extremely hard to section. All subsequent steps are preferably be carried out at 4ºC.

d) After fixation, the specimen is dehydrated with methyl cellosolve followed by two changes of absolute ethanol. The duration of dehydration depends on the size of the specimen. Dehydration should take place at 4ºC to minimize extraction of macromolecules from cells. After the completion of dehydration, if the specimens are not processed immediately, they can be stored in the freezer and used at a later date. In order to ensure that there is no more air present within the specimen, we routinely vacuum the specimen one more time after the first change of absolute ethyl alcohol. Since absolute ethanol is hygroscopic, be sure to allow the vials to warm up to room temperature before opening to avoid unnecessary condensation of moisture.

e) The tissue is ready for the infiltration of the plastic embedding solution. Although there are many formulae based on the glycol methacrylate, we prefer the formulation manufactured by Kulzer & Co. Gmbh (Bereich Technik, D-6393 Wehrheim, Germany). This embedding medium is sold under the trade name Technovit 7100. In North America, Leica markets the same embedding medium under the trade name, Historesin. The advantage of this embedding medium is that it can be polymerised at or below room temperature. This, together with microtomes which allow for retraction during the return stroke, enables us to obtain serial sections easily.

Prepare the Technovit/Historesin infiltration according to the manufacturer's instruction. To prepare the "infiltration solution", dissolve one packet of activator (benzoyl peroxide powder, moistened with 20% H_2O, supplied in packets of 0.5 gm) in 50 ml of the basic resin. Alternatively, if one foresees that many samples will be processed, all powder packages could be dissolved in the entire bottle of basic resin. The infiltration solution should be kept at 4 ºC. However, if the solution is not going to be used frequently, it can be stored in a freezer (-20ºC). This will prolong the "life" of the solution. Be sure to allow the bottle to warm up to room temperature prior to its use in order to prevent condensation of water vapor from the air. Infiltration of the "infiltration solution" should be carried out gradually

with a mixture of absolute ethyl alcohol and infiltration solution in a ratio of 2:1, 1:1, 1:2 before transferring to the pure infiltration solution. The duration of infiltration depends on the size and the density of the specimens. For embryos with a large amount of storage product deposition, a longer infiltration time is necessary. Usually, a 24-hour period is sufficient in each of the intermediate solutions. A rotator, if available, can be used to facilitate the infiltration process.

f) Plastic molding cups are used for the embedding of specimens. Molding trays with the well size of 6x12x5 mm are suitable. Somatic embryos are small and many embryos can be placed within the same well. Since embryos are bipolar structures, they lie flat onto the bottom of the mold and longitudinal sections can be obtained easily.

For embedding, pour the embryos together with the infiltration solution into one or more wells. Once the embryos are in place, the embedding solution is prepared. This is done by mixing 15 ml of infiltration solution with 1 ml of the hardener. In order to increase the "stickiness" of the section, 0.6 ml of polyethylene glycol 400 can be added to 15 ml of the embedding medium (Yeung and Law 1987). This solution should be used immediately because polymerization begins as soon as it is prepared. In our experience, each well takes approximately 2 ml of the embedding solution. One can prepare the proper volume depending on the number of wells used.

The next few steps should be carried out carefully and quickly. First, remove most of the infiltration solution from the embryo-containing wells. The embryos are rinsed briefly with the embedding solution by adding a small quantity of the embedding solution into each well and then removed immediately. After rinsing, the embedding solution is added so that it fills the wells close to their rim. Working quickly, the orientation of the embryos within the wells should be checked using a stereomicroscope if necessary, prior to the addition of the round plastic specimen adapter. It is important that embryos form a “monolayer” resting at the bottom of the mold. Furthermore, it is desirable to move the embryos to the center of the well. This will facilitate ribbon formation later on. The embryos can be moved gently using a tooth pick. Once the embryos are properly arranged within the well, a plastic block holder is placed gently on top of each well to exclude air from the surface of the mold as oxygen interferes with the polymerization process. We prefer the “specimen adapter round Histomold” from Leica (Junghistoshop, part number: 702218310). Once the wells are all capped, the entire tray is left on the bench for at least two hours, by which time the embedding solution should be polymerized. The polymerized blocks should be sectioned as soon as possible as the humidity could alter the properties of the block. In countries where the humidity is high, it is preferably to store the specimen blocks in a desiccator.

g) Serial sections can be obtained readily with a Ralph type glass knife (Bennett et al. 1976) using a microtome with a retractable return stroke such as the Leica 2040 rotary microtome. Occasionally, when ribbons fail to form, a small amount of liquid PEG 400 can be painted on the top and bottom edges of the block to aid in the formation of ribbons. The long ribbon can be cut into 4 cm long pieces (about 9 sections). The short chains of sections are placed on water on a regular glass slide. The plastic sections stretch once they are in contact with water. The slides are then allowed to dry using a slide warmer at 50ºC or at room temperature. If the slides are very clean, such as the J. Melvin Freed Brand microscope slides (Cat. No. 301M) obtained from VWR Canlab, (Mississauga, Ontario, Canada), slides can be used without the need of any treatment. Sections will not detach from clean slides. However, many so called "pre-cleaned" slides are still quite dusty and sometimes feel greasy. In this case, these new slides can be cleaned by soaking overnight in a 70% ethyl alcohol solution containing 0.5% of 1N HCl, followed by thorough washing with distilled water. The cleaned slides are then taken directly (without drying) from distilled water, dipped in an adhesive solution, and placed in a dust free area to dry. The adhesive solution is prepared by dissolving 5.0 gm gelatin in 1 litre of warm distilled water and adding 0.5 gm chrome alum (chromium potassium sulfate). After the solution has cooled, it is filtered through Whatman no. 1 filter paper. This solution may be stored at 4ºC for up to 48 hours, but should be discarded after that time (Pappas 1971). Further information on handling sections can be obtained in the papers of Bennett et al. (1976) and Yeung and Law (1987).

Notes on sectioning: Kulzer, the maker of Technovit resins has developed a disposable histoknife for plastic sectioning. For details, see EBSciences web site (www.ebsstore.com). The Ralph glass knife maker can be obtained from Ted Pella (www.Tedpella.com).

6. STAINING

There are numerous recipes for staining of sections. The readers are referred to the text by Jensen (1962) and O'Brien and McCully (1981). It is beyond the scope of this chapter to detail problems and artifacts dealing with the processing and staining of specimens. The author would like to draw attention to the fact that fixation, processing, and the type of embedding medium used could extract and alter the staining properties of the specimen. Care should be taken to interpret one's own finding. For a detailed discussion on the problems associated with staining, see Horobin (1982).

Serially sectioning generates a large number of slides. In order to facilitate the staining process, we use the Tissue-Tek® slide staining set and the 24-place slide holders (VWR International). Many slides can be processed within a short time.

• The staining of paraffin sections

For paraffin sections, the safranin and fast green staining procedure is commonly used (see Jensen, 1962). This procedure is often time consuming as the safranin stain is rapidly replaced by the fast green stain; hence, the specimens need to be stained for a long time in the safranin staining solution. A modified safranin mixture, i.e. safranin, crystal violet, and basic fuchsin (0.5%, 0.2%, 0.2% respectively in 50% ethyl alcohol, w:v) can be used as an alternative to safranin alone. This mixture will stain the specimen intensely and the staining time is reduced to 20-30 mins. If the staining intensity is too intense, simply counterstains the slides slightly longer in fast green. The result is pleasing and reproducible (see Yeung and Peterson, 1972). Meristematic areas are easily identified as they always have a more intense red colour.

a) The safranin and fast green staining procedure for general histology

1. Remove the paraffin from sections by placing the slide in xylene for 5 minutes and then in a 1:1 mixture of xylene and absolute alcohol for another 5 min. All solutions are kept in Coplin stain jars or in the Tissue-Tek® slide staining container.

2. Partially hydrate sections by passing through a series of ethyl alcohols of decreasing concentration: absolute, 95%, 70%, and 50% (about 5 min each). Agitate the slides occasionally.

3. Stain in the following safranin mixture for about 20 min (minimal time).

Safranin, basic fuschin, and crystal violet – 0.5%, 0.2%, 0.2% respectively in 50% alcohol.

4. Rinse briefly in distilled water to remove excess stain on slides.

5. Dehydrate very quickly (a few seconds each) from 50% to absolute alcohol as the alcohol solution will extract the stain from sections.

6. Counterstain and differentiate in fast green solution – 3-4 dips (a few seconds in total). The staining time is usually very short or else the entire section simply stains green. The time for this step is determined by trial and error. If the sections appear too green, they can be re-stained with the safranin staining solution.

Fast green FCF – a 0.5% solution in absolute alcohol. The traditional fast green staining solution is made up in methyl cellosolve (2-Methoxyethanol), absolute alcohol and clove oil. However, clove oil is very expensive, an alcoholic solution of fast green is adequate for general staining purposes.

7. Pass through absolute alcohol and xylene (1:1) – 2 min each.

8. And then into 2 changes of xylene - 5 min each.

9. Mount in Cytoseal® mounting medium (VWR International) or other types of mounting media.

Note: In order to obtain reproducible results, once the dehydrating solutions are intensely colored, they should be changed, i.e. steps #d, e, and g.

Results: Nuclei, phenolic substances, cuticle, and lignified elements stain red to purplish red. Cytoplasm and non-lignified cell walls stain green.

• The staining of plastic sections

One of the advantages of the GMA-type embedding medium is that the plastic does not interfere with the staining procedure and, therefore, its removal is not necessary. Furthermore, since GMA is hydrophilic, the sections can be stained easily as it is compatible with a majority of aqueous staining recipes. This greatly simplifies the staining procedures. In general, slides are stained with an appropriate stain, rinsed in de-ionized water, dried, and mounted. For detailed staining recipes, consult Feder and O'Brien (1968), O'Brien and McCully (1981), and Yeung (1984). For the staining of lipid using the Technovit 7100, consult van Goor et al. (1986) for tissue processing and staining. The following are procedures used routinely in our laboratories for the staining of GMA sections.

a) TBO stain for general histology

Toluidine blue O is a polychromatic, cationic dye that binds to negatively charged groups. This is an excellent stain for free hand sections. An aqueous solution of this dye is blue, but different colours are generated when the dye binds with different anionic groups in the cell. The generation of colour requires the section to be mounted using water as the mountant. For the staining of plastic or any other type of "processed" sections, since the specimen has undergone different treatments, i.e. fixation, dehydration, and infiltration, some components might have been extracted or modified. Hence the "histochemical colours" may not be as reliable as fresh free hand sections. Care should be taken to interpret the results. Furthermore, water has to be used as the mountant.

If histochemical information is not required, TBO is an excellent counterstain for the periodic acid- Schiff's (PAS) reaction (see the next section). TBO greatly enhances the contrast and the staining intensity of the specimen as it masks the red colour of the PAS reaction. The specimen looks blue once the sections are dried.

Stain preparation: Dissolve 0.1 g of toluidine blue O in 100 ml of 0.1 M benzoate buffer, pH 4.4. (benzoic acid 0.25 g, sodium benzoate 0.29 g, water 200 ml). This buffer is recommended for histochemical purposes. If benzoate buffer is not available, for general use, de-ionized water can be used as the solvent for TBO.

1. Place slides directly into the stain and stain for 2-3 min. Wash the slide in slow running water until the water is clear. Rinse in de-ionized water to remove salt from the usual, "hard" running water. Dry and mount.

2. If histochemical information is required, the slides should be mounted using water as the mountant and examined at once.

Results: For slides using an aqueous mount, the following colours are possible: pectin, red or reddish purple; lignin, blue; other phenolic compounds, green to blue-green. Thin-walled parenchyma, reddish purple; collenchyma, reddish purple; lignified elements such as tracheary elements and sclerenchyma, green to blue-green; sieve tubes and companion cells, purple; middle lamella, red to reddish purple; callose and starch, unstained (O'Brien et al. 1964).

b) Periodic acid - Schiff's reaction for total insoluble polysaccharides

1. Place slides into a 0.15% freshly prepared periodic acid solution for 15 min.

2. Rinse in slow running water for 5 min.

3. Gently block dry to remove excess water from slides and place them directly into Schiff's reagent for 30 min. Schiff's reagent can be prepared by dissolving 1 gm basic fuschin (C.I. 42510) in 200 ml boiling distilled water. Cool to 50ºC and add 30 ml 1N HCl and then 3 gm potassium metabisulfite. This combination of HCl and metabisulfite generates sulfur dioxide. This serves to reduce the pararosaniline to a colourless form. Therefore, it is important that the container is tightly capped once the metabisulfite is added. Gently shake for 2 min. Leave in the dark for 24 hr and then add 1 gm decolorizing activated charcoal. Shake for 5 min and filtered quickly through filter paper. Preferably, a Buchner funnel is used and filtering can be aided by a mild vacuum. This is to minimize the escape of sulfur dioxide. The solution should be clear and colourless. Store it at 4ºC when not in use. This solution can be stored for months in a well-capped dark bottle. One can also purchase Schiff's reagent directly from a commercial source. Since sulfur dioxide is generated during the preparation of the Schiff's reagent, all the above steps should be carried out in the fumehood.

4. Wash the slides in slow running water for about 10 min, rinse with de-ionized water, dry and mount. The sections usually turn intense red when washing in running water. The slides can be examined directly for insoluble carbohydrates once dried and mounted or they can be counterstained with Toluidine blue O for improving the contrast or with Amido black 10B for proteins.

Results: Insoluble carbohydrates, i.e. cell wall and starch granules stain intensely red.

c) Amido black 10B stain for protein

1. Place slides directly to the staining solution for 2-10 min depending on the cell types studied. Generally, vacuolated cells require longer staining time and a shorter washing time. The staining solution is prepared by dissolving 1 gm of amido black (C.I. 20470) into 100 ml of 7% acetic acid. The solution should be filtered before use. If the sections are over-stained, they can be destained by placing in one or more changes of 0.7% acetic acid solution.

2. Wash slides briefly with running water to remove excess stain on the slide, rinse in de-ionized water, dry and mount. Since a majority of plant cells are vacuolated, the staining intensity may be low. If necessary, the slides can be re-stained to improve the staining intensity.

Results: Proteins stain blue.

7. SOME COMMENTS ON PHOTOGRAPHY

The general approach in slide examination has been discussed by Yeung (1999). Photographing the sections during the course of slide examination provides an important means of demonstrating structural changes, as well as keeping a record of the study. Prior to taking any photographs, be sure to understand your own photomicroscope equipment and the film characteristics. Readers are advised to consult Bradbury and Evennett (1995), Lacey (1989), and Smith (1994) for photomicrography procedures.

• Black and white films

For normal black and white photography, the Kodak Technical Pan film (Cat. No. 129 7563) is preferred as this film has extremely fine grains and a pleasing contrast. We set the ASA of the film to 80. For developing this film after photography, first prepare a stock developer solution (Kodak HC-110, Cat. No. 140

8938) by dissolving the entire bottle 473 ml into a final volume of 1.9 litre or portion thereof. From this stock solution, prepare a developing solution using 1 part of the stock developer to 19 parts of water. Be sure the temperature is at 20ºC. Develop the film for 7.5 minutes, rinse in water and fix with a Kodak Rapid fixer (Cat. No. 146 4106), full strength for 2 min. Open the developing tank and wash the negatives in slow running water for 10 minutes, rinse with de-ionized water with a few drops of Kodak Photoflow (Cat. No. 146 4502) to remove water spots and hang dry.

For printing of this film, the Kodak RC Polycontrast papers can be used. Contrast filters No. 3 and 3.5 usually give excellent results.

For fluorescent images, a faster speed film is required to capture images with a lower light intensity. The Kodak TMAX and Tri X Pan films are suitable. The film characteristics and developing schedules are available from the Kodak website (www.Kodak.com). In taking fluorescence images, be sure to use the "dark field" feature of the camera system. If unavailable, it is necessary to calibrate the shutter speed for proper exposure time.

- **Colour films**

For colour prints, the Fuji and Kodak Color ASA 100 films are suitable. However, the Fuji Professional 100ASA film is preferred as it gives a truer color reproduction. For fluorescent images, a faster speed film with ASA 200/400 may be needed. Since the colour print films are balanced for "daylight", it is essential that a daylight conversion filter be used when taking bright field images through the microscope with a tungsten light source.

For positive slide films, both tungsten and daylight films are available. For microscopic work, the Kodak Ektachrome 160T tungsten film is suitable. Kodak also manufactures other Professional tungsten films with different speeds, i.e. 64T and 320T. A daylight conversion filter is not necessary when taking photographs through the microscope. However, for proper colour reproduction, one has to ensure that the images are captured with the proper light quality and intensity. Slide positive films balanced for daylight are also available such as the Kodak Chrome films. Again, if this type of film is chosen, a daylight conversion filter has to be used. There are different manufacturers of colour films. Film technology continues to change. It is advisable to check websites of different manufacturers to get new information and updates on films.

- **Iris diaphragm**

One of the most common mistakes during the course of photography is that viewer tends to close down the iris diaphragm of the microscope to improve contrast for the ease of viewing. However, dust and other imperfections will appear and be captured by the film and the images usually look dull.

- **Low magnification images**

Low magnification specimens are difficult to focus. If in doubt, take a series of photographs with a slightly different focus. This will ensure that at least one photo will be in perfect focus.

- **Digital cameras**

Digital cameras are becoming more popular. It will not come as a surprise to see that the film based photography will become obsolete in the near future. The advantage of having a good quality digital camera is that images can be captured, stored in one's computer, processed using a variety of softwares and printed using a colour printer. The operator is in full control of his/her artistic composition. Furthermore, a darkroom is not required for the production of digital photographs. On the other hand, in order to produce good quality photographs, hardwares of considerable expense are needed.

If resources are a problem, one can combine the traditional film based technology with the current software manipulation techniques. One can first capture the images using photographic film. The film can be developed by commercial companies and the negatives scanned using a high quality film scanner such as the Nikon Coolscan® ED 4000. The scanned images can then be processed using a computer with appropriate softwares. Although photographic prints can be scanned directly using a simple scanner, the images is usually of a lesser quality as prints developed by commercial vendors tend to be sub-optimal in colour and sharpness. A good negative scanner can capture the information from the negatives and the images can be reproduced in a more accurate and pleasing fashion. We find the quality of micrographs produced using this approach to be excellent.

8. RESOURCES AND SUPPLIERS

More detailed treatments are available in texts dealing specifically with histotechniques. Readers are urged to consult the text by Gahan (1984), Jensen (1962), O'Brien and McCully (1981), Razin (1999). In addition, many websites also provide useful information dealing with specific products, especially those provided by the manufacturers. For example, using the keyword "Technovit" will locate information dealing with procedures and related information on different plastic embedding media manufactured by the Kulzer & Co.

The following are useful web addresses for different suppliers:
General supplies: Fisher Scientific (www.Fishersci.com), Sigma (www.sigmaaldrich.com) and VWR International (www.vwr.com).

Microscopy supplies: Electron Microscopy Sciences (www.emsdiasum.com/ems), Energy Beam Sciences (www.ebsstore.com), Leica Microsystems (www.leica-microsystems.com), Leica Comsumables (www.junghistoshop.de), PolySciences (www.Polysciences.com), Ted Pella (www.Tedpella.com), and TAAB (www.taab.co.uk).

9. CONCLUSION

A carefully designed and executed histological study always provides valuable information on any biological system. The methodologies provided in this chapter serve as an introduction to the most common histological techniques used. Many additional protocols are available in the literature. The readers are urged to consult the literature for additional methods that can further their studies. The key to a successful structural investigation is to make sure the study is complete and detailed. In a structural study, one should always strive for that perfect section. This involves making hundreds and thousands of slides and indeed, this is a labor intensive process. Perfect sections, together with good imagination and observation guarantee success to a research program.

10. REFERENCES

Bennett, H. S., A.D. Wyrick, S.W. Lee & H.J. Jr. McNiel, 1976. Science and art in preparing tissues embedded in plastic or light microscopy, with special reference to glycol methacrylate, glass knives and simple stains. Stain Technol. 51: 71-97.

Berlyn, G.P. & J.P. Miksche, 1976. Botanical Microtechnique and Cytochemistry. The Iowa State University Press, Ames, Iowa.

Bracegirdle, B., 1995. Scientific Photomacrography. Bios Scientific Publishers, Oxford.

Bradbury, S. & P.J. Evennett, 1995. Contrast Techniques in Light Microscopy. Bios Scientific Publishers, Oxford.

Cameron, R., R. Harrison-Murray, M. Fordham, H. Judd, Y. Ford, T. Marks & R. Edmondson, 2003. Rooting cuttings of *Syringa vulgaris* cv. Charles Joly and Corylus avellana cv. Aurea: the influence of stock plant pruning and shoot growth. Trees Struc. Func. 17: 451-462.

Cassells, A.C., 2000. Contamination detection and elimination. In: R.E. Spier (Ed). Encyclopedia of Plant Cell Biology, 577-586. Wiley, Chichester.

Coetzee, J., 1985. Fixation of plant cells for electron microscopy. In: A.W. Robards (Ed). Botanical Microscopy 1985, pp. 17-38. Oxford University Press, Oxford.

Dong, Z., M.E. McCully & M.J. Canny, 1994. Retention of vacuole contents of plant cells during fixation. J. Microscopy 175: 222-228.

Feder, N. & T.P. O'Brien, 1968. Plant microtechnique: some principles and new methods. Amer. J. Bot. 55: 123-142.

Gahan, P.B., 1984. Plant histochemistry and cytochemistry, an introduction. Academic Press, London.

Goodbody, K.C. & C.W. Lloyd, 1994. Immunofluorescence techniques for analysis of the cytoskeleton. In: N. Harris & K.J. Oparka (Eds). Plant cell biology, pp. 221-243. IRL Press, London.

Horobin, R.W., 1982. Histochemistry. Gustav Fischer, Stuttgart, Germany.

Jakstys, B. P., 1988. Artifacts in sampling specimens for biological electron microscopy. In: R.F.E. Crang & K.L. Klomparens (Eds). Artifacts in Biological Electron Microscopy, pp. 1-12. Plenum Press, New York.

Jensen, W.A., 1962. Botanical Histochemistry. Freeman, San Francisco.

Lacey, A.J. (ed.), 1989. Light Microscopy in Biology. IRL Press, Oxford.

Mersey, B. & M.E. McCully, 1978. Monitoring the course of fixation of plant cells. J. Microscopy 114: 49-76.

O'Brien, T.P., J. Kuo, McCully, M.E.& S.Y. Zee, 1973. Coagulant and non-coagulant fixation of plant cells. Aust. J. Biol. Sci. 26: 1231-1250.

O'Brien, T. P. & M.E. McCully, 1981. The Study of Plant Structure, Principles and Selected Methods. Termarcarphi Pty. Ltd., Melbourne, Australia.

Pappas, P. W., 1971. The use of a chrome alum-gelatin (subbing) solution as a general adhesive for paraffin sections. Stain Technol. 46: 121-124.

Raghavan, V., 2000 Developmental Biology of Flowering Plants. Springer-Verlag, New York.

Redenbaugh, K., 1993. SynSeeds: Applications of Synthetic Seeds to Crop Improvement. CRC Press, Boca Raton, FL.

Ruzin, S.E., 1999. Plant Microtechnique and Microscopy. Oxford University Press, New York.

Smith, R. F., 1994. Microscopy and Photomicrography, a working manual. CRC Press, Boca Raton, FL.

Thorpe, T.A., 1994. Morphogenesis and regeneration. In: I.K. Vasil & T.A. Thorpe, (Eds). Plant Cell and Tissue Culture, pp. 17-36. Kluwer Academic Publishers, Dordrecht.

Thorpe, T.A. & C. Stasolla, 2001. Somatic embryogenesis. In: S.S .Bhojwani; & W.Y. Soh, (Eds). Current Trends in the Embryology of Angiosperms, pp. 279-336. Kluwer Academic Publishers, Dordrecht.

Van Goor, H., P.O. Gerrits & J. Grond, 1986. The application of lipid-soluble stains in plastic-embedded sections. Histochemistry 85: 251-253.

Yeung, E.C., 1984. Histological and histochemical staining procedures. In: I.K. Vasil (Ed). Cell Culture and Somatic Cell Genetics of Plants, Vol. 1. Laboratory procedures and their applications, pp. 689-697. Academic Press, Orlando.

Yeung, E.C., 1998. A beginner's guide to the study of plant structure. In: S.J. Karcher (Ed). Tested Studies for Laboratory Teaching, Vol. 19,. Proceedings of the 19th Workshop/Conference of the Association for Biology Laboratory Education (ABLE), pp. 125-141. Purdue University, Lafayette, Indiana.

Yeung, E.C., 1999. The use of histology in the study of plant tissue culture systems – some practical comments. In Vitro Cell. Dev. Biol. – Plant 35: 137-143.

Yeung, E.C. & S.K. Law, 1987. Serial sectioning techniques for a modified LKB Historesin. Stain Technol. 62: 147-153.

Yeung, E.C. & R.L. Peterson, 1972. Studies on the rosette plant *Hieracium floribundum*. I. Observations related to flowering and axillary bud development. Can. J. Bot. 50: 73-78.

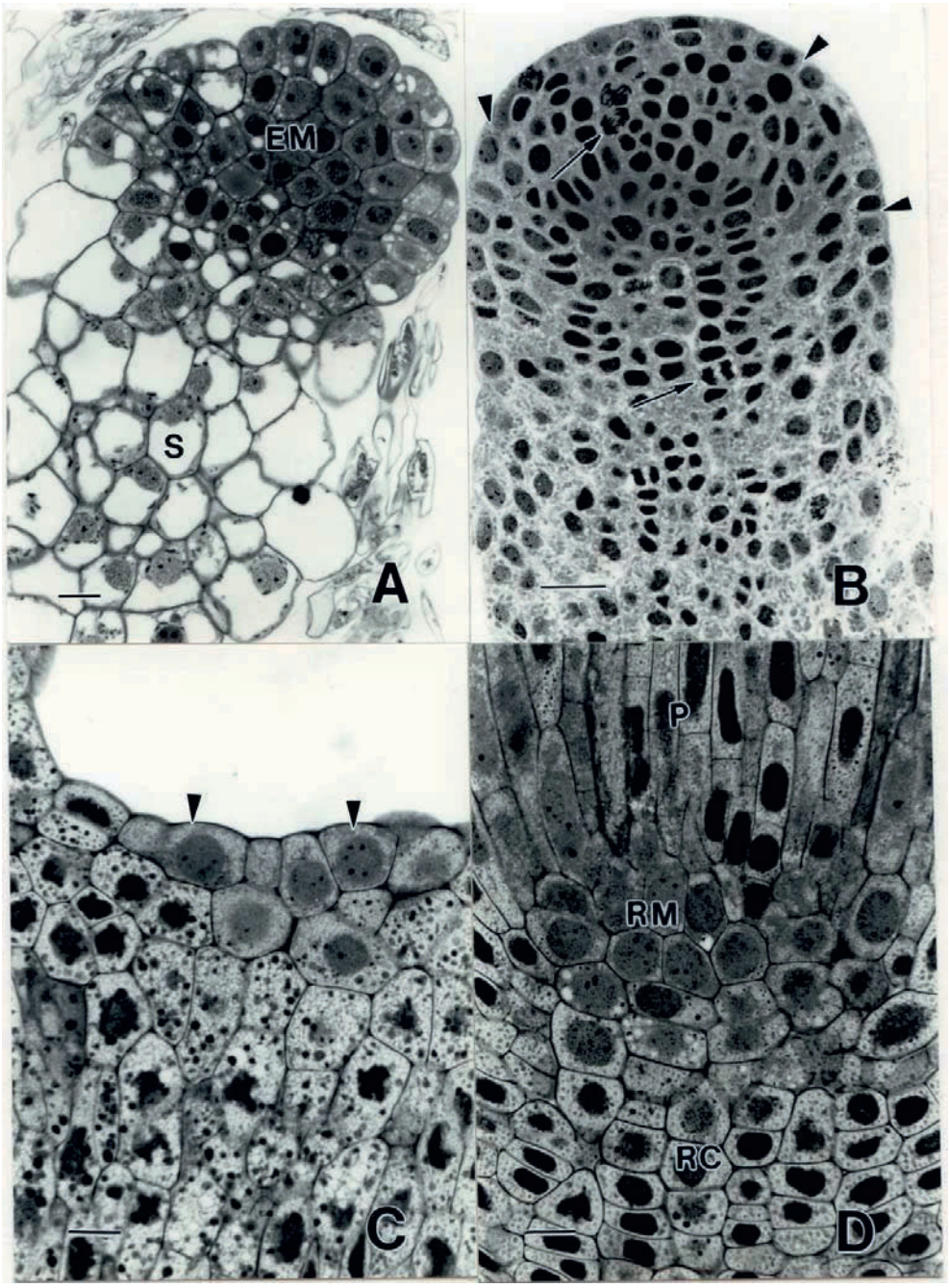
EM
S
A
B
P
RM
RC
C
D

Figure 1. Micrographs of developing white spruce somatic embryos prepared using Historesin as the embedding medium. The sections were first stained with the periodic acid –Schiff's procedure followed by counterstaining with toluidine blue O (Figs. 1A and 1B) or amido black 10B (Figs. 1C and 1D).

A. Developing white spruce somatic embryo soon after transfer onto the maturation medium. The embrogenic head (EM) cells have a dense cytoplasm with small vacuoles. The subtending suspensor-like (S) cells are highly vacuolated. Scale bar = 50 μm.

B. After 10 d on the maturation medium, globular-shaped embryos appear. The globular embryo has a well-defined protoderm (arrowhead). Cells within the central core also have a dense cytoplasm and mitotic figures (arrows) are common at this time. Scale bar = 10 μm.

C. At the time of maturation, the shoot apex is composed of a surface layer of large apical meristem initials (arrowheads). The subtending cells have dense storage deposits. Scale bar = 40 μm.

D. The root pole is composed of large root apical meristem initials (RM). This group of cells is located between the procambium (P) and the root cap (RC). Scale bar = 40 μm.

BIOENCAPSULATION OF SOMATIC EMBRYOS IN WOODY PLANTS.

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1. INTRODUCTION

Application of synthetic seed technology in the field of micropropagtion, storage and transport has been well recognized in several agronomically important crops and woody species. Despite the spurt in synthetic seed research in the recent past, there is need for more studies mainly on the physiological and biochemical aspects of synthetic seeds, especially the factors affecting their germination and subsequent plant growth in the soil (Redenbaugh, 1990, 1993). Establishment of an efficient somatic embryogenesis system is a major prerequisite for a successful program on synthetic seeds. However, this has not been achieved very well in several important woody plants. Long life cycle, extended juvenility, poor and inconsistent seed yield and constraints in establishment of *in vitro* cultures are some of the major hurdles for woody plant species. It is always desirable to raise cultures from mature plants with known features and desired traits but explants from mature trees exhibit recalcitrance under aseptic conditions and therefore most studies on tree tissue culture use seedling parts. However in this, efficacy of progeny is not known.

The regeneration of plants in culture and their subsequent acclimatization and delivery to the field especially woody plants pose many problems and do not make tissue culture technology a viable proposition. The successful

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germination of encapsulated somatic embryos has initiated a new line of research on synthetic seed technology (Onishi *et al*., 1994, Rao *et al.,* 1998). The main thrust of research on synthetic seeds is on the direct sowing of encapsulated embryos under field condition. The concept of synthetic seeds or artificial seeds is based on the assumption that somatic embryos can be encapsulated and handled like a real seed for transport, storage and sowing. Alternatively, the encapsulation of *in vitro* derived propagules (buds, bulbs or any form of meristematic tissue) has ushered in a new era in synthetic seed research (Bapat *et al*., 1987, Piccioni and Standardi, 1995). The current broader definition of synthetic seeds hence is "an artificially encapsulated somatic embryo, shoot or any other mersitematic tissue which can develop into a plant under *in vitro* or *in vivo* conditions. Microbulbs, microtubers, rhizomes, corms, microcuttings, shoot apices, axillary buds, meristemoids, cell aggregates, clumps and primordia can be used as explants for the preparation of synthetic seeds. The concept of incorporating nutrients, biofertilizers, antibiotics or other essential additives to the matrix allowing easy handling, storing, shipping and planting makes the synthetic seed a unit of delivery for *in vitro* plants. The uniform and simultaneous production of somatic embryos and their germination after encapsulation could possibly minimize these disadvantages associated with natural seeds. Trees produce seeds only in certain months of the year whereas synthetic seeds can be made available throughout the year.

Success in the synthetic seed technology relies upon several major steps (Redenbaugh and Ruzin, 1989). These include establishment of efficient systems of somatic embryogenesis, synchronization of somatic embryos leading to plantlet regeneration, selection of non toxic encapsulating matrix, increased storage capability of synthetic seeds and then germination under *in vivo* conditions. These steps, however, are not easy to achieve in woody plants due to the inherent characters associated with trees.

2. PROTOCOL FOR SOMATIC EMBRYO ENCAPSULATION

2.1. Factors controlling somatic embryogenesis

Explants, generally young shoots, zygotic embryos or seedling parts are cultured in woody species after surface sterilization. Secretion of phenolic compounds detrimental to the culture is very common in majority of woody plants. Like herbaceous species, induction of embryos in woody plants is determined by a variety of factors such as age, proper developmental stage of explant, effective medium and other appropriate physical and chemical conditions (Stasolla and Yeung, 2003). Formulation of suitable medium for callus induction is genotype dependent. In several cases the callus induction medium may not support embryo induction. Hence, altered nutrient medium has to be used (Von Arnold *et al.,* 2002). Conversion of embryos to plants also may require nutrient medium changes

Among auxins, 2,4-D is widely used for the induction of callus although cytokinins in conjuction with auxins also stimulate callusing. Charcoal can also be a component in the medium mainly for reducing phenolic exudates. In an established embryogenic system, quantification of embryos is not easy due to lack of synchronous growth of the cultures. Techniques such as control of cell cycle, sieving of cultures, use of abscisic acid are available to achieve certain level of synchrony in cultures as well as useful to identify highly embryogenic cell lines among phenotypically similar plants. Several basal media, from relatively simple to more complicated have been employed but these cannot be generalized for any plant. It has been observed that embryogenic cells secrete proteins into the medium which stimulate to induce somatic embryogenesis in non embryogenic cells. Repetitive embryogeny for the continuous supply of embryos have been reported in woody plants essential for preparation of synthetic seeds continuously. Maturation of somatic embryos prior to encapsulation is essential for better germination (Stasolla and Yeung, 2003). Maturation and desiccation of somatic embryos has been carried out in woody plants for enhanced conversion of embryos to plants. Only morphologically mature embryos which have accumulated enough storage materials and have acquired desiccation tolerance develop into normal plants. The benefits of high sucrose, high nitrogen content or abscisic acid or drying of the tissue are well known. Compared to other herbaceous plants, much less work has been carried out on molecular mechanism of embryogenesis of woody plants, however, the utilization of methods such as cDNA

microarray, could lead to understanding of genes responsible for embryogenesis in woody genera. Characterization of gene expression during embryo development, maturation and germination has led to the identification of five distinct classes of developmentally regulated genes. Three sets of genes are presumed to influence the process of embryogenesis in woody species. First set expresses throughout the process, second set is involved during maturation and in late stages of development and the third set is responsible for conversion of embryos to plants. All these genes appear to express in a sequential pattern.

2.2. Encapsulation of somatic embryos

Germination of synthetic seeds depends upon the somatic embryos having functional shoot and root apices that can germinate like a zygotic embryo. For the encapsulation process, it is often necessary to encase the embryo in a matrix, which serves as a synthetic endosperm containing mineral nutrients, source of carbon, growth regulators and antimicrobial agents. The coating should be non toxic to the propagule, protect from mechanical damage during handling and allow its development and conversion to occur without any variation (Redenbaugh and Ruzin, 1989). Several agents including sodium alginate, sodium alginate with gelatin, potassium alginate, guar gum, agar, tragacanth gum, sodium pectate, carboxymethyl cellulose, carrageenan with locust bean gum, gerlite have been used for encapsulation (Redenbaugh *et al*., 1986, 1987). Amongst these, sodium alginate has been extensively used because it is less toxic and offers sufficient rigidity to the capsule allowing easy handling. In addition, polyethylene oxide homopolymers, acrylic copolymer, carboxyl methylized cold soluble swelling starch, synthetic trotachedral smectite, synthetic sodium magnesium lithium silicate, starch plus synthetic polymer of acrylamide and sodium crylate, anionic flocculant have been also used as coating agents in some studies (Janick *et al*., 1993). The calcium alginate capsule is generally wet and sticky causing problems in handling. Redenbaugh *et al*., (1987) tested various hydrophobic coatings and found Elvax polymer coating to be very effective for shoot emergence. Dupuis *et al*., (1994) used pharmaceutical type capsules as coating systems in which the capsule body was covered on its inner surface with watertight film

composed of polyvinylchloride (PVC), polyvinylacetate (PVA) and bentone as a thickener. This allowed a controlled and steady nutrient supply to the developing somatic embryos of carrot.

Somatic embryos have to be carefully isolated and blot dried on a filter paper prior to dipping for a few seconds in a mixture of sodium alginate gel (100ml) prepared in a basal medium preferably MS basal medium. The embryos are then picked up by a pair of forceps and dropped into a solution of $CaCl_2$.$2H_2O$ (1.036g / 150 ml). Each drop contains a single embryo. These are then kept in this solution for 40 to 50 minutes on a gyratory shaker (80rpm) in light (1000 lux). After the incubation period, the $CaCl_2$.$2H_2O$ solution is decanted, the beads are washed 3 to 4 times with hormone free MS medium. The alginate complexes in the presence of $CaCl_2$ forms a firm coating on the embryo. Such encapsulated embryos are then either cultured on the nutrient medium or stored at 10^oC in parafilm sealed petri dishes.

There are two types of synthetic seeds: hydrated and desiccated. Hydrated synthetic seeds consist of somatic embryos individually encapsulated in hydrogel such as calcium alginate. Use of polyoxyethylene glycol for encapsulation produces desiccated synthetic seeds. In this method, the coating mixture is allowed to dry for several hours on a Teflon surface in a sterile hood. The resultant wafers are then hydrated and placed on a medium for scoring embryo survival. Both these matrices have their own merits and demerits. Hydrated capsules are difficult to store because the embryo requires respiration. These capsules dry out unless kept in a humid environment or coated with a hydrophobic membrane. In case of dehydrated or desiccated capsules, the process of desiccation itself could cause damage to the embryo restricting survival.

2.3. Encapsulation of embryogenic cell suspension

For the encapsulation of embryogenic cell suspension, actively growing cells are selected and filtered through a nylon net (100 μ) prior to washing twice in hormone - free nutrient medium. Washed cells are then mixed with 2.5% sodium alginate in 1:1 proportion. The mixture is then pipetted and dropped into 50 ml of $CaCl_2$.$2H_2$ 0 (1.036g/150ml) prepared in

hormone free nutrient medium. Beads of 5-10mm formed by this technique are shaken in $CaCl_2$.$2H_20$ solution for 1 hour and washed twice with the culture medium. The beads are then suspended in 25ml medium (10 beads/flasks) and are shaken in continuous light (1000 lux) at 25 ± 2^0 C.

3. CASE STUDIES

3.1.Cocoa

Cacao is a major tropical crop, grown for its oil rich seeds which are the major source of cocoa solids and butter. Somatic embryogenesis has been reported from a number of tissues of cacao including zygotic embryos, nucellar tissue, young bud petals and leaves (Figueira and Janick, 1995). However, somatic embryos from zygotic embryos and nucellar tissue are mainly used for establishing cultures. Synthetic seeds, 4.5 to 5 mm were prepared from the excised embryos of mature seeds (approximately 120 days old pods) collected from ten year old cocoa trees by encapsulating the embryos in a medium containing 4% sodium alginate and complexed using75mM $CaCl_2$.$2H_20$ (Sudhakara *et al.* 2000). They were tested for their germination characteristics after a storage of 25 days at 10^oC and subsequently grown on either wet or dry cotton under aseptic conditions. Germination of synthetic seeds was 97.3% if tested immediately after encapsulation. This declined to 71% and 49% respectively, when stored in either wet or dry cotton medium for 25 days. Germination percentage of the seeds extracted from fresh pods was 90% and declined to 76% at the end of five days storage of pods at 27 ± 2^0 C. At the end of 10 days in storage, complete mortality was observed. A significant observation in this study was that the time taken for the initiation and completion of germination of root and shoot were shorter in the case of synthetic seeds compared to the normal seeds.

3.2. Camellia

Camellia is a genus of tropical or subtropical trees and shrubs whose leaves are used to prepare tea. In *Camella*, somatic embryogenesis has mainly been investigated in *C. sinensis, C. japonica and C. reticulata*. Cotyledons

are the main source of explants for establishing the cultures (Vieitez, 1995). In *C. japonica,* the establishment of somatic embryo cultures and subsequent plantlet regeneration was obtained on MS+BA(4.4µM)+IBA(0.4µM) and on MS+GA(14.4µM)+IAA(28.5µM) respectively (Janeiro *et al.,*1997).Somatic embryos of *Camellia japonica* were encapsulated using 3% sodium alginate and 0.1M Calcium chloride to produce synthetic seeds (Janeiro *et al*., 1997). Both germination and embryogenic capacity of the encapsulated embryos was investigated. The frequency of *in vitro* germination of artificial seeds was dependent on various nutrient additives to the encapsulation matrix. On a calcium free MS basal medium containing 3% sucrose, 14.4µM GA_3 and 28.5µM IAA, 63% plant recovery rate was obtained. This result was similar to that of non encapsulated embryos. This implies that encapsulation of somatic embryos did not negatively affect their embryogenic competence. The mean number of secondary embryos were significantly increased when the alginate beads were supplemented with growth regulators (4.44 µM BAP and 0.41µM IBA). Storage at 4^oC significantly reduced the survival and germination frequencies of both encapsulated and non encapsulated somatic embryos. However, the reduction was much greater for non encapsulated embryos. Plant recovery of encapsulated embryos was 40% and 30% following storage for 30 and 60 days respectively (Janeiro *et al*., 1997).

3.3. Mango

Mango (*Mangifera indica* L.) is an economically important fruit crop. Several groups working on tissue culture of mango have reported somatic embryogenesis and plantlet regeneration. Successful induction and subsequent regeneration of somatic embryos from nucellar explants of mango cv. Amrapali has been obtained (Ara *et al*., 1999). In this study, somatic embryos were induced on MS+ 2,4-D (4.5µM) + L glutamine (2.74mM) + sucrose (175mM) and plant regeneration was obtained on B5 medium. Somatic embryos were encapsulated in calcium alginate (2%) capsules prepared in liquid nutrient medium containing B5 macrosalts, MS microsalts and organics (quarter strength each). $CaCl_2$. $2H_20$ (100mM) solution was used for complexing. The encapsulated somatic embryos germinated on a medium containing B5 macrosalts(half strength), MS

microsalts (full strength), sucrose (87mM) and gibberellic acid (2.9µM). Encapsulated somatic embryos germinated at a higher rate than naked somatic embryos of the same size, on the same medium. The germination ability of encapsulated somatic embryos was increased when the medium was supplemented with full strength B5 macrosalts. Approximately 46% encapsulated embryos developed into plantlets. Incorporation of abscisic acid (0.004 or 0.02µM) did not enhance germination percentage. Instead, it delayed germination. Plants were successfully established in this study (Ara *et al*., 1999).

3.4 Sandalwood

Sandalwood is a major forest tree of India, well known for its oil and fragrant wood. Its seeds lose viability upon storage. Callus was raised from stem internodes of young shoots of a 20 year old sandalwood tree (*Santalum album* L) on MS medium containing sucrose (87.6mM) and 2,4-D (4.52 µM) . Subsequent transfer of the callus to MS basal medium containing IAA (2.85µM), BA (2.22µM) and sucrose (87.6mM) resulted in the development of a highly regenerative embryogenic callus which consisted of somatic embryos of all stages from globular to torpedo (Rao and Bapat, 1995).

Somatic embryos of late torpedo stage from several cultures were manually picked and mixed in 3% w/v sodium alginate. The mixture of somatic embryos and alginate was dropped in $CaCl_2$ $2H_20$ (1.036/150ml) solution and allowed to stand in this for 1h. After decanting off the $CaCl_2$ solution, the beads were washed with sterile water and were stored at 4^oC or cultured on nutrient medium.

The callus containing embryos of all stages was transferred to petridishes on a sterile filter paper and left to dry in the laminar air flow for 8 h at 28 ^0C in light. After this the petridishes containing the desiccated somatic embryos were sealed and kept in the dark at room temperature for various periods from 10 days to 30 days. Following this the tissue was cultured on fresh MS medium containing IAA (2.85µM) + BA (2.22 µM) + sucrose (87.6mM). Some desiccated embryos were encapsulated as mentioned

earlier in sodium alginate and their performance was compared to non-desiccated encapsulated ones. Both desiccated and non desiccated embryos showed revival of growth upon rehydration on White's medium (1954) and developed into plants. The tolerance to desiccation and regeneration of viable plantlets depended upon the pretreatment given to somatic embryos. Embryogenic callus subjected to dry state for 30 days showed revival of somatic embryogenesis upon transfer to fresh medium. Somatic embryos of sandalwood are therefore desiccation tolerant and an excellent material for preparation of synthetic seeds analogous to true botanic seeds. The viability of synthetic seeds too is observed to be better than normal sandalwood seeds (Bapat and Rao, 1988).

3.5. Eucalyptus

Eucalyptus trees are a significant source of fuel wood, timber, and raw material for the paper/pulp industry, honey, tannins and essential oils. *Eucalyptus citriodora* L. is grown extensively for its timber and essential oils (Muralidharan and Mascarenhas, 1995)..Induction of somatic embryos in *Eucalyptus* and their regeneration has been widely reported by several groups. Somatic embryogenesis from seeds has been established on MS+NAA (26.88µM) or on B5 +NAA (16.13µM). Germination of somatic embryos was obtained on B5 medium containing 2% sucrose. Studies have been done using a variety of matrices to encapsulate *Eucalyptus* embryos, storing the synthetic seeds at various different temperatures and testing their germination (Muralidharan and Mascarenhas, 1995). Sodium alginate (1 and 2 % w/v, complexed with 0.2% calcium nitrate solution), gerlite (0.2%) and agar (1 and 2% w/v) gelling at ambient temperatures were tested for encapsulation of isolated embryos. The use of sodium alginate gave the best results and formed a seed coat suitable to preserve the viability of isolated somatic embryos. Alginate (1%) formed a soft bead that eased handling of embryos with a forceps and that resulted in 30% germination of encapsulated embryos on a sterile medium, whereas all the other matrices were found to be too soft to handle.

A semi-automatic method has been adopted to encapsulate *Eucalyptus* somatic embryos. This method involves the use of a peristaltic pump to

control the rate of droplet formation of sodium alginate (1% w/v of Protanal LF, proton, Norway) and a magnetic stirrer to ensure uniform mixing of the calcium nitrate (0.2%) solution used for complexation. Individual embryos were picked with a pair of forceps and introduced automatically into the droplets, which were then allowed to fall into the stirred complexing agent. After 20min of complexation, all the beads were removed, washed with sterile water and stored in a test tube at room temperature or at 4^{o}C for varying period of time. Encapsulated embryos were then scored for germination capacity on a variety of media/substrates both under sterile as well as non-sterile conditions. Rate of germination of encapsulated embryos, stored at 25^{o}C (room temperature) declined on sterilized germination medium. After 1 day of storage, 38% of the embryos germinated and after 7 days storage only 28% germinated. No embryo germination was recorded when stored for 10 days. Embryos stored at 4^{0} C decreased their germination rate more rapidly and after 7 days none could germinate. Encapsulated embryos stored at room temperature were grown on a sterilized water agar medium, sterilized sand and non- sterilized soil. On sterilized water, he germination rate was 30% of which 98% of the plantlets survived. On sterile sand (irrigated with a solution of B_5 salts) germination was only 4%, but all the plantlets survived. On non- sterilized soil, none of the encapsulated embryos germinated, and were infested with microbial contamination (Muralidharan and Mascarenhas, 1995).

3.6. Aegle marmelos (L) CORR

Aegle marmelos is an important medicinal tree extensively planted for its fruits and roots, which are ingredients for Ayurvedic medicines. Seedling parts mainly cotyledons and hypocotyls have been used to establish cultures. Embryogenic cultures were established on MS+IAA (1.4µM) +BA (0.44µM) or on MS+ 2,4-D (1µM) +BA (0.88 µM)+glutamine (68.5µM) (Arumugam and Rao, 2000). Sodium alginate (O.5-5%) was complexed with $CaCl_2$ (2.5%) for making synthetic seeds (Arumugam and Rao, 2000). Amongst these, 3% sodium alginate was most suitable for encapsulation. The highest survival response was 70.4% and the percentage of synthetic seeds germinated was 45.5% on medium containing suitable growth regulators. At lower and higher concentrations of sodium alginate,

within 25 days of culture, 75% encapsulated embryos germinated when stored for 1-3 days at 4^0C. Storage at more days drastically inhibited the germination rate. The effect of storage of synthetic seeds on germination was statistically significant at 5% level. Various substrates were tested for germination on half strength solidified MS medium, moist cotton, and sterile soil moistened with half strength MS containing NAA (5.4μM) and BA(2.5μM).

On half strength MS medium, 60% seeds germinated within 30 days. Among the three substrates used, MS solid medium was most effective. Combinations of growth regulators (BA and NAA) were responsible for highest germination response. The germination response was poor on sterilized soil moistened with MS nutrients and on cotton moistened with growth regulators. The highest germination response (75.5%) was observed in BA (2.5μM)+ NAA(1μM) of which 23.4% germinated and 16.2% plantlets survived. The encapsulated embryos germinated within 15 days of culture. The alginate matrix ruptured, green leaves emerged and roots developed from encapsulated embryos. The plantlets obtained from encapsulated embryos were observed to be normal (Arumugam and Rao, 2000).

4. CONCLUSIONS AND FUTURE PROSPECT

Synthetic seed technology offers many useful advantages on a commercial scale for propagation of variety of crops. Key factors determining the success of somatic embryogenesis and synthetic seeds production, control the practical application of synthetic seed technology especially in trees. Induction of high quality somatic embryos followed by corresponding conversion of somatic embryos to plants is currently a major problem in woody species. The process of somatic embryogenesis and synthetic seeds is interlinked and depends on each other. The lack of synchrony of somatic embryos is the single most important hurdle to be overcome before advances leading to widespread commercialization of synthetic seeds can occur (Saiprasad, 2001). Non availability of well standardized protocol in several tree species and problems in developing an easy encapsulation procedure for making synthetic seeds are the other major hurdles in woody

plants. Germination of encapsulated embryos, contamination free of synthetic seeds and mechanical damage to seeds play a significant role once the embryos are encapsulated. Correct formulation of the medium in the coating complex helps to enhance the germination frequency of encapsulated embryos and requires elaborate studies. Automation of the technique depends upon all these factors and also upon imparting shelf life that is as long enough as a normal botanic seed and inherent tolerance against drying after sowing (Onishi *et al*., 1994). Use of encapsulation of somatic embryos in woody plants is limited because of lack of basic research and necessity for years of field testing to ensure clonal fidelity (Zimmermann, 1985). Finally, the issue of cost benefits needs to be addressed in each plant. Every plant has its own requirements and problems and therefore each has to be judged case wise. Nevertheless, the encapsulation technique using somatic embryos as propagules remains an attractive proposition, especially for tree species, which are known for loss of seed viability. The technique finds use for germplasm conservation of elite and unique species as well as products of wild hybridization and to be extinct species of tree crops.

5. REFERENCES

Ara H., Jaiswal U. and Jaiswal V. S. (1999). Germination and plantlet regeneration from encapsulated somatic embryos of mango (*Mangifera indica* L.). Plant Cell Rep. 19, 166-170.

Arumugam S. and Rao M. V. (2000). Somatic embryogenesis in *Agel marmelos* (L) Corr. A medicinal tree. In: Somatic embryogenesis in woody plants (Eds. S. M. Jain, P. K. Gupta and R. J. Newton). Kluwer Acad. Pub. Netherlands, pp 663 –655.

Bapat V. A., Mhatre M. and Rao P. S. (1987). Propagation of *Morus indica* L. (mulberry) by encapsulated shoot buds. Plant Cell Rep. 6, 393 – 395.

Bapat V. A. and Rao P.S. (1988). Sandalwood plantlets from synthetic seeds. Plant Cell Rep. 7, 434 – 436.

Dupuis J. M., Roffat C., Derose R.T., Molle F. (1994). Pharmaceutical capsules as coating systems for artificial seeds. Bio/Tech. 12, 385 – 389

Fernandes P.C. 1993. Investigations on the factors controlling the production of artificial seeds and their conversion into plants from somatic embryos of

Santalum album L. (Sandalwood). Ph. D. thesis submitted to University of Mumbai, Mumbai, India.

Figueira A. and Janick J. 1995. Somatic embryogenesis in cacao (*Theobroma cacao* L.). In: Somatic embryogenesis in woody plants (Eds. S. M. Jain, P.K. Gupta and R. Newton), Kluwer Acad. Publ, Netherlands, Vol. 2, pp. 291-310.

Gamborg O.L., Miller R.A. and Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exptl. Cell Res. 50, 151-158.

Janeiro- Laura V., Ballester – Antonio, Vieitez.A.M. (1997). *In vitro* response of encapsulated somatic embryos of *Camellia*. Plant Cell, Tissue & Org. Cult. 51(2), 119 –125.

Janick J, Kim Y.H, Kitto S., Saranga Y. (1993). Desiccated synthetic seeds. In ; Synseeds : Application of synthetic seeds to crop improvement. (Ed. K. Redenbaugh)., CRC press, Boca Raton, USA, pp 11 – 33.

Muralidharan E. M. and Mascarenhas A. F. (1995). Somatic embryogenesis in *Eucalyptus*. In: Somatic embryogenesis in woody plants. (Eds. S. M. Jain, P. K. Gupta and R. J. Newton) Kluwer Acad Pub, Netherlands Vol. 2, pp. 23 – 40.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15, 473-497.

Piccioni E. and Standardi A. (1995). Encapsulation of micropropagated buds of six woody species. Plant Cell Tissue & Org. Cult. 42, 221 – 226.

Onishi N. Sakamoto Y. and Hirosawa T. (1994). Synthetic seeds as an application of mass production of somatic embryos. Plant Cell, Tissue & Org. Cult. 39(2), 137-145.

Rao P. S. and Bapat V. A. (1995). Somatic embryogenesis in sandalwood (*Santalum album* L.). In: Somatic embryogenesis in woody plants. (Eds. S. M. Jain, P. K. Gupta and R. J. Newton). Kluwer Acad Pub, Netherlands, Vol. 2, pp 153-170.

Rao P.S., Suprasanna P. and Bapat V. A. 1998. Synthetic seed technology in horticultural crops. In: Biotechnology in Horticulture Crops. (Ed. K. L. Chadha), ICAR, Malhotra Pub. House, Delhi, pp 38-72.

Redenbaugh K., Paash B.D., Nichol J. W., Kossler M.E., Viss P.R., Walker K. A. (1986). Somatic seeds: encapsulation of asexual embryos. Bio / Tech. 4, 797 – 801.

Redenbaugh K., Slade D., Viss P., Fijii J. (1987). Encapsulation of somatic embryos in synthetic seeds coats. Hort Sci. 22, 803 –809.

Redenbaugh K. and Ruzin S. (1989). Synthetic seed production and forestry. In: Application of biotechnology in forestry and horticulture. (Ed. V. Dhavan), Plenum press, NY, pp. 57 –71.

Redenbaugh, K. (1990). Application of artificial seed to tropical crops. Hort. Science 25, 251-255.

Redenbaugh K. (1993). Synseeds. Application of synthetic seeds to crop improvement. CRC Press, Boca Raton, USA.

Saiprasad, G. V. S. (2001). Artificial seeds and their applications. Resonance. May 2001, 39 – 47.

Stasolla C. and Yeung E. C. (2003). Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell, Tissue & Org. Cul. 74, 15 – 35.

Sudhakara K., Nagaraj B. N., Santhoshkumar A.V., Sunilkumar K., Vijaykumar N.K. (2000). Studies on the production and storage potential of synthetic seeds on Cocoa (*Theobroma cacao* L.). Seed Research 28 (2), 119 – 125.

Von Arnold Sara, Sabala I., Bozhkov P., Dyachok J. and Filonova L. (2002). Development pathways of somatic embryogenesis. Plant Cell, Tissue and Org. Cult. 69, 233 –249.

White P. R. (1954). The cultivation of animal and plant cells. The Ronald Press. New York.

Zimmermann, R. (1985). Application of tissue culture propagation to woody plants. In: Tissue Culture in Forestry and Agriculture. (Eds. R. Henke, K. Hughes, M. Constantin and A. Hollaender). Plenum Press, New York. Pp. 165-177.

PROTOPLAST ISOLATION AND CULTURE OF WOODY PLANTS

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1. INTRODUCTION

Most of the woody plants are characterized with long juvenility periods, a high degree of heterozygosity, along with inadequate information concerning genetic background. Conventional breeding has made great contributions to genetic improvement of the woody plants. However, certain difficulties exist in conventional breeding, such as cross or self-incompatibility, polyembryony, male and/or female sterility and differential flowering periods. So, exploration of other breeding alternatives is imperative as necessity to meet the needs of genetic improvement of woody plants. Much progress has been made in protoplast culture of woody plants since the first attempt was made on protoplast isolation from Acer pesudoplatanus. Vardi et al (1975) regenerated plants for the first time from cultured protoplasts of ovule-derived embryogenic callus of sweet orange. Later on, plants were regenerated from other woody plants, including fruit crops and forest trees (Saito and Suzuki, 1999; Witjaksono et al, 1998; Zhu et al, 1997; Zhang et al, 1998; Yu et al, 2000; Ara et al, 2000; Qiao et al, 1998; Sushamakumari, 2000). The present chapter is devoted to a general protocol for protoplast isolation and culture of woody plants.

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2. PROTOPLAST ISOLATION

Protoplast culture and genetic manipulation depend on the production of large number of viable and healthy protoplasts. Earlier, protoplasts were isolated mechanically and did not produce high yield consistently. This method is not used any more for protoplast work after introduction of enzymatic method to protoplast isolation. Enzymatic method is efficient and reliable for protoplast work.

2.1. Explants preparation

Protoplasts can be isolated from different types of explants. But only young and tender leaf, and actively growing callus or cell suspension cultures give high yield of healthy protoplasts. Leaves are excised from plants grown in the greenhouse, out door field, plant growth chamber or *in vitro*. For consistent results, *in vitro* grown plants are most suitable for protoplast isolation and culture. The leaves excised from the former three sources are first washed in tap water, then surface disinfested with 70% alcohol (V/V) for 30 s, followed by 10% sodium hypochloride (V/V) for 10 min or 0.1% $HgCl_2$ (V/V) for 5-10 min, which are then washed with double distilled water for three to five times. With seeds sampled from mature fruits they are first immersed in 1 mol/L NaOH to remove the pectin on the surface. Then they are treated with 10% sodium hypochloride (V/V) for 10-15 min, followed by wash with sterilized distilled water for 3 times. The seed coats are subsequently removed and the sterile seeds are sown in the test tubes or flasks, from which *in vitro* leaves are to be harvested. The in-vitro leaves should be excised just after they are fully developed or expanded, generally 20 days or so after the *in vitro* germination. The leaves are cut into 2 mm pieces with sharp scalpel for protoplast isolation. Callus can be induced from different explants (e.g. meristem, zygotic or somatic embryo and nucellus, seed, immature inflorescence, leaf, root and anther etc.) in basal medium supplemented with phytohormone (e.g. MS or MT + 2,4-D). The callus is subcultured for 7-10 cycles at intervals of 7-15 days depending on the species prior to protoplast isolation. Actively growing cell suspension cultures can be established by culturing the callus in liquid medium. The ingredients of medium for suspension

culture are more or less similar to the medium for growing callus cultures. Similarly, the suspension cultures are subcultured at intervals of 7-15 days and cultured in gyratory shaker at 110 rpm. At subculture the cells or cell clusters from previous suspension culture are pipetted into a new container with fresh medium. The callus and the suspension cultures are always sampled in the logarithmic phase for protoplast isolation.

2.2. Enzyme digestion for protoplast isolation

The leaves and the callus or cell suspension culture are directly incubated in the enzyme solution containing 1-2% cellulase (Cellulase Onozuka R-10 or RS and Driselase) and 0.1-0.2% pectinase (Pectolyase Y-23, Serva and Macerozyme), supplemented with osmoticum regulators (glucose, fructose, sucrose, sorbitol and mannitol, with a concentration of 0.6-0.8 mol/l), pH stabilizer [2-(N-morpholino) ethanesulfolic acid, MES], membrane protectants ($CaCl_2{\cdot}2H_2O$, KH_2PO_4 or dextran sulfate potassium), etc. The enzyme solution is sterilized by membrane filtration (0.22-0.45 μm). This enzymatic digestion of the tissue is usually finished in a single step. In two-step incubation, explant is treated with pectinase and cellulase consecutively and has also been used to isolate protoplasts from apple cotyledon (Yamaki 1981). The ratio of explants to enzyme mixture is possibly different, depending on the physiological status, the age of the explants and composition of enzyme mixture. For citrus protoplast isolation, usually 1 g callus or 0.1 g leaflets is incubated in 3 ml enzyme solution (Grosser and Gmitter, 1990). The mixture containing explants and enzyme mixture is put in petri dishes or in small flasks (50 ml capacity). With leaves, 15 min vaccum treatment is sometimes needed to stimulate the enzyme's infiltration into tissue. The petri dish or flask is generally kept in the dark on a gyratory shaker at 25-28ºC for several hours or overnight. Generally, tissue is incubated in enzyme solution in light for some plants and for others in darkness. The optimum revolution speed differs among species, for instance, 25-30 rpm for citrus and 40-100 rpm for apple, pear and peach.

2.3. Protoplast purification

After incubation, the mixture is first passed through a stainless steel or nylon sieves with pore size of 45-70 μm to discard debris and undigested tissue. The mixture is washed with CPW salt solution (see Table 1). For the removal of enzyme mixture and washing solution, the filtered protoplasts are centrifuged for 6 min at 30-100 g with either of two methods, sinking or floating. By sinking method, the washing solution is added to the enzyme mixture and protoplasts will sediment at the bottom of the centrifuge tube. With floating method the mixture is layered on a discontinuous density gradient (25% sucrose and 13% mannitol), and by centrifugation protoplasts will float to form a band in the interface of the two gradient solutions, and the debris or the multiple-cell clusters will settle to the bottom or remain suspended in the solution due to difference in the specific gravity. The resulting sediment (in the former method) or the protoplast band (in the latter method) should be carefully collected and transferred to another centrifuge tube in which washing solution or culture medium is added. The resuspended protoplasts are then centrifuged for 5-6 min at 100g to form a pellet. The supernatant is then removed and culture medium is added again to dilute the protoplasts and the protoplasts are adjusted to an appropriate density, for example 5×10^5-10^6/ml with hemocytometer.

Table 1 Composition of the washing solution used for protoplast purification (mg/l)

	KH_2PO_4	KNO_3	$CaCl_2{\cdot}2H_2O$	$MgSO_4{\cdot}7H_2O$	KI	$CuSO_4{\cdot}5H_2O$
Concentration	27.2	101	1480	246	0.16	0.025

pH of the washing solution is 5.6.

2.4. Protoplast viability analysis

Viability of the purified protoplasts is usually determined by FDA (fluorescein diacetate) staining. FDA is prepared in acetone at 2 mg/ml and stored as a stock

solution at 4°C. It accumulates only inside the plasma membrane of viable protoplasts. Therefore, under UV light using a fluorescent microscope, green fluorescence can be observed in the viable cells.

3. PROTOPLAST CULTURE

3.1. Culture medium and method

The most widely used basal media for woody plants are MS, B_5, LP, LM, DCR, WPM, K_{8p} and KM8p, and other media are modified from these, such as BH_3

Table 2. Media composition of macro elements, trace elements, vitamins and organic acids and growth regulators (mg/l); sugars (g/l); organic supplements (ml/l)

Compostion	KM_{8p}	MS	B_5	Compostion	KM_{8p}	MS	B_5
Macro elements (mg/L)				*Trace elements (mg/L)*			
NH_4NO_3	600	1650	134	H_3BO_3	3	6.2	3
KNO_3	1900	1900	2500	$MnSO_4 \cdot H_2O$	10	22.3	10
$CaCl_2.2H_2O$	600	440	150	$ZnSO_4 \cdot 7H_2O$	2	8.6	2
$MgSO_4.7H_2O$	300	370	250	$NaMoO_4 \cdot 2H_2O$	0.25	0.25	0.25
$(NH_4)_2SO_4$	-	-	134	$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025
$NaH_2PO_4 \cdot H_2O$	-	-	150	$CoCl \cdot 6H_2O$	0.025	0.025	0.025
KH_2PO_4	170	170	-	KI	0.75	0.83	0.75
KCl	300	-	-	*Vitamins and organic acids (mg/L)*			
$FeSO_4 \cdot 7H_2O$	-	28	28	Inositol	100	100	100
Na_2EDTA	-	37	37	Nicotinic acid	-	0.5	1
Sequestrene 330 Fe	28	-	-	Pyridoxin HCl	1	0.5	1

Sugars (g/L)				Thiamine HCl	10	0.1	10
Sucrose	0.25	30	34	D-Ca-Pantothen-ate	1	-	-
Mannose	0.25	-	-	Folic acid	0.4	-	-
Glucose	68.4	-	-	p-aminobenzoic acid	0.02	-	-
Fructose	0.25	-	-	Biotin	0.01	-	-
Ribose	0.25	-	-	Cholinechloride	1	-	-
Xylose	0.25	-	-	Ascorbic acid	2	-	-
Rhamnose	0.25	-	-	Vitamin A	0.01	-	-
Cellobiose	0.25	-	-	Vitamin D_3	0.01	-	-
Sorbitol	0.25	-	-	Vitamin B_{12}	0.02	-	-
Mannitol	0.25	-	-	Glycine	2	-	-
Growth regulators (mg/L)				Na-pyruvate	20	-	-
2,4-D	0.2	-	2	Citric acid	40	-	-
NAA	1	-	-	Malate	40	-	-
IAA	-	-	-	Fumarate	40	-	-
Zeatin	0.5	-	-	*Organic supplements (ml/L)*			
Kinetin	-	3	0.75	Edamin	-	1000	-
				Casien hydrolysate	250	-	-

(Fowke LC and Fonstabel F (eds.). Plant Protoplast, CRC Press, 1985)

medium for citrus protoplast culture (Grosser and Gmitter, 1990). Components of MS, B_5 and WPM are listed in Table 2. There are several ways to culture protoplasts , such as liquid culture, solid culture and liquid plus solid

culture (liquid over solid culture), nurse culture, feeder layer culture and droplet culture. Take liquid thin layer as an example, 2-3 ml protoplast suspension is added in a petri dish (6 cm × 6 cm), which is sealed with parafilm. When solid culture or also called agarose bead culture or embedding culture is used, protoplasts are mixed with 1.2% agar or low melting temperature agarose (LMT), which is kept in water bath at 30-40ºC at a certain ratio (1:2). The cultured protoplasts are placed in the growth chamber at 25-28ºC. In addition liquid plus solid culture is always used for protoplast culture. With this culture system, the solid medium is poured in the petri dish on which protoplasts are added. Without exception of culture method, protoplasts are cultured initially in the dark.

3.2. Callus formation

Cell wall regenerates in cultured protoplasts after 24-72 hrs depending on the plant species. When protoplasts become elliptical, it is a signal of cell wall regeneration, which can be investigated by Calcofouor White (CW). This dye binds to cell wall material and exhibits fluorescence by irradiation with blue light. A stock solution of CW can be prepared from 50 mg CW dissolved in 5 ml buffer (Tris, pH 9.0). The working solution contains 0.1 ml stock solution in 9.9 ml buffer. Both solutions are stable and are stored at room temperature in the dark. When protoplasts are stained by CW, cells with resynthesized cell walls appear yellow or green under a fluorescent microscope, while those without cell walls appear red. The time for cell wall regeneration is not fixed for different plants, and ranges from several hours to several days. Shortly after the regeneration of cell walls, protoplasts recover their first mitotic division (Fig. 1, A). Species, genotype, and explant all affect the relative time required for first division. For some plants the division can be observed within 1-2 days, while others may require 5-7 days or even longer. The protoplasts keep dividing and develop into multi-cell clusters or micro-calli or protcalli (Fig 1, B and C). At this time it is usually necessary to supply fresh culture medium to the cultures because nutrition and osmotic pressure in the culture system cannot meet the needs of growing cells. The protoplasts keep dividing form cell clusters and subquently micro calli, which further grows for another 2-3 weeks

and are transferred to shoot or embryo differentiation culture medium.

3.3. Shoot formation

The callus can be differentiated into shoots via either organogenesis (apple, pear, chinar, poplar and mango, etc.) or somatic embryogenesis (*Abies alba*, *Pinus*, *Larix*, *Picea*, papaya, citrus, avocado and banana) (Fig. 1, D-F). As for organogenesis usually shoot primordias arise directly from the callus, which are frequently featured with areas of darker green pigmentation. As for somatic embryogenesis, green embryoids are developed around the callus. Four phases are always detected during somatic embryo formation: globular shape, heart shape, torpedo shape and cotyledon shape. Usually proembryos that show early developmental stage prior to formation of globular embryos could be observed. Shoot primordias can develop from the embryoids on the shoot induction medium. The shoot primordias from both cases develop further into normal shoots. For the development of protoplast- derived calli, different media are needed, with (Saito and Suzuki, 1999) or without plant growth regulators (Vardi and Spiegel-Roy, 1982). Herein media used for citrus protoplast culture are listed as follows. BH_3 (Grosser and Gmitter, 1990) is used for initial protoplast culture, and MT +malt extract 500mg/L is used for callus growth, MT + malt extract 1500 mg/L+Sucrose 50 g/L and MT +KT 0.5 mg/L+BA 0.5mg/L + NAA 0.1 mg/L for regeneration of embryhoids and shoots, respectively.

3.4. Root and plant regeneration

The shoots are cut and transferred to the root-induction medium containing 1/2 MT supplemented with some phytohormones, mainly auxins (NAA and IBA). In addition, activated charcoal (AC) is always needed. For example root-induction medium for citrus protoplast-derived shoots is 1/2 MT + IBA 0.5mg/L + NAA 0.1 mg/L + AC 0.5g/L + Sucrose 30 g/L+agar 7g/L. White root primordias appear at the bottom of the shoots several days after the transfer. Later on the roots grow longer and stronger (Fig.1, G). If the shoots are recalcitrant to rooting, *in vitro* grafing can be employed to get complete plantlets.

3.5. Hardening and transplantation of the plantlets

The rooting or the micrografted plantets are usually cultured in the root-induction medium for a certain period. Then the plantlets are cultured in a medium containing the same basal medium supplemented with sugars but without phytohormornes, aiming at fully hardening of plantlets. The plants grow under high light intensity (33 μmol/m^2·s) at the room temperature. The lids of jars are removed when plantlets are well developed, and their roots are washed thoroughly to remove culture medium with running tap water before transferring to the greenhouse or growth chamber. Initially the plants should be covered with glass flasks or transparent plastic cups in order to maintain the high relative humidity (over 90%). These plants are nourished with a nutrition solution containing 1/3 MS macroelements at every one week interval for 2 weeks. Flasks or plastic cups are removed when plants show new growth, and are allowed to grow in the pots filled with soil (Fig.1, H) until they are ready to go to the field.

For summary a flow chart of protoplast isolation, purification, culture and regeneration is given in Figure 2.

4. CONCLUSIONS AND PROSPECTS OF IMPROVEMENT

Since the first report on protoplast manipulation of woody plants was reported great successes have been accomplished during the past decade. Protoplasts are useful for germplasm cryopreservation, protoplast fusion, selection of *in vitro* mutants, and genetic transformation. However, high yield of protoplasts is affected by several factors such as plant species and genotype (Schum et al, 2001; Assani et al, 2002), physiological status and source of explant (Matsuta et al, 1986; Schum et al, 2001; Sticken et al, 1986), enzyme condition (Matsuta et al, 1986), and pretreatment (Marchant et al, 1997). Similarly, protoplast culture is affected by genotype (Sasmoto et al, 1989), explant source (Assani et al, 2002), culture medium (Chen et al, 1993; Ochatt et al, 1992; Grosser, 1994; Qiao et al, 1998; Witjaksono et al, 1998), culture density (Qiao et al, 1998; Sticken et al, 1986),

culture methods (Assani et al, 2001; Park and Son, 1992; Sushamakumari et al, 2000) and pretreatment (Ochatt et al, 1988). Therefore, all these factors should be taken into considerations while working on protoplast isolation and culture. In addtion, reliable protocols are needed in large number of plant species for protoplast work.

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5. REFERENCES

Afonso CL, Harkins KR, Thomas-Compton MA, Krejci AE, Galbraith DW. Selection of somatic hybrid plants in Nicotiana through fluorescence-activated sorting of protoplasts. Bio/tech, 1985, 3: 811-816

Ara H, Jaiswal U, Jaiswal VS. Plant regeneration from protoplasts of mango (Mangifera indica L.) through somatic embryogenesis. Plant Cell Rep, 2000, 19: 622-627

Assani A, Haicour R, Wenzel G, Cote F, Bakry F, Foroughi-Wehr B, Ducreux G, Aguillar ME, Grapin A. Plant regeneration from protoplasts of dessert banana cv. Grande Naine (Musa spp., Cavendish sub-group AAA) via somatic embryogenesis. Plant Cell Rep, 2001, 20: 482-488

Assani A, Haicour R, Wenzel G, Foroughi Wehr B, Bakry F, Cote FX, Ducreux G, Ambroise A, Grapin A. Influence of donor material and genotype on protoplast regeneration in banana and plantain cultivars (Musa spp.). Plant Sci, 2002, 162: 355-362.

Chen MH, Chupeau MC, Lemoine M, Chupeau Y. Requirement of thidizauron for healthy protoplast development to efficient tree regeneration of a hybrid popular (Populus trmula × P. alba). J Plant Physiol, 1993, 141: 601-609

Dornelas MC, Tavares FCA, Oliviera JC de, Vieira MLC. Plant regeneration from protoplast fusion

in Passiflora spp. Plant Cell Rep, 1995, 15: 106-110

Grosser JW. Observation and suggestions for improving somatic hybridization by protoplast isolation, fusion and culture. Hort Sci, 1994, 29: 1241-1243

Grosser JW, Gmitter FG Jr. Protoplast fusion and citrus improvement. Plant Breed Rev, 1990, 8: 339-374

Grosser JW, Ollitrault P, Olivares-Fuster O. Somatic hybridization in citrus: an effective tool to facilitate variety improvement. In Vitro Cell Dev Biol Plant, 2000, 36: 434-449

Marchant R, Davey MR, Power JB. Isolation and culture of mesophyll protoplasts from Rosa hybrida. Plant Cell Tissue Organ Cult, 1997, 50: 131-134

Matsuta N, Hirabayashi T, Akihama T, Kozaki I. Callus formation from protoplasts of peach cell suspension culture. Sci Hort, 1986, 28: 59-64

Ochatt SJ, Chevrearu E, Gallet M. Organogenesis from 'Passe Crassane' and 'Old Home' pear (Pyrus communis L) protoplasts and isoenzymatic trueness-to-type of the regenerated plants. Theor Appl Genet, 1992, 83: 1013-1018

Ochatt SJ, Rech EL, Davey MR, Power JB. Long term effect of electroporation on enhancement of growth and plant regeneration of colt cherry (Prunus avium× pseudocerasus) protoplasts. Plant Cell Rep, 1988, 7: 393-395

Ochatt SJ. The development of protoplast-to-tree system for Prunus cerasifera and Prunus spinosa. Plant Sci, 1992, 81: 253-259

Olivares-Fuster O, Pena L, Duran-Vila N, Navarro L. Green Fluorescent Protein as a visual marker in somatic hybridization. Annals of Bot, 2002, 89 (4): 491-497

Park YG, Son SH. In vitro shoot regeneration from leaf mesophyll protoplasts of hybrid poplar (Populus nigra×P. maximowiczii). Plant Cell Rep, 1992, 11: 2-6

Pattanavibool R, Klimaszewska K, Von Anderkas P. Interspecies protoplast fusion in Larix: comparison of electric and chemical methods. In Vitro Cell Dev Biol-Plant, 1998, 34: 212-217

Qiao J, Kuroda H, Hayashi T, Sakai. Efficient plantlet regeneration from protoplasts isolated from suspension cultures of poplar (Populus alba L.). Plant Cell Rep, 1998, 17: 201-205

Saito A, Suzuki M. Plant regeneration from meristem-derived callus protoplasts of apple (Malus × domestica cv. Fuji). Plant Cell Rep, 1999, 18: 549-553

Samoylov VM, Sink KC. The role of irradiation dose and DNA content of somatic hybrid calli in producing asymmetric plants between an interspecific tomato hybrid and eggplant. Theor Appl Genet, 1996, 92: 850-857

Sasmoto H, Hosoi Y, Ishii K, Sato T, Saito A. Factors affecting the formation of callus from leaf protoplasts of Populus alba. J Jpn For Soc, 1989, 71: 449-455

Schum A, Hofmann K, Ghalib N, Tawfik A. Factors affecting protoplast isolation and plant regeneration in Rosa spp. Gartenbauwwissenschaft, 2001, 66: 115-122

Sticken MB, Domir SC, Lincberger RD. Shoot regeneration from protoplast of Ulmus×pioneer. Plant Sci, 1986, 47: 29-34

Sushamakumari S. Plant regeneration from embryogenic cell suspension-derived protoplasts of rubber. Plant Cell Tissue Organ Cult, 2000, 61: 81-85

Tamura M, Tao R, Sugiura A. Production of somatic hybrids between Diospyros glandulosa and D. kaki by protoplast fusion. Plant Cell Tissue Organ Cult, 1998, 54: 85-91

Vardi A, Spiegel-Roy P, Galun E. Citrus cell culture: isolation of protoplasts, plating densities, effects of mutagens and regeneration embryos. Plant Sci Lett, 1975, 4: 231-236

Vardi A, Spiegel-Roy P. Plant regeneration from citrus protoplasts: variability in methodological requirements among cultivars and species. Theor Appl Genet, 1982, 62: 171-176

Witjaksono, Litz RE, Grosser JW. Isolation, culture and regeneration of avocado (Persea americana Mill.) protoplasts. Plant Cell Rep, 1998, 18: 235-242

Yamaki, S. Subcellular localization of sorbitol-6-phosphate dehydrogenase in protoplast from apple cotyledons. Plant Cell Physiol, 1981, 5: 359-367

Yu CH, Chen ZG, Lu LX, Lin JW. Somatic embryogenesis and plant regeneration from litchi protoplasts isolated from embryogenic suspensions. Plant Cell Tissue Organ Cult, 2000, 61: 51-58

Zhang YJ, Qian YQ, Mu XJ, Cai QG, Zhou YL, Wei XP. Plant regeneration from in vitro-cultured seedling leaf protoplasts of Actinidia eriantha Benth. Plant Cell Rep, 1998, 17: 819-921

Zhu YM, Hoshino Y, Nakano M, Takahashi E, Mii M. Highly efficient system for plant regeneration from protoplasts of grapevine (Vitis vinifera L.) through somatic embryogenesis by using embryogenic callus culture and activated charcoal. Plant Sci, 1997, 123: 151-157

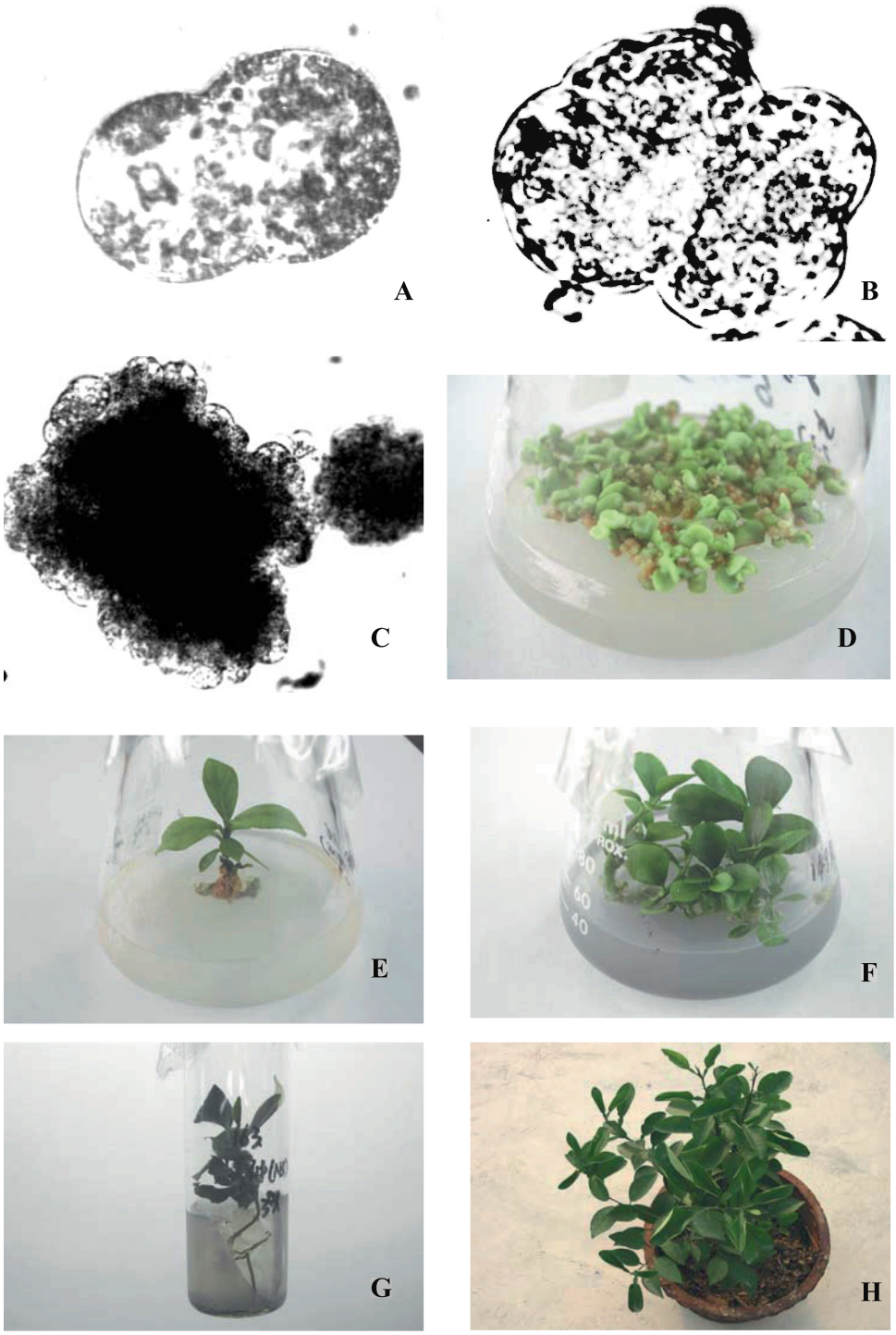

Fig. 1. Regeneration of plantlets from citrus protoplasts. A. Recovery of the first division. B. A cell showing the second division. C multi-cell cluster. D. Regeneration of embryoids. E-F. Regeneratino of shoots. G-H. Regeneration of plants.

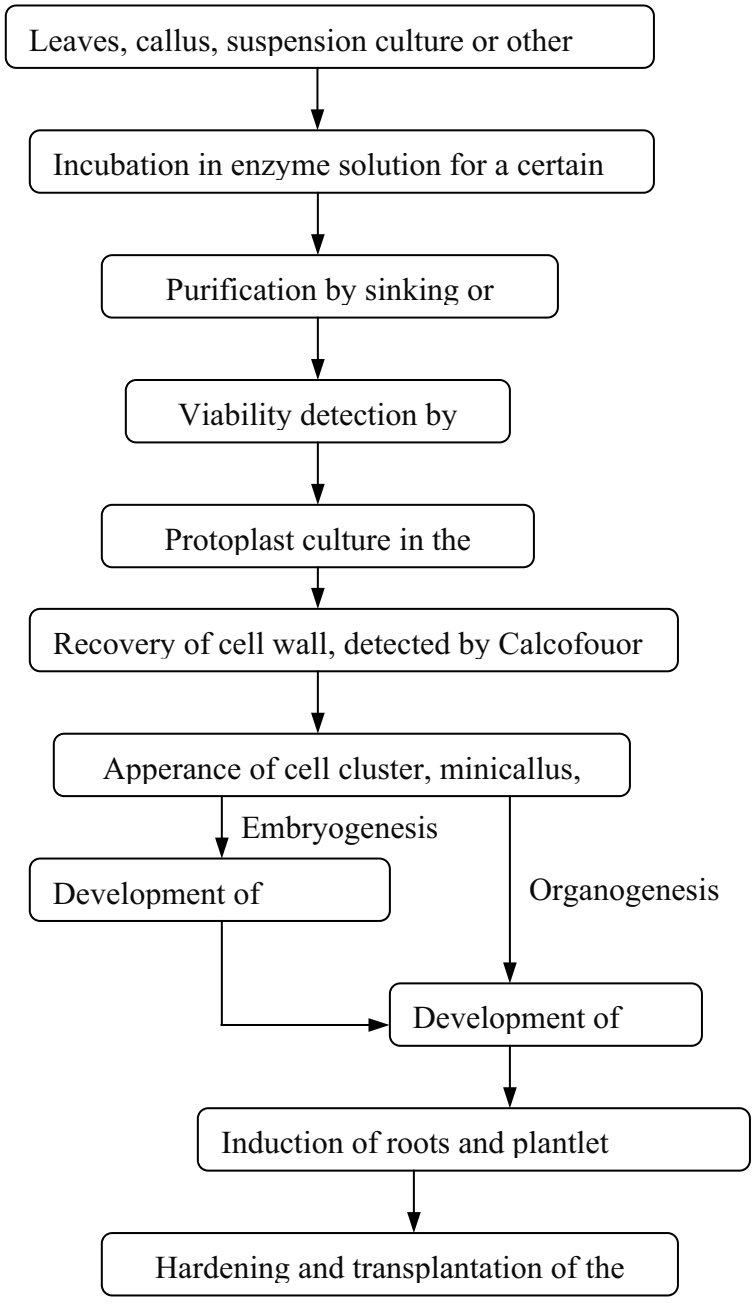

Figure 2. Flow chart of protoplast isolation, purification, culture and regeneration.

CRYOPRESERVATION OF EMBRYONAL CELLS

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1. INTRODUCTION

Cryopreservation of plant cells and organs has become an important tool for long-term preservation of germplasm or experimental material (Kartha 1985). Plant cell suspension cultures are widely used for regeneration of somatic embryos. However, these types of cultures are known for their genetic instability.

They are subject to epigenetic changes leading to, for instance, loss of regeneration potential, loss of embryogenic nature and somaclonal variation. In addition, costs of maintaining these cultures are high and the risk of losing material because of contamination, technical or human errors is always present. Cryopreservation of embryogenic cultures is an attractive option for long-term storage. Cryopreservation allows tissues to potentially be stored indefinitely with minimal maintenance and risks.

Successful cryopreservation procedures aim to reduce water content to a level at which ice crystal formation is non-lethal. To cryopreserve plant cells successfully it is necessary to avoid the formation of ice crystals, which would physically disrupt the cells and cause death. The general strategy is the removal of free water by pretreatment with higher osmoticum medium, followed by the addition of a cryoprotectant. The cells are then subjected to slow cooling at a rate which further minimizes the amount of intracellular water and consequently, intracellular ice. Then the cells are rapidly cooled by direct immersion in liquid nitrogen (LN_2, -196^{o}C). Thawing must be sufficiently rapid to again avoid ice crystal formation. Cryoprotectants are removed from the thawed cells by gradual elution. Only cryopreservation in liquid nitrogen can presently ensure the conservation of cell strains for theoretically unlimited time duration, without alternations and sheltered from contamination. Cryopreservation of embryogenic cells has been achieved with large number plant species, both angiosperm and gymnosperm (Cry 1999).

S.M. Jain and P.K. Gupta (eds.), Protocol for Somatic Embryogenesis in Woody Plants, 567–572.

The protocol for cryopreservation of embryonal cells is based on that developed by Kartha, *et al*. (1988). The process for cryopreservation of embryonal cells starts with two 24-hour culture periods in sorbitol to reduce cell vacuole and water contents osmotically.

This more dehydrated tissue is then treated with a cryoprotectant (DMSO), which stabilizes cell membranes against the severe desiccation that subsequently occurs. Tissue is frozen slowly in a programmable freezer to minus 35^{o}C to remove most of the remaining water from inside the cells. This water accumulates as ice crystals outside the cells, which can be tolerated. Next the tissue is plunged into liquid nitrogen (LN_2) which solidifies remaining cell contents. To thaw the tissue rapidly, the cryo-vials are plunged into warm water. The thawed embryogenic tissue is then detoxified of DMSO by three transfers to semi-solid media before being allowed to re-grow for 2 – 4 weeks.

2. MATERIALS

1. Actively growing embryonal suspension cultures
2. Liquid media (BM1), BM2 (BM1+0.2M sorbitol), BM3 (BM1+ 0.4M sorbitol) and semi-solid (BM4) re-growth media. See Table 1.

TABLE 1: LIQUID MULTIPLICATION MEDIA (BM1)

Constituent	mg/L
Basal Salts	
KNO_3	1250
$CaCl_2.6H_2O$	200.0
$Ca(NO_3)_2.2H_2O$	200
KH_2PO_4	340.0
$MgSO_4.7H_2O$	400.0
$MnSO_4.H_2O$	20.8
$ZnSO_4.7H_2O$	8.0
$CuSO_4.5H_2O$	0.024
$FeSO_4.7H_2O$	27.85
Na_2EDTA	37.25
H_3BO_3	5.0
$NaMoO_4.2H_2O$	0.20
$CoCl_2.6H_2O$	0.025
KI	1.00
$AlCl_3$	0.02
Organic Additives	
Myo-Inositol	5000
Thiamine.HCl	1.00
Nicotinic acid	0.50
Pyridoxine.HCl	0.50
Glycine	2.00
L-Glutamine	1000
Casamino acids	500.0
Sucrose	30000
pH 5.7	

BM2 = BM1 + 0.2M Sorbitol
BM3 = BM1 + 0.4M Sorbitol
BM4 = BM1+5000mg/L TC Agar

3. Programmable temperature controller, freezer chamber, temperature recorder (CryoMed)
4. Cryostorage tank with canisters to accommodate cryovials, with inventory system and liquid nitrogen cylinder
5. Measuring cylinder, pipette, forceps

6. Water bath
7. Regrowth plates with BM1 medium and Whatman # 2 filter paper
8. Crushed ice
9. Filter sterilized DMSO. Sterilize with a 0.2-micron solvent filter one-week in advance and store at 3-6^{o}C. DMSO is toxic. Refer MSDS sheet before use.
10. Safety equipment: Goggles, cryo gloves, and face shield

3. METHOD

To maintain sterility of cultures, all appropriate manipulation should be performed in a Laminar flow hood using aseptic techniques and sterile materials. The method below is divided into three parts: cryoprotection, cryopreservation, and culture thawing. Steps in the method correspond to steps in Figures 1, 2 and 3.

3.1. Cryoprotection

1. Settle the suspension culture for 15-30 minutes and remove the supernatant. Transfer 5 ml settled cells to BM2 (with 0.2M sorbitol) medium (25 ml in 250 ml Erlenmeyer flask) at a 1:9 density. Place the flask on a shaker (rotating at 100 RPM) for 24 hours.
2. After 24 hours, settle the cells again and transfer 5 ml settled cells to BM3 (with 0.4M sorbitol) medium at 1:9 density and return the flask to the shaker.
3. After 24 hours, place the flask with culture in ice. Add DMSO 10 times over a 30 min period to a final concentration of 5% (v/v).
4. Shake the flask continuously when adding DMSO to thoroughly mix cryoprotectant with culture medium.

3.2. Cryopreservation

5. Adjust the cell density by removing supernatant to achieve 30% (v/v) packed cell volume. Dispense 1.0-ml aliquots of cell suspension into 1.2-ml cryovials. Place the cryovials into canisters standing in crushed ice.
6. Load the canisters into a CryoMed programmable freezer and follow the instructions for operation. The cooling rate is from 0.3^{o}C to 1 ^{o}C per minute, which means the process can take up to 2.5 hours to bring the temperature down to minus 35^{o}C. When cooling is complete, open the programmable freezer door and remove the canister.
7. Place the canister in desired rack in the cryostorage tank. Store racks in liquid nitrogen immersion phase. Record the date and location in your inventory.

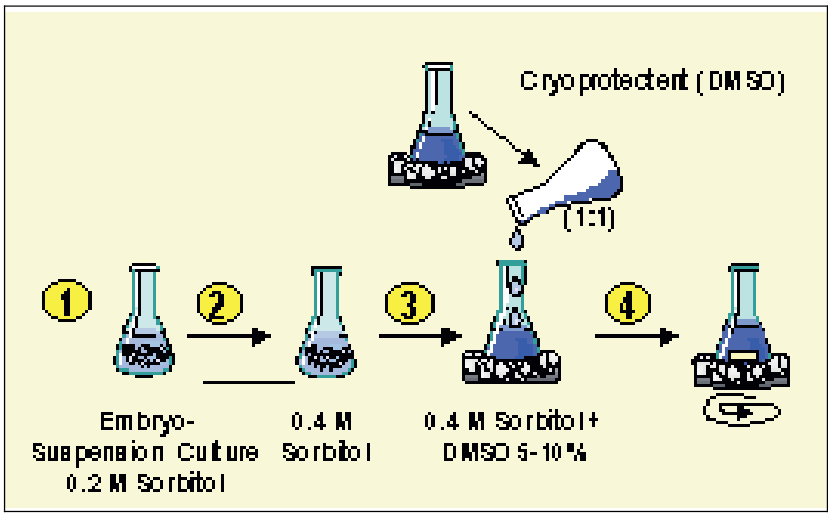

Figure 1. Cryoprotection of embryonal cells, method steps 1 – 4.

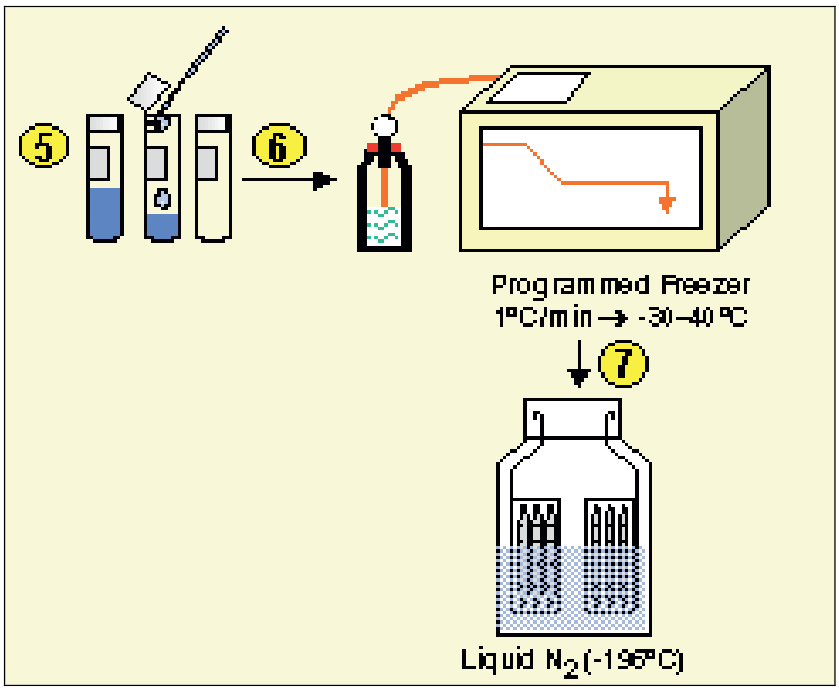

Figure 2. Cryopreservation of embryonal cells, method steps 5 – 7.

3.3. Culture Thawing

8. To thaw, remove the cryovials from the canister in the cryostorage tank, and transfer to a beaker of sterile water placed in a water bath at 37°C. Agitate until all the ice has melted, and then transfer immediately to a rack at room temperature.
9. Surfaces sterilize cryovials one by one by wiping each with 70% isopropyl alcohol. Pour thawed cultures on Whatman # 2 filter placed on semi-solid BM4 regrowth medium in petri dishes.
10. After 1 – 2 hours and again after 24 hours, transfer the filter paper with cells onto fresh regrowth media.
11. When most of the filter paper is covered with a thick layer of cells, transfer the cell mass to a fresh BM4 plate without filter paper. Subculture onto fresh medium at two-week intervals.

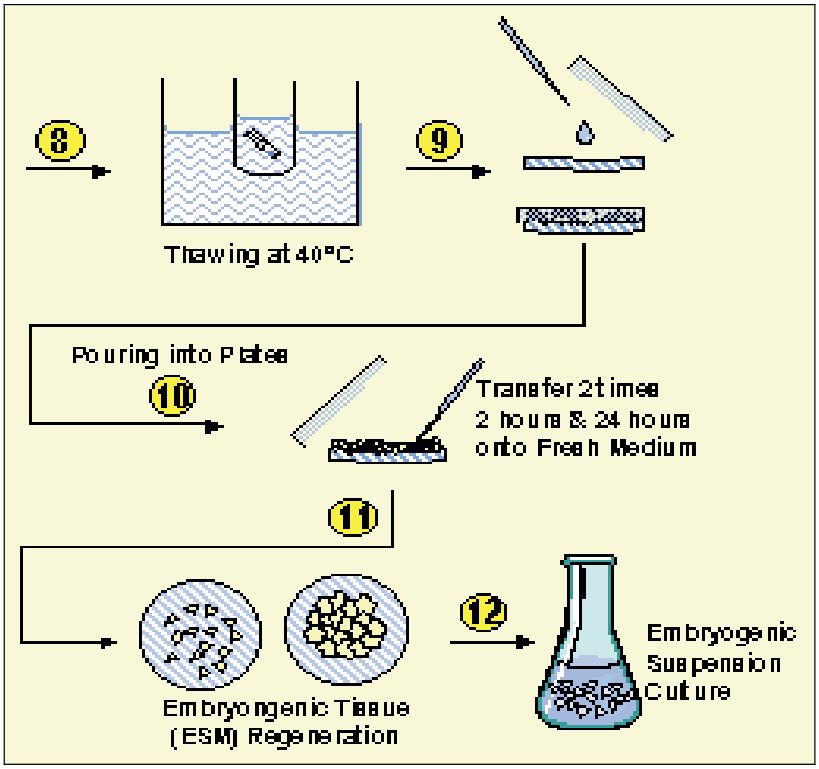

Figure 3. Cryo-thaw of embryonal cells, method steps 8 – 12.

12. After about 4-6 weeks growth, there will be sufficient embryonal cell mass (1-2 g fresh weight) to start a suspension culture as described in book chapters.

This protocol has successfully been applied in our laboratory as well as in other groups engaged in conifer embryogenesis work (Cyr 1999). If the problems are encountered for poor rate of recovery after cryostorage, alternate methods may be tested (Withers 1990, Schrijnemakers and Van Iren 1995).

4. REFERENCES

Kartha KK (1985) Meristem culture and germplasm preservation. In Cryopreservation of plant cells and organs. Kartha KK (ed) CRC Press Inc, USA. p,115

Kartha KK, Fowke LC, Leung NL, Caswell KL, and Hakmann I (1988). Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce. J. Plant Physiol. 32. 329.

Cyr DR (1999). Cryopreservation of embryogenic cultures of conifers and its application to clonal forestry. In Somatic embryogenesis of woody plants. Vol. 4. Jain SM, Gupta PK & Newton RJ (eds.). Kluwer Academic Publ., Netherlands, p. 239.

Schrijnemakers WM and Van Iren F (1995) A two-step or equilibrium freezing procedure for the cryopreservation of plant cell suspension culture, In Cryopresrvation and freezing drying protocol. Day JG & Mclellan MR (eds.). Human Press Inc. Totowa, NJ, p 103

Timmis R, Timmis K & Budworth D (1994). Placement of embryonal suspensor mass cultures into the retrieval from cryogenic storage. (Unpublished). Weyerhaeuser R & D Forest Biotechnology Dept., Reliable Process No. 16A.

Withers LA (1990). Cryopreservation of plant cells. In Plant cell and tissue culture. Pollard JW and Walker JM (eds.) Human Press Inc.Totowa, NJ, p. 39.

DOUBLE STAINING TECHNOLOGY FOR DISTINGUISHING EMBRYOGENIC CULTURES

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1. INTRODUCTION

It is very difficult to distinguish embryogenic cells from non-embryogenic cells. A double staining technique has been developed to differentiate embryogenic material from non-embryogenic (Gupta et al. 1987). This method has been called double staining because two stains, acetocarmine and Evan's blue have been used for staining cells. Embryogenic tissue is easily distinguished by double staining. First, embryogenic cells have large nuclei and dense cytoplasm. These nuclei stain an intense, bright red with acetocarmine. Strands in the cytoplasm also show an affinity for acetocarmine and stain bright red. Acetocarmine is normally used to detect glycoproteins, chromatin and DNA in cytochemical studies (Sharma and Sharma 1980). Second, smaller nuclei, which are associated with formation of suspensors derived from embryonal cells, react with Evan's blue to further differentiate the embryogenic mass. The exclusion of Evan's blue determines the viability of cells. By contrast, less viable cells are vacuolated with small nuclei that permit Evan's blue dye to enter (Gahan 1984). In cells of non-embryogenic callus, nuclei are very small. The acetocarmine-stained red material is difficult to locate and whole cells stain blue with Evan's blue.

2. METHOD

Double staining: A two-step staining process with the addition of 2% acetocarmine to the callus first, followed by 0.5% Evan's blue.

2.1 Preparation of 2% Acetocarmine

Measure out 55 ml H_2O into a 100-ml cylinder and pour into a 300-ml beaker containing a stir bar. Measure out 45 ml of glacial acetic acid using a pipette and add to beaker containing H_2O. This gives a 45% acid solution. Weigh out 2.0 grams of carmine and add to the 45% acid solution. Place a beaker on a stir plate in a fume hood. Boil gently for 5 minutes on highest setting, stir,

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cool and filter by funnel using Whatman filter paper. Store it at room temperature.

2.2 Preparation of 0.5% Evan's Blue

Measure out 100 ml of H_2O into a 250-ml flask. Weigh out 0.5 grams of Evan's blue and add to the 100-ml H_2O in the flask. Cap flask and by hand swirl to mix solution.

2.3 Double staining procedure

1. Place a small piece of callus (2- 5 mm in size) on a glass slide.
2. Add few drops of 2% acetocarmine to the callus until it is submerged.
3. Gently divide the callus with forceps into very small pieces in the acetocarmine.
4. Hold the slide with forceps or a slide holder and heat over a low flame for a few seconds. Do not boil it.
5. Wash the callus 2 to 3 times with water and remove all liquid.
6. Add two to three drops of 0.5% Evan's blue to acetocarmine stained cells.
7. Wait for 30 seconds.
8. Wash 2 – 3 times with water and then remove all water.
9. Add one to two drops of glycerol to the stained cells. This will prevent the preparation from drying.

Observe the slide under the microscope using illumination from beneath the specimen. The embryonal head cells stain bright red (acetocarmine) and suspensor cells stain blue in an embryogenic mass *(Figure 1)*.

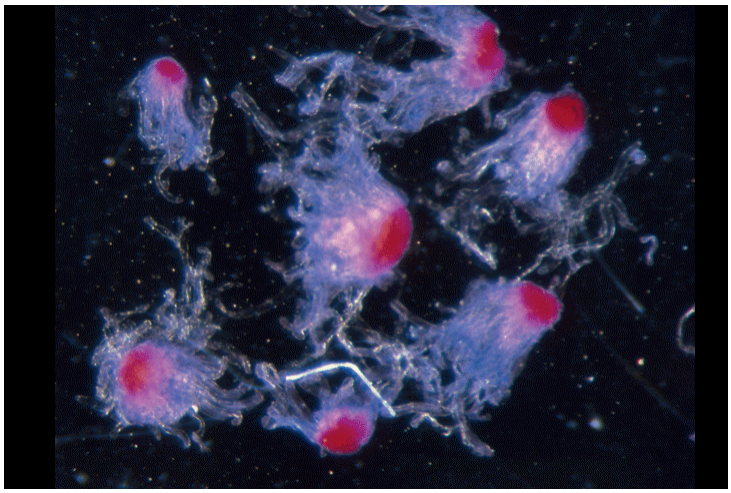

Figure 1: Early stage embryos after double staining, embryonal heads stained red (acetocarmine) and suspensors stained blue (Evan's blue).

If the mass is non-embryogenic callus, cells do not show any organization of head and suspensor and will stain blue with Evan's blue *(Figure 2)*.

This method will distinguish embryogenic masses from non-embryogenic callus.

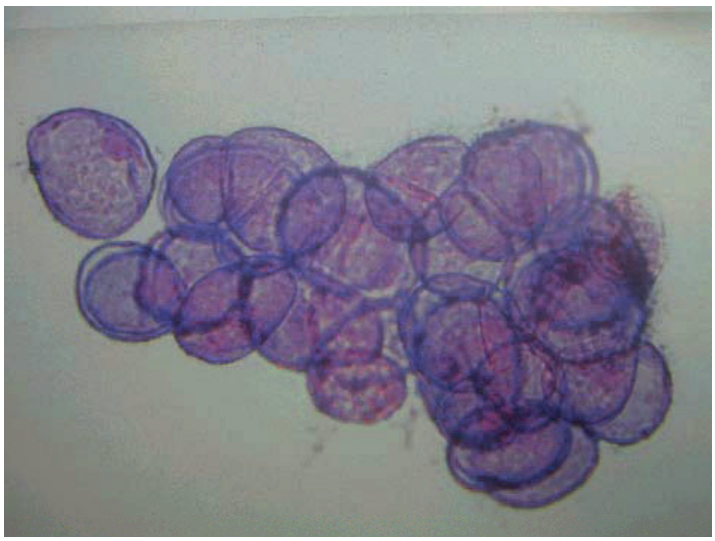

Figure 2: Double stained non-embryogenic cells, showing only Evan's blue stain.

3. REFERENCES

Gupta PK and Durzan, DJ (1987) Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Biotechnology 5, 147 – 151

Sharma AK and Sharma A (1980) Chromosome techniques, theory and practice. Frakenham Press, Ltd., Norfolk., P. 121

Gahan PB (1984) Reversible and irreversible damage in plant cells of different ages. In: Cell Aging and Cell Death. Davis I., Sigee, DC (eds) Cambridge Univ. Press, p. 271-287.

THIN CELL LAYER SECTIONING FOR INDUCING SOMATIC EMBRYOGENESIS IN WOODY PLANTS

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1. INTRODUCTION

Thin cell layer (TCL) technology allows controlled production of somatic embryos in otherwise recalcitrant species, has enhanced the importance of somatic embryogenesis in plant (micro) propagation and mass propagation, (cryo) preservation and genetic transformation. TCL technology also provides an ideal experimental basis for investigating differentiation and for understanding the mechanisms of totipotency and differentiation in plant cells, tissues and organs.

Thin cell layers (TCLs) are small explants, excised longitudinally (lTCL) or transversely (tTCL) from different plant organs including floral parts, specific root/rhizome, stems, leaf vein, floral stalk, petiole, pedicel, and bulb-scales. lTCL are used when a definite cell type (epidermal, sub-epidermal, cortical cambial or medullar cell) is to be analyzed. As for tTCL, other organs can be used too: leaf blade, root/rhizome, floral organs (sepal, petal, anther, filament, pistil), young spikelet, meristem, stem-node, *inter alia* TCLs are important for the programming of developmental and morphogenetic processes, which can be altered by making changes in organ/tissue/cell correlation and size to be uniformly exposed to the medium (Compton and Veuilleux, 1992).

Recently, TCL culture of various explants has emerged as a useful tool for the study of cellular, biochemical and molecular mechanisms controlling *in vitro* morphogenesis in plants, and to overcome recalcitrance to regeneration of leguminous, lignous (Tran Thanh Van et al., 1985) and monocotyledoneous species (Jullien and Tran Thanh Van, 1994). The advantages of TCL methodology are a high induction frequency of organogenesis/embryogenesis within a short-time frame. This chapter deals with: (i) TCL method to study the morphogenic progress of embryogenesis in woody plants and (ii) the usefulness of TCLs in transformation studies of woody plants involving somatic embryos.

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2. PROTOCOLS FOR SOMATIC EMBRYOGENESIS OF SOME SELECTED WOODY PLANTS

2.1. Bamboo

2.1.1. *Bambusa* spp.

This popular woody monocot is a landscape ornamental and is usually vegetatively propagated by division of culms, but has been used for inducing somatic embryos by the TCL method.

tTCLs from young leaves sectioned through a bud from the base to the apex are cultured on a medium supplemented with 18 μM 2,4-dichlorophenoxyacetic acid (2,4-D) in darkness. After 5 weeks, friable and mucilaginous callus or compact and epidermized embryonic nodules, depending on the horizontal and vertical gradient: the inner leaf tTCLs and the tTCLs of the basal part of this leaf are highly responsive for somatic embryogenesis (Jullien and Tran Thanh Van, 1994). The pale yellow nodules are similar to embryonic nodules differentiated on *Iris pallida* leaf tTCLs. The embryonic nodules of *Bambusa* form embryo-like structures (ELSs) that fail to develop any further.

Remove several outer layer leaves, and cut 5 mm semi-thin transverse sections (STTSs) from *in vivo* young leaves of *B. glaucescens*. *In vitro* shoots are obtained by allowing dormant buds to grow by cutting stems of 4-8 mm diameter into two internodes giving fragments of 5-7 cm long including node-bearing dormant buds, and two internodes. Leaf sheaths covering axillary buds are removed. Explants are surface sterilized by three chemical treatments: benlate 0.1% for 3 h, 75% ethanol for 2 min and 8% sodium hypochlorite for 20 min, followed by thorough washing in sterile distilled water.

The basal medium contains Murashige and Skoog (1962) (MS) medium amended with: 100 mg 1^{-1} myo-inositol, 0.5 mgl^{-1} nicotinic acid, 0.5 mgl^{-1} pyridoxine-HCl, 1 mg 1^{-1} thiamine-HCl and 2 mgl^{-1} glycine. Micropropagation media contains 15 gl^{-1} sucrose and ⌴-naphthaleneacetic acid (NAA) in various concentrations. After 2 weeks, elongated shoots are obtained and use as explants for callus initiation and embryogenesis. The callus medium contains MS basal medium supplemented with 30 gl^{-1} sucrose, 250 mgl^{-1} casein hydrolysate, 40 ml^{-1} coconut milk, 8 gl^{-1} Bacto-agar and 500 mgl^{-1} polyvinylpolypyrrolidone (PVPP). Plant growth regulators (PGRs) are added to the medium at different concentrations: 9-18 μM 2,4-D, 1.14-2.85 μM NAA, 0.22-2.2 μM 6-benzyladenine (BA) and 0.6 μM abscisic acid (ABA). Cultures are kept in the dark or under fluorescent light 10-40 μE s^{-1} m^{-2} on a 16 h photoperiod and at a constant temperature of 26±1°C.

Alternative protocol: After removal of the outer leaves, four inner leaves from the apex, approximately 2 cm long, are cut into STTSs; culture on a modified MS medium containing 18 μM 2,4-D; keep in the dark. After 1 week, cells beneath the epidermal layer in contact with the medium and cells surrounding vascular bundles start proliferation. The four STTSs of each explant do not show the same competence for cell proliferation and a gradient is observed from the first STTS (closer to the apex, producing more cell division) to the fourth (upward). The dissection of each STTS reveals a second gradient from the inner leaves to the outer leaves. In addition, the type of container and sealing influence the cell proliferation rate. The highest cell proliferation can be obtained when explants are cultured in hermetically sealed plastic tubes.

After 3-5 weeks of culture in the dark, STTSs produce three types of calli on culture medium supplemented with 8 μM 2,4-D. The first type is soft, friable, composed of loose and large cells. The second type is white to pale yellow color, smooth and compact and potentially embryogenic. The third type is mucilaginous, fast growing and is interspersed between the first two types. The appearance of the three types of calli depends on the position of the initial explant on the donor plant. The intensity of the proliferation and the type of callus obtained are correlated to the vertical and horizontal gradients. Compact callus can be only initiated in the two first STTSs from the two inner leaves. Other STTSs produce only friable or mucilaginous calli. From 5-8 weeks of culture, 2,4-D concentration is reduced to 9 μM. Morphological changes are observed; nodule-like structures or root primordia are obtained from the compact zones of the mixed three calli. Within 18 μM 2,4-D alone, only friable callus is produced. After 8 weeks, calli are exposed to 10 $\mu E\ s^{-1}\ m^{-2}$ light intensity, BA and indole-3-acetic acid (IAA) concentrations are doubled. More nodular structures are obtained under light conditions. After 10 weeks, embryogenic callus and polyembryo-like structures (PELs) are formed in 5% explants. Two weeks later, PELs develop into ELSs, become green and show polarity when ABA is added. Plantlets don't develop any further.

Axillary buds of *B. glaucescens* are initiated to grow in a modified liquid MS medium supplemented with 20 μM NAA, following a complete immersion of the explants in the liquid culture medium containing 500 mgl^{-1} PVP.

2.1.2. *Dendrocalamus* spp.

Semi thin tTCLs (3-5 mm) of *D. bransdisii* Krus single-node stem segments with unsprouted buds from new proliferated branches of 2-year-old field-grown culms are used to initiate cultures, after removal of the leaf sheath. Sterilized shoot tips of the nodal explant are then excised for use as explants, and place on MS basal medium supplemented with 30 gl^{-1} sucrose, 7 gl^{-1} agar and BA at various

concentrations (1, 5, 10, 20, 50μM). Cultures are kept at 4000 lux with a 16 h photoperiod at 26ºC. Cultures are subcultured onto fresh medium every 3 weeks to induce axillarly shoot proliferation. Shoots are separated into clusters bearing 2-3 shoots (propagules) and transfer them to root induction medium containing various (1-10μM) concentrations of indole-3-butyric acid IBA and coumarin. Multiple shoots are not uniform and show an increase in proliferation by increase BA concentration (1 –50 μM). MS with 10 μM BA induce the highest percentage of shoot regeneration (62.8%). The incorporation of 10 μM IBA and 10 μM coumarin induce shoot elongation and highest root induction (95%) after 4 weeks of culture, while 10 μM IBA, 10 μM coumarin and controls result in 75%, 88% and 1.2% rhizogenesis, respectively. The mean root number is also higher, 3.8 roots per propagule for medium containing 10 μM IBA and 10μM coumarin. All propagules are well developed when transfer to the soil in polybags. Rhizome forms within 2 months and plants eventually establish in soil.

2.2. *Cocos nucifera* (Coconut palm)

Coconut palm trees have commercial importance fruits and many by-products, but have an equally weighty value as landscape plants. Gupta et al. (1984) regenerated coconut plants by using zygotic and somatic embryos. Thin transverse slices (0.2-0.5 mm thick) from different size young inflorescences are also used to induce nodular structures and that later develop into shoot-like structures. A series of inflorescences is collected from 30-year-old *Cocos nucifera* L. 'Typica'. Immature inflorescences ranging from 0.4-7.8 cm long are selected. The corresponding rachillae (inflorescence branches) are isolated from the rachis, and their basal portions are discarded to exclude the female flower meristems, and the remaining portion of rachilla with numerous male flower meristems is used as a source of explants. For surface sterilization, inflorescences enclosed by outer and inner spathes are dipped in 3% H_2O_2 for 15 min and 80% ethanol for 5 s under aseptic conditions. After thorough washing with sterilized water, the outer and inner spathes are peeled off. The rachilla tissues are then sliced transversely (0.2-0.5 mm thick) in a medium composed of Y3 formulation supplemented with 3% sucrose and vitamins at half strength for callus induction. Cultures are kept in darkness inside an incubator controlled at 28±2°C until well-developed calli are obtained.

Initially, to determine a suitable explant stage, tissue slices are taken from a series of inflorescence lengths ranging from 0.4-7.8 cm and evaluated for callus induction and browning. Our observations show that: (i) explants taken from inflorescences more than 3.8-4.7 cm long has slight callusing and a higher rate of browning; (ii) explants derive from inflorescences shorter than 0.4-0.8 cm has minimal browning, but the expansion of tissues is very slow and induction of callus seldom occurs; and (iii) explants (0.8-3.8 cm long) taken from inflorescences has relatively higher callus induction and show minimal browning.

On the basis of preliminary results, inflorescences of five stages comprising 0.98, 1.4, 1.6, 2.5, and 4.1 cm long are considered. For each stage, one hundred tissue slices are inoculated into media containing a wide range of 2,4-D concentrations (5-100ppm) in the presence of 0.25% activated carbon (AC). Discoloration of tissues and initiation of callus are closely monitored during the initial culture period of 1-1.5 months. A high rate of callus induction can be obtained with minimal browning. Stages 1, 2 and 3 show a lower frequency of callus induction. Induction and growth of callus is best accomplished with the addition of 20-30 ppm 2,4-D in the medium. At higher concentrations, 50 and 100 ppm, 2,4-D, severe browning of tissues is observed regardless of the stage and size of cultured tissues. When explants are culled with 10 ppm 2,4-D without AC, all explants turn brown. The inclusion of AC is essential in minimizing browning. Although addition of AC may reduce the availability of 2,4-D at higher concentrations, these high concentrations are still fatal to rachilla tissues.

Rachilla callus continues to develop with 20 ppm 2,4-D. Airing the initial culture period allow explants to expand quickly, followed by the initiation of small nodular calli at the regions of male flower meristems. Sub-culturing of calli at 1-month interval on the initial medium, nodular masses from intervals. When transfer to a reduced concentration of 2,4-D (5-10 ppm), more defined structures such as white nodular and translucent masses appeared after three or four subcultures. During further subculturing on low concentrations of 2,4-D (1-5 ppm), green shoot-like structures developed under fluorescent illumination.

3.3. *Citrus* spp. (Citrus)

Citrus spp. is the most widely grown fruit crop worldwide, with several species serving also as ornamental trees, such as orange, lemon, and mandarin.

Experiments were conducted in six different *Citrus* species (*Citrus deliciosa* Ten. 'Avana', *C. limon* L. Brum. 'Berna', *C. madurensis* Lour. 'CNR 19', *C. medica* L. 'Vedrodi Trabia', *C. tardiva* Hort. ex Tan 'CNRP6', *C. sinensis* L. Osb. 'Ugdulena 7') to investigate the influence of explant type (stigma, style or ovary) and culture media on callus formation and embryo regeneration by using tTCL methodology (Carimi et al., 1999).

Unopened flowers are collected and surface-sterilized stigmas, styles and ovaries are cut perpendicularly to the longitudinal axis, 0.4-0.5 mm thick sections, and are cultured on MS medium with 146 mM sucrose. Three different media are used: growth regulator free MS medium (MSO), MS supplemented with 500 mg/l malt extract (MSI), or with 500 mg/l malt extract and 13.3μM BA (MSII). Cultures are maintained at 25±1^{o}C under 16 h day length, and a photosynthetic photon flux

(PPF) of 100 μmolm^{-2}s^{-1}. All the species regenerate somatic embryos but show different embryonic frequencies in the stigma, style and ovary tTCLs 2-5 days after culture initiation. Somatic embryogenesis is significantly dependent on genotype, culture medium and explant type.

C. limon and *C. medica* show highest embryogenic potential from stigma and style tTCLs, while lemon and orange ovary tTCLs never regenerate embryos; *C. sinensis* and *C. madurensis* stigma tTCLs show lower embryogenic potential, while attempts to induce somatic embryogenesis in *C. sinesis* and *C. madurensis* and *C. deliciosa* occur only from ovary tTCLs.

The average number of embryos produce per responsive tTCLs is ranged from 5.0 (stigma tTCL of *C. sinensis* on MSI) to 42.0 (ovary tTCLs of *C. deliciosa* incubated on MSI). The embryonic response do not vary after 1 year of sub-culturing, the only exception being *C. sinensis*. Twelve weeks after their transfer to germination medium somatic embryos develop into plants with varying percentages.

3.4. *Pinus radiata* (Monterey pine)

Pinus radiata D. Don is an economically important conifer in several southern-hemisphere countries. In the latter studies, it was found that excised cotyledons of 5-7 day-old germinated seeds are the most efficient, and after treatment with a cytokinin (CK), shoot induction takes place along the entire length of the cotyledon in contact with the medium (Aitken et al., 1981), leading us to use cotyledon explants instead of tobacco callus or other bulky explants to examine physiological aspects of *de novo* shoot primordium initiation, in particular since in cotyledons the process takes place more synchronously and includes a higher proportion of the tissue.

P. radiata seeds from open-pollinated trees are supplied by the Forest Research Institute, Rotorua, New Zealand. Seeds are surface sterilized following the method of Reilly and Washer (1977) and germinate them aseptically. Excise 3-5 mm long cotyledons and culture them horizontally on a modified Schenk and Hildebrandt medium in the presence or absence of 25 μM BA. All cultures are maintained in a 16 h photoperiod with an approximate photon fluency rate of 80 μE m^{-2}s^{-1} at 28±1°C (Aitken et al., 1981). The presence of CK is an absolute requirement during the first 3 days in culture, but for optimum shoot formation, CK and light are required during the first 21 days in culture (Biondi and Thorpe, 1982; Villalobos et al., 1984a). In the absence of CK, the excised cotyledons elongate, so that by day 10, they are some 5 times longer than shoot-forming cotyledons. Histological examination of the shoot-forming cotyledons indicates cytological changes within 24 h in culture (Yeung et al., 1981). While mitotic activity remains

random in cotyledons cultured without CK, it becomes restricted to the epidermal and subepidermal cell layers in cotyledons in contact with the CK-containing medium, supported by macromolecular (nucleic acid and protein) synthesis and distribution determined autoradiographically (Villalobos et al., 1984a) and histochemical staining patterns (Patel and Thorpe, 1984).

Pattern of cell divisions leading to organized structures in the subepidermal regions of the cotyledonary face in contact with BA-containing medium became apparent very early in culture, as previously shown (Yeung et al., 1981). Initially, cell division in cotyledons is random in the presence of BA. However, by day 3, mitotic activity is concentrated in the epidermis and subepidermal parenchyma cells in contact with the medium. At the same time, organized structures can be detected, each having their origin from a single subepidermal cell that begin to divide in a periclinal direction after the 3rd day in culture.

After 10th day of culture, the cotyledons develop nodular morphology as a result of increase in size of the organized structures underneath the epidermis and by day 21, leaf primordia are evident. In radiata pine epidermal cells of the cotyledon explants do not rupture and they become the protoderm of the shoot primordium, unlike other systems such as the bud-forming cotyledons of Douglas-fir in which developing shoot primordia rupture the explant epidermis.

Shoot formation in radiata pine cotyledon explants can be divided into two developmental phases. One phase includes the events of induction and differentiation and the other involves development of the primordia, that is, organized structures are plastic and can be channeled into other pathways of development. The first phase is completed with the formation of the organized cluster of 6-8 cells by day 5 in culture. The formation of this structure, for which we propose the name promeristemoid, is the most significant finding of this present study. In radiata pine cotyledons, promeristemoids arise in the first subepidermal cell layers on the side of the cotyledon in contact with the BA-containing medium. This position is probably influenced in part by physiological gradients of nutrients, including CK, from the medium into the tissue.

The promeristemoids are spherical or polarized and remain stable for several days when the cotyledons are transferred to non-shoot-forming conditions (Villalobos et al., 1984b). Sometime after day 10, they are disorganized and rapidly continue to undergo mitotic activity and give rise to meristemoids by day 10 in the presence of CK. It is interesting to note that promeristemoids do not arise from a typical meristematic cell. The cells contain vacuoles and have abundant chloroplasts. Densely plasmatic cells are conspicuously absent in radiata pine cotyledons (Yeung et al., 1981) as a result of using method of tissue preparation.

3.5. *Rosa hybrida* (Rose)

Rose is one of the four major cut flower species commercially explored worldwide. Micropropagated plants are superior to budded plants in both yield and flower quality. Explants are cultured on a full-or half MS medium supplemented with 0.05-5 mgl^{-1} 2,4-D or NAA usually in combination with a BA, Zeatin or Kin. Pre-incubation at a high (100μM) 2,4-D concentration increase the frequency of both organogenic and embryogenic callus from *Rosa hybrida* leaf explants (Hsia and Korban, 1996). Sucrose at 2-3% is used as the sole carbon source in most cases. However, replacement of sucrose by galactose or fructose increase somatic embryogenesis from leaf explants of some *R. hybrida* cultivars (Hsia and Korban, 1996). Breaking of bud dormancy in roses is important for rapid multiplication of roses using grafting. TCLs are excised longitudinally from dormant buds from *R. hybrida* 'Baccara' floral stalks and cultured on medium with 10 μM BA and 3μM gibberellic acid (GA_3). After one week on culture medium, bud breaks and development begin, with more than 7 buds per lTCL being obtained after 4 weeks culture. The synergetic effect of dormancy break and bud development is observed in the presence of BA and GA_3. This TCL protocol can be applied for rapid multiplication of rose species used for grafting onto rootstocks.

4. FUTURE PERSPECTIVE AND CONCLUDING REMARKS

Somatic embryogenesis is a fascinating aspect of plant growth and development, and it addresses problems dealing with micropropagation, and genetic transformation. An increasing understanding of the cellular and genetic mechanisms underlies somatic embryogenesis more practical technique to micropropagate woody plants. TCL technology allows the control of somatic embryogenesis in a regulated manner, and has allowed for the successful mass production of certain species that were once thought to be recalcitrant to *in vitro* tissue culture and micropropagtion. Somatic embryogenesis can be used in conjunction with bioreactor technology and cryopreservation, and provides an important basis for woody plant germplasm selection and conservation. TCLs, due to their small size and reduced cell number, are important building blocks to developmental models, and also provide perfect platforms for biolistic or *Agrobacterium*-mediated transformation since their reduced number reduces the chance of obtaining a chimeric transgenic plant.

6. REFERENCES

Aitken, J., Morgan, K.J. and Thorpe, T.A. (1981) Influence of explant selection on the shoot-forming capacity of juvenile tissue of *Pinus radiata, Can. J. For. Res.* **11**, 112-117.

Biondi, S. and Thorpe, T.A. (1982) Growth regulator effects, metabolite changes and respiration during shoot initiation in cultured cotyledon explants of *Pinus radiata, Bot. Gaz.* (*Chicago*) **143**, 20-25.

Carimi F., De Pasquale F. and Crescimanno F.G. (1999) Somatic embryogenesis and plant regeneration from pistil thin cell layers of Citrus, *Plant Cell Rep.* **18**, 935-940.

Compton M.E. and Veuilleux R.E. (1992) Thin Cell Layer morphogenesis. **Hort. Rev. 14**, 239-264.

Gupta, P.K., Kendurka, S.V., Kulkarni, V.M., Shigurka, M.V. and Mascarenhas, A.F. (1984) Somatic embryogenesis and plants from zygotic embryos of coconut (*Cocos nucifera* L.) *in vitro*, *Plant Cell Rep.* **3**, 222-225.

Hsia, C.N. and Korban, S.S. (1996) Factors affecting *in vitro* establishment and shoot proliferation of *Rosa hybrida* L. and *Rosa chinensis minima*, *In Vitro Cell. Dev. Biol. – Plant* **32**, 217-222.

Jullien F. and Tran Thanh Van K. (1994) Micropropagation and embryoid formation from young leaves of *Bambusa glaucescens* "Golden Goddess". *Plant Sci.* **98**, 199-207.

Patel, K.R. and Thorpe, T.A. (1984) Histochemical examination of shoot initiation in cultured cotyledon explants of radiata pine, *Bot. Gaz.* (*Chicago*) **145**, 312-322.

Reilly, K.J. and Washer, J. (1977) Vegetative propagation of radiata pine by tissue culture: plantlet formation from embryonic tissue, *N.Z. J. For. Sci.* **7**, 199-206.

Tran Thanh Van K., Yilmaz A. and Trinh T.H. (1985) How to programme *in vitro* different morphogenetic expression in some conifers, *In*: Bonga, Durzan (Eds.) Tissue culture and forestry. Elsevier Science, 168-182.

Villalobos, V.M., Leung, D.W.M. and Thorpe, T.A. (1984a) Light-cytokinin interactions in shoot formation in cultured cotyledon explants of radiata pine, *Physiol. Plant.* **61**, 497-504.

Villalobos, V.M., Oliver, M.J., Yeung, E.C. and Thorpe, T.A. (1984b) Cytokinin-induced switch in development in excised cotyledons of radiata pine cultured *in vitro*, *Physiol. Plant.* **61**, 483-489.

Yeung, E.C., Aitken, J., Biondi, S. and Thorpe, T.A. (1981) Shoot histogenesis in cotyledon explants of radiata pine, *Bot. Gaz.* (*Chicago*) **142**, 494-501.

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