

Cell suspension cultures

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1. Introduction

Somatic embryogenesis is the process by which the somatic cells give rise to bipolar structure, which develop to whole plants without gamete fusion. It is strongly depends on the plant genotype, type and physiological state of the explants, the composition of the culture medium and the conditions of cultivation. Somatic embryogenesis in *Medicago truncatula* is strongly genotype-dependent. The process could be direct when the embryogenic structures develop directly from the initial explant, or indirect through a callus stage. Regeneration via direct somatic embryogenesis in liquid media for *M. truncatula* line R 108 1 from leaf and root explant has been established (Iantcheva et al. 2001; Iantcheva et al. 2005). These procedures based on suspension culture for regeneration of this model species offer new advantages: omit callus stage that allow shortening of the process of somatic embryogenesis and provide basis for morphological, biochemical and molecular studies of the nature of somatic embryogenesis from single cell to whole plant.

In *Medicago truncatula* cv. Jemalong only few selected lines/genotypes are described to have embryogenic capacity. A highly embryogenic line (M9-10a) was obtained *via* somatic embryogenesis from a line with very low embryogenic potential (Neves et al., 1999). Although an efficient method for transformation-regeneration through somatic embryogenesis using leaf explants have been established for this M9-10a Jemalong line (Araújo et al., 2004) an alternative embryogenic cell suspension culture protocol represents an *in vitro* attractive system for mutant selection, mass propagation, and gene transfer experiments. Using the method here described long-term cell suspension cultures of *M. truncatula* line M9-10a were obtained and plants successfully regenerated *via* somatic embryogenesis. Constant availability of embryogenic-competent cells, the possibility of scaling-up and the reduction of time needed for plant regeneration are advantages of this protocol.

2. Cell suspension culture of *M. truncatula* cv. R 108 1 initiated from leaf and root explants

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Plant material

For initiation of suspension culture plant explants from 30 days old *in vitro* plant material from *Medicago truncatula* line R 108 1 established from seeds are used. The seeds of line R 108 1 were kindly provided by Institut des Sciences du Végétale, Gif-sur Yvette, France where it was established (Hoffman et al. 1997). They are rinsed with water prior surface sterilization with 6% solution of sodium hypochlorite (commercial bleach) for 15 min. and then rinsed three times with sterile distilled water. Seedlings developed on MS (Murashige and Skoog, 1962) basal medium are propagating *via* cuttings. All plant material is cultivated in magenta box in a growth chamber at 24 °C, 70% humidity, and photoperiod of 16-h with a light intensity of 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white fluorescent light.

Culture media

Initiation of direct somatic embryogenesis is performed on modified Gamborg B5 (Gamborg et al. 1968) liquid medium (EIM) containing dichlorophenoxyacetic acid (2,4-D) 1 mg/l (for root explants) and 4 mg/l (for leaves) plus 0.2 mg/l kinetin, 1 mg/l adenine, 500 mg/l casein hydrolysate and 500 mg/l myo-inositol, 3 % sucrose.

For embryo development and maturation MS liquid medium supplemented with 0.05 mg/l BAP (6-benzylaminopurine), 250 mg/l casein hydrolysate, 3% sucrose (EDM) is utilized.

Embryos in the late cotyledonary stage are plated onto MS basal (2% sucrose) or MS solid medium supplemented with 0.05 mg/l BAP (6-benzylaminopurine), 250 mg/l casein hydrolysate, 3% sucrose (ERC) for rooting and conversion to plants (Tab.1).

The pH of all media is adjusted to 5.8 with 1M NaOH before autoclaving. Suspension culture is maintained on a horizontal shaker (120 rpm) in a growth chamber at the above-mentioned conditions.

Explant preparation

Leaves and roots from 30 –35 day old seedlings or *in vitro* grown plants are used as initial explants. Twelve leaflets are cut on small pieces (2–4 mm) by razor blade and transfer in 100 ml Erlenmeyer flasks containing 15 ml liquid medium. Roots (24-28 cm total length) are cut by surgical blade on 2–4 mm pieces and explants are transferred in flasks (100 ml) with 10 ml liquid medium (EIM).

Observation, Results and Maintenance

Induction of somatic embryogenesis in suspension culture

Leaves: After 20-25 days of the initiation of suspension culture globular embryos appeared on the explants or released into liquid medium (Fig. 1a). Structures developed directly without a callus phase (Fig.1b). About 90 % of the explants are capable to regenerate and formed 5-15 embryos per explant (Fig.1c).

Roots: The period for induction of globular embryos from root explants is 25 days. For this period 65-70% of explants reacted and gives rise to 4-15 embryos per explant (Fig.1d). Induction period could be shortened to 10 –15 days if the initial root explants are pre-treated with 1 M solution of sucrose for 72 h (Iantcheva et al. 2005). Initiated embryos continued their development to torpedo stage on modified B5 medium.

Leaves and roots: Part of the globular embryos detaches from the explants and released in the liquid medium. The rest of them stay attached to the explants (Fig.1a). Embryos are with white to light yellow color. First refreshment of medium is after 15 days of initiation and after that every 10 days. Remove the all amount of spent liquid medium with a sterile tip (5 ml) and add the same volume of fresh medium (Tab. 2).

Formation of cell fraction and initiation of fine suspension culture

Leaves and roots: During the somatic embryo induction period from day 15-20 many single cells, clusters of 3-5 cells, small (up to 20 cells) and big (more than 20 cells) aggregates are released from the explants. Using dilution ration 1:1 mother culture to fresh EIM is possible to separate and maintain cell suspension culture. Fine cell fraction could be transferred with sterile tip (5 ml). Sterile meshes (Sigma cell dissociation kit) could be used in order to separate large aggregates and established a suspension culture only with single cells, clusters and small aggregates. Use 60 mesh in order to initiate a suspension culture composed of big and small aggregates, clusters and single cells; 80 mesh – small aggregates, clusters and single cells; 100 mesh – clusters and single cells; 150 mesh almost single cells.

The work with cell dissociation kit requires careful sterile techniques. The meshes with different size have to be preliminary autoclave, each of them in aluminum foil. Put the sterile meshes on the top of the empty sterile 100 ml flask and using a sterile tip transfer 5 ml from the fine fraction through it. Add the same volume of EIM. Keep the dilution ratio 1:1. For the period of 20 days cell mass almost doubled and then split the culture into two flasks with an equal volume of the fresh medium.

Fine cell fraction from suspension culture is composed from clusters and cells with different shape – spherical, elliptical or elongated. These cells are with dense cytoplasm

and part of them possessed high embryogenic potential and once separated in new flasks with fresh induction medium they started to divide. Approximately 10-15 % of the single cells and cell from the clusters are capable to divide asymmetrically (Iantcheva et al. 2005) and start the process of embryo formation (Fig.1e,f). Globular embryos appeared again after 20-25 days (Fig.1g). In order to complete their development to whole plantlet follows the protocol for cell suspension culture. The embryogenic potential of this culture could be maintained for 2-3 passages if the medium is refreshing every 15-20 days.

In order to follow the development of suspension culture microscope observations are necessary. Aseptically take small samples of the suspension or fine fraction and transfer them to a petri dish or microscope slide. Observations with inverted microscope confirm the formation of structures, single cell, clusters or cell divisions.

Induction of fine cell suspension culture is possible if callus tissue is resuspended in flasks. Transfer callus from 3-4 folioles in 15 ml of EIM liquid medium or callus from 3 petioles in 10 ml medium. Fine cell suspension culture induced from callus tissue is also composed from single cells, clusters and aggregates. Maintain and separate fine suspension culture with the same manner as is mentioned above.

Embryo maturation

Embryos induced in initial suspension culture continued their development until torpedo stage into EIM (Fig. 1h). The torpedoes are thick, white to light yellow. Further on their growth and development continued in liquid EDM medium. Remove the spent EIM using sterile (5 ml) tip and replace it with EDM liquid medium (15 ml for leaves and roots). To synchronize the suspension culture transfer it in sterile petri dish and separate only well developed torpedoes from the other explants containing embryos and globular structure. Well-developed torpedoes continued on EDM liquid medium the rest of suspension culture continued on EIM. Period for embryo maturation is about 25-30 days for embryos originated from leaves and 35-40 days for embryos induced from roots. Refresh this medium every 15 days using a sterile tip and replace with the same volume fresh medium for EDM.

During this period about 55-65% of the torpedo embryos reach cotyledonary stage and develop their cotyledonary leaves (Fig. 1i,j) with well-formed root part (in the case of leaves as initial explant). Embryos originated from leaves possessed fresh green color while these originated from roots are dark green.

Conversion of somatic embryos

Suspension culture at this stage is composed from torpedo and cotyledonary embryos. Transfer the suspension culture in a sterile petri dish and pick out the embryos with well-developed cotyledonary leaves. Planted them on petri dish containing MS basal solid or ECR solid medium for conversion. The conversion rate for suspension culture initiated from leaves explants is 45% and 35 % from suspension culture initiated from roots. Embryos converted to healthy and vigorous plantlets from the period of 30-35 days.

Application of cell suspension culture

Cell suspension cultures are particularly suitable for physiological, biochemical and molecular studies of the process of somatic embryogenesis and its different stage – induction development, maturation and conversion. The development of a genome and

proteome database of model *Medicago truncatula* species together with the presence of protocol of cell suspension will give the opportunity to identify and characterize genes involved in the whole process of somatic embryogenesis. The use of fine suspension culture offers the opportunity to confirm single cell origin of somatic embryos and the asymmetry of the first cell division, which starts the process. The other advantage of cell suspension culture is the use of single embryogenic cells and somatic embryos as a source for direct gene transfer via particle bombardment, transient gene expression and confocal microscopy observation.

Equipment required:

Laminar flow cabinet, forceps, scalpel blade, blades, sterile tips, Petri dishes, Erlenmeyer flask 100 ml, rotary shaker, cell dissociation kit Sigma, inverted microscope.

All chemicals used are ordered from Duchefa company.

3. Long-term embryogenic cell suspension cultures of *Medicago truncatula* cv. Jemalong line M9-10a

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Micropropagation of Medicago truncatula

Plants of the M9-10a genotype of *Medicago truncatula* cv Jemalong are maintained in *in vitro* culture conditions and micropropagated in growth-regulator-free medium: MS0A - MS (Murashige and Skoog, 1962) basal salts and vitamins, 3 % (w/v) sucrose, 0.7% (w/v) agar (Microagar, Duchefa, The Netherlands) by sub-culture of 2-5 cm stem segments every 30-40 days (Neves et al., 2001).

Callus induction from leaf explants

Well-developed leaflets from 30 day *in vitro* cultured M9-10a plants are used as explants for *callus* induction. Leaflets are placed onto a wet sterile filter paper in a Petri-dish to prevent desiccation and wounded perpendicularly to the midrib using a scalpel blade. Explants are transferred with the abaxial side-down to Petri-dishes containing *Callus* Induction Medium: CIM - MS basal salts and vitamins, 3 % (w/v) sucrose, 0.2% (w/v) gelrite (Merck, USA) supplemented with 1 mg.l⁻¹ of 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg.l⁻¹ of Zeatin (*Zea*) (Tab.1). *Calli* are maintained at 23°C in the dark and sub-cultured to fresh medium every 4 weeks.

Establishment of long-term embryogenic cell suspension cultures

To initiate cell suspension cultures, 2 g of *callus* portions are placed in 50 ml of liquid Suspension Culture Medium: SCM - MS basal salts and vitamins, 3 % (w/v) sucrose, supplemented with 1 mg.l⁻¹ of 2,4-D and 1 mg.l⁻¹ Kinetin (Kin) (Tab. 1). Until an adequate cell density is obtained, the cells are pellet and medium substituted every week. The cell suspension cultures are then maintained by sub-culturing in liquid SCM medium supplemented with 0.5 mg.l⁻¹ of 2,4-D and 0.5 mg.l⁻¹ of Kin (Tab. 1). Sub-cultures are carried out by transferring 10 ml of a cell suspension at the end of the exponential growth phase (every 7 days) to 100 ml of fresh medium (in 500 ml Erlenmeyer flasks). Cell suspension cultures are maintained in an orbital shaker at 124 rpm (Innova 4900, New Brunswick Scientific, Germany) in the dark, at 24°C.

Somatic embryogenesis and plant regeneration

Seven days old M9-10a cell suspension cultures are filtered through a 1050 µm nylon mesh and the filtrate is transferred to growth-regulator-free liquid MS medium (after

being rinsed twice with this medium) and grown for one week. Cell suspension cultures are then transferred to solid Embryo Proliferation Medium: EPM – MS basal salts and vitamins, 3 % (w/v) sucrose and 0.2% (w/v) gelrite (Merck, USA) (Tab. 1) where somatic embryos developed until late torpedo/dicotyledonar stages (after 4 weeks). Embryos ready to be isolated (late torpedo/dicotyledonar stage) are transferred to an Embryo Conversion Medium (Tab.1) (ECM=MS0A, similar to EPM but solidified with 0.7% (w/v) agar instead of gelrite) for conversion to plants.

The regenerated plants with well-developed root system are potted in a mixture of 2:1 soil-vermiculite, covered with a plastic film for acclimatisation and placed in a growth chamber. After 1-2 weeks, the plants can be transferred to pots containing soil and grown in the greenhouse.

For embryo development, plant regeneration and acclimatisation, cultures are maintained under a 16 hours photoperiod of $100 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ applied as cool white fluorescent light and a day/night temperature of 24°/22°C (Phytotron Edpa 700, Aralab, Portugal).

The pH of all media is adjusted to 5.8 before autoclaving (121°C, 20 min.). Growth regulators are filter sterilised through 0.2 μm Whatman filters and added to autoclaved (cooled to 40°C) media.

Observation, Results and Maintenance

Callus induction from leaf explants

Callus development is observed, at the edges of the wounded tissues, 10-15 days after culture initiation. Generally, 100% of the explants developed friable dark-yellow *callus*. *Callus* cultures can be maintained in Petri-dishes and sub-cultured (6 portions of 1-1,5 cm per dish) every 4 weeks. Friable portions of 4 week grown *callus* can be separate and transferred to liquid medium to initiate a cell suspension culture (Duque et al., submitted).

Establishment of long-term embryogenic cell suspension cultures

After transfer to liquid SCM medium and submitted to constant agitation *callus* begin to dissociate into small cell clumps and single cells. Typically, two months are needed before cell suspension cultures become stable. Cells suspension cultures obtained with this process are dark-yellow and consist of single cells and cell aggregates up to 2.5 mm. Small spherical cells containing dense cytoplasm can be observed (this type of cells are usually described as embryogenic competent) together with few elongated cells. These cultures can be maintained for long-time periods without lost of embryogenic capacity by subculture at the end of the exponential growth phase to fresh medium (in our hands, three-year-old cultures maintained by weekly sub-culture preserve their embryogenic competence) (Duque et al., submitted).

Somatic embryogenesis and plant regeneration

Green proembryogenic masses are observed after 1 week in MS liquid growth-regulator-free medium. For somatic embryo development 1 ml of cell suspension is transferred to Petri-dishes containing gelrite solidified MS medium (EPM) and the excess of liquid is removed with a sterile Pasteur pipette. A mean number of 84.5 ± 9.1 (mean \pm SD) somatic

embryos (SE) can be obtained per ml of suspension culture (corresponding to 5.8 ± 1.0 (mean \pm SD) SE per mg of dry-weight of the suspension culture) ($p < 0.05$) (Duque et al., submitted). Torpedo/dicotyledonar stage somatic embryos are observed after 3-4 weeks on EPM. Conversion of somatic embryos to plants is dependent on the replacement of gelrite by agar (Araújo et al., 2004). In these conditions typical conversion rates are $54.5 \pm 1.6\%$ (mean \pm SD, $p < 0.05$) (Duque et al., submitted). The development of somatic embryos is asynchronous and several stages of embryo development can be observed simultaneously. All green somatic embryos in a late-torpedo/dicotyledonar development stage are isolated and transferred to fresh ECM until conversion to plantlets. Some somatic embryos developed secondary embryos and usually fail convert to plants because of arrested shoot and root development (Neves et al., 1999). The same culture medium is used for root growth (with no requirement for growth-regulators) and transfer to soil is not a limiting step when plants with *in vitro* well-developed radicular system are chosen. The appearance of proembryogenic masses in liquid culture and the development of somatic embryos on solid medium can be followed under a stereomicroscope (Leica Wild MZ8, Germany).

Advantages of using embryogenic cell suspension cultures

Advantages of this procedure when compared to protocols of somatic embryogenesis from leaves are: a) the decrease in the time to obtain plants (no requirement for an induction step); b) the constant availability of embryogenic-competent cells; and c) the easiness for scaling-up.

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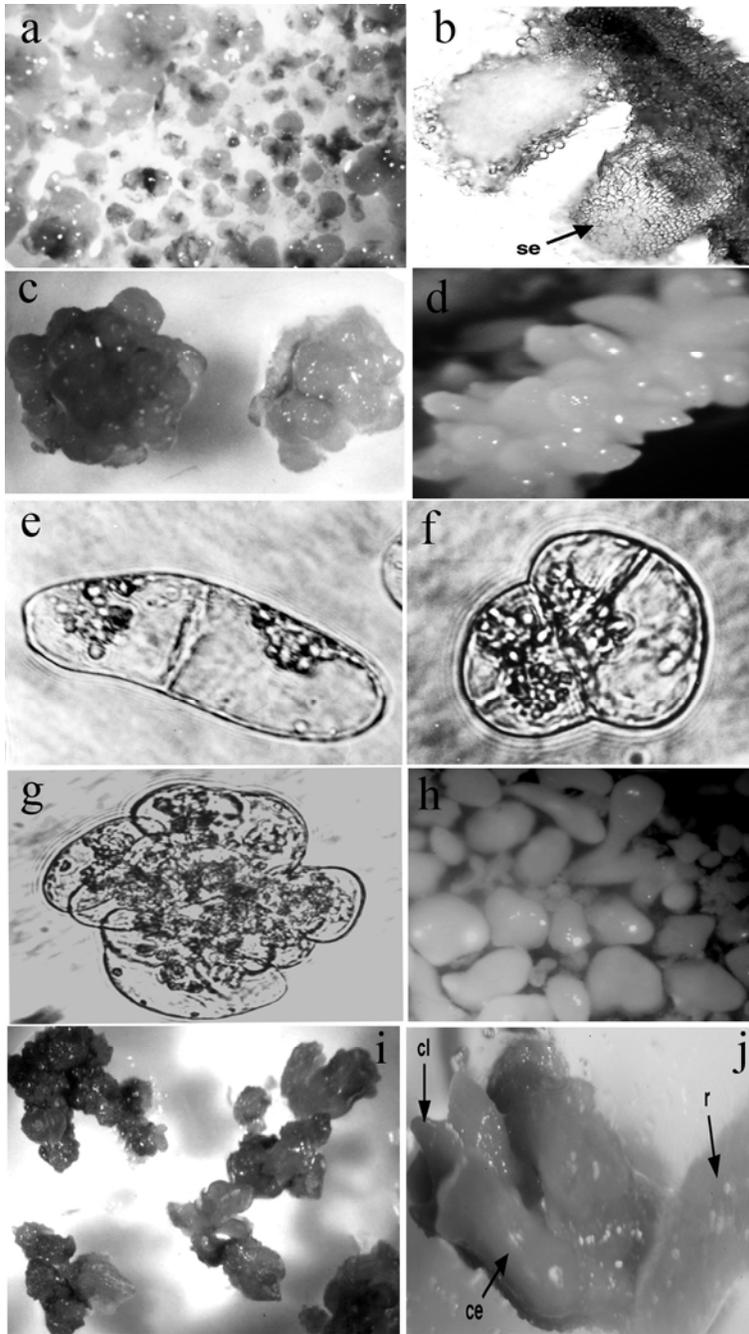


Figure 1. Cell suspension culture of *Medicago truncatula* R 108 1. a-induction of suspension culture, explants with embryos and released globular embryos; b- histological section of somatic embryo (se); c-leaf explant with induced globular embryos; d- root explant with induced embryos; e- asymmetric division in elliptical cell; f- three cell proembryo; g- globular embryo from fine cell suspension culture; h- torpedo embryos; i – cotyledonary embryos originated from root explant; j- cotyledonary embryos originated form leaf explant (cl-cotyledonary leaves, ce-cotyledonary embryo, r-root)

Tab. 1. Media composition for cell suspension culture of *Medicago truncatula*

<i>M. truncatula</i> line R 108 1				<i>M. truncatula</i> cv. Jemalong M9-10a				
MS	EIM	EDM	ECR	MS	CIM	SCM	EPM	ECM=MS0A
MS Major Salt	B5 Major Salts	MS Major Salts	MS Major Salts	MS Major Salt	MS Major Salts	MS Major Salts	MS Major Salts	MS Major Salts
MS Minor Salts	B5 Minor Salts	MS Minor Salts	MS Minor Salts	MS Minor Salts	MS Minor Salts	MS Minor Salts	MS Minor Salts	MS Minor Salts
Iron EDTA	Iron EDTA	Iron EDTA	Iron EDTA	MS Vit.	MS Vit.	MS Vit.	MS Vit.	MS Vit.
MS Vit.	B5 Vit.	MS Vit.	MS Vit.	Sucrose	Sucrose	Sucrose	Sucrose	Sucrose
3%	3 %	3%	3 %	3%	3 %	3%	3 %	3 %
Agar			Agar		Gelrite		Gelrite	Agar
0.7%			0.7%		0.2%		0.2%	0.7%
	2.4D	BAP	BAP		2.4D	2.4D		
	1or 4 mg/l	0.05 mg/l	0.05 mg/l		1 mg/l	0.5 or 1 mg/l		
	kinetin	Casein hydr.	Casein hydr.		Zeatin	Kinetin		
	0.2 mg/l	250 mg/l	250 mg/l		2 mg/l	0.5 or 1 mg/l		
	adenine							
	1 mg/l							
	<i>myo</i> -Inositol							
	500 mg/l							
	Casein hydr.							
	500 mg/l							

Tab. 2. Summarized data of the protocol cell suspension culture of *M. truncatula* cv. R 108 1.

Plant material	Induction					Maturation					Conversion			
	Media	Vol. ml	Period days	React. Ex %	Refresh. days	Media	Vol. ml	Period days	Cotyl. %	Refresh. days	Media	Period days	Refresh days	Convert. %
<i>M.truncatula</i> R108 1														
Leaf	EIM	15	20-25	90	15/10	EDM	15	25-30	65-70	15	MS	30	14	45
Root	EIM	10	25	65-70	15/10	EDM	15	35-40	55-60	15	ECR	35	14	35