

# **SOMATIC EMBRYOGENESIS FROM LEAF EXPLANTS 1 - SOMATIC EMBRYOGENESIS FROM LEAF EXPLANTS OF *MEDICAGO TRUNCATULA* CV. JEMALONG GENOTYPE 2HA**

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## **INTRODUCTION**

Somatic embryogenesis is a process whereby a cell or group of cells from somatic tissue forms an embryo. The development of somatic embryos nearly replicates the process of zygotic embryo formation. Somatic embryogenesis mostly occurs indirectly via an intervening callus phase or directly from initial explant.

## **PLANT MATERIAL**

Leaves explants from 30 days old *in vitro* plants originated from seeds of highly embryogenic genotype 2HA cv. Jemalong (Nolan et al. 1989) is used for the initiation of somatic embryogenesis. Seeds are kindly provided by Dr. Pascal Ratet, Institut des Sciences du Végétale, Gif-sur Yvette, France. They are rinsed with water and surface sterilized for 15 min in 6% solution of sodium hypochlorite (commercial bleach) followed by three times rinse with sterile distilled water. Seedlings are maintained on MS (Murashige and Skoog, 1962) basal medium. They are propagated via cuttings. *In vitro* plant material is cultivated in Magenta boxes in a growth chamber at 24 °C, 70% humidity, and a photoperiod of 16-h with a light intensity of 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white fluorescent light.

## **CULTURE MEDIA**

MS medium (Murashige et al. 1962) supplemented with 1 mg/l 2,4-D, (2,4-Dichlorophenoxyacetic acid) 0.2 mg/l BAP (6-benzylaminopurine) and 1 g/l casein hydrolysate, 3 % sucrose, 0.7% agar (EID) is used for embryo induction and development. Liquid medium with the same composition is successfully used for direct induction of somatic embryos in carnation (Yantcheva et al. 1998). Embryos converted to plantlets and rooted on medium (ECR), which is MS containing 0.1 mg/l NAA ( $\alpha$ -Naphthaleneacetic acid), 250 mg/l casein hydrolysate, 1 % sucrose, 0.7%agar.

The pH of all media is adjusted to 5.8 with 1M NaOH before autoclaving. Embryo cultures are maintained in a growth chamber at 24 °C, 70% humidity, and a photoperiod of 16-h with a light intensity of 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white fluorescent light.

## **EXPLANT PREPARATION**

Leaves are detached from well-developed 28-30 days old *in vitro* plants. Every foliole of the trifoliate leaves is wounded 3-4 times by scalpel blade across main vein. Leaves prepared that way are transferred abaxial side up to a Petri dish containing EID medium. Each Petri dish contains around 12-15 leaflets. Gently push the explants to the medium to ensure good contact.

## **OBSERVATION, RESULTS AND MAINTENANCE**

During first 14 days, leaflets become yellowish and turn brownish to day 20. The process of embryogenesis starts with formation of very slight callus tissue or sometimes almost directly. First embryos appear 20 days on induction medium at the edges of the leaflets or at the wounded places. Up to day 40 their number increases 2-3 times. (Fig. 1a, b). Induced embryos possess fresh green color. 80-90 % of the explants react positively. Approximately 5-15 embryos per explant are developed.

In this first step of the protocol induction of somatic embryos are promoted by 2,4-D in combination with the cytokinin BAP. Formation of vigorous embryos almost without callus is supported by the presence of casein hydrolysate (Fig. 1c). Induction and development of the structures on EID medium take around 40 days. This first step of the protocol resemble of the first two steps of the protocol described by (Chabaud et al. 1996) but the period for induction and development is shorter with 20 days.

Subculture explants every two weeks on fresh EID medium. If embryos already appeared at the surface of the explant transfer them very carefully. In order to complete their development embryos should stay attached to the explants.

First step of the protocol on EID medium finishes with the formation of embryos possessing well-developed cotyledonary leaves (Fig. 1c).

The second step of the protocol is related to embryo conversion to plantlets and formation of roots. This step is performed on ECR medium. Embryo structures with well-developed cotyledonary leaves are detached very carefully from the explants and planted on petri dishes containing ECR medium (Fig. 1d). For a period of 40 days embryos develop to plantlets and form roots. Subculture structures every 14 weeks. In some cases a necrotic tissue appears at their base and should be removed carefully with a scalpel blade. Approximately 50 % of the embryos per explant convert to normal plantlets.

This two-step protocol is characterized by quick and efficient embryo formation. The total period for regeneration is around 80 days. The efficiency of the protocol is the production of 65-70 vigorous plantlets starting from 15 foliole explants. Regenerated plants possess normal phenotype and set seeds with good fertility.

The process of embryo formation using this protocol is summarized in Table 2.

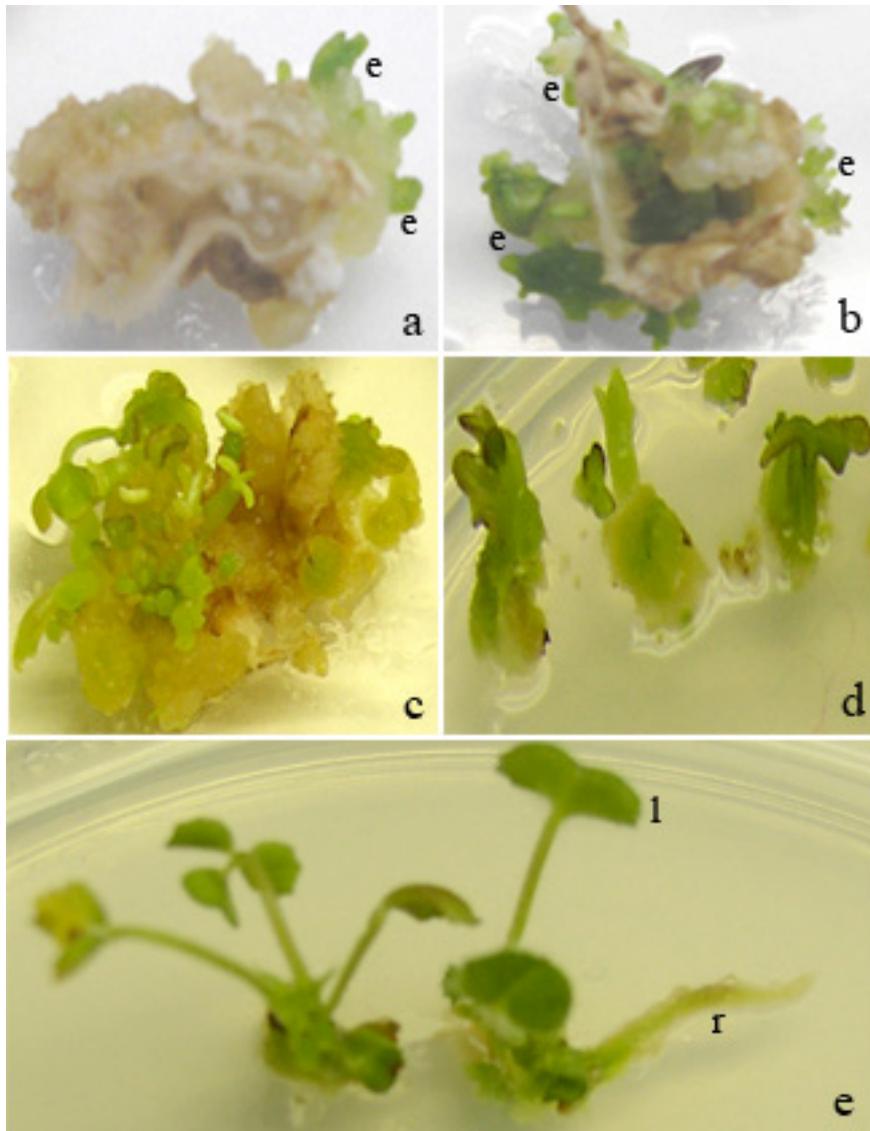
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**Figure1.** Process of somatic embryo formation in *M. truncatula* cv. Jemalong 2HA. a- first appeared embryos (e) after slight callus phase; b-increased number of somatic embryos (e); c- embryos in cotyledonary stage; d-embryo conversion; e-plantlets (l-leaf, r-root).



**Table 1.** Media composition.

MS medium	EID medium	ECR medium
MS Major Salts	MS Major Salts	MS Major Salts
MS Minor Salts	MS Minor Salts	MS Minor Salts
Iron EDTA	Iron EDTA	Iron EDTA
MS Vitamins	MS Vitamins	MS Vitamins
Sucrose 3%	Sucrose 3%	Sucrose 1%
Agar 0.7%	Agar 0.7%	Agar 0.7%
	2.4D 1 mg/l	NAA 0.1 mg/l
	BAP 0.2 mg/l	Casein hydr 250 mg/l
	Casein hydr. 1 g/l	

**Table 2.** Summarized data of the protocol.

<i>M.truncatula</i> cv.Jemalong 2HA	Embryo induction and development					Embryo conversion and rooting			
	explant	Media	Period days	React.ex. %	Embr/expl Subculture days	Media	Period days	Conv/expl %	Subculture days
Leaf	EID	40	80-90	5-15	14	ECR	40	50	14

# SOMATIC EMBRYOGENESIS FROM LEAF EXPLANTS 2 - SOMATIC EMBRYOGENESIS FROM LEAF AND PETIOLE EXPLANTS OF *MEDICAGO TRUNCATULA* CV. JEMALONG GENOTYPE 2HA

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## PLANT MATERIAL

Leaves explants from 30 days old *in vitro* plants originated from seeds of highly embryogenic genotype 2HA cv. Jemalong (Nolan et al. 1989) are used for the initiation of somatic embryogenesis. Seeds are kindly provided by Dr. Pascal Ratet, Institut des Sciences du Végétale, Gif-sur Yvette, France. They are rinsed with water and surface sterilized for 15 min in 6% solution of sodium hypochlorite (commercial bleach) followed by three times rinse with sterile distilled water. Seedlings are maintained on MS (Murashige and Skoog, 1962) basal medium. They are propagated via cuttings. *In vitro* plant material is cultivated in Magenta boxes in a growth chamber at 24 °C, 70% humidity, and a photoperiod of 16-h with a light intensity of 30  $\mu\text{mol m}^{-2} \text{s}^{-1}$  white fluorescent light.

## CULTURE MEDIA

*Medium for callus induction* (CIM) is B5 (Gamborg et al. 1968) solid medium containing 2,4-D (2,4-Dichlorophenoxyacetic acid) 1 mg/l, 0.2 mg/l kinetin, 1 mg/l adenine, 500 mg/l casein hydrolysate and 500 mg/l *myo*-Inositol, 3 % sucrose, 0.7 % agar (Table 3). Liquid medium with the same composition is used for direct induction of somatic embryos in suspension culture of *M. truncatula* R 108 1 (Iantcheva et al. 2001).

*Medium for embryo induction* (EIM) (Table 3) - MS supplemented with 0.9 mg/l BAP (6-benzylaminopurine) and 0.3 mg/l NAA ( $\alpha$ -Naphthaleneacetic acid), 3 % sucrose and 0.7 % agar.

*Embryo development medium* (EDM) (Table 3) - MS1 solid medium (MS supplemented with 0.05 mg/l BAP (6-benzylaminopurine), 250 mg/l casein hydrolysate, 3% sucrose, 0.7 % agar).

*Embryo conversion and rooting medium* (ECR) (Table 3) - MS containing 0.1 mg/l NAA, 250 mg/l casein hydrolysate, 1 % sucrose, 0.7 % agar.

*The level of auxin 2,4-D in CIM could be increased up to 5 mg/l depending on genotype. Callus tissue can be obtained at light and dark conditions. Calli induced at dark are more white and friable.*

## **EXPLANT PREPARATION**

Trifoliolate leaves and petiole explants from 30-35 days old *in vitro* plants are used as initial explants for induction of somatic embryos. The leaves and petioles are cut from well-developed plants. Petioles are cut on 1 cm long pieces and wounded 3-4 times by scalpel blade. Every foliole from trifoliolate leaves is wounded 3-4 times by scalpel blade across main vein. Such are prepared leaflets are transferred abaxial side up to petri dish containing CIM medium. Each petri dish contains around 12-15 leaflets and 30-40 petiole explants. Contact of the explants with the medium has to be very strong. Gently push the explants to stick closely to the medium.

## **OBSERVATION, RESULTS AND MAINTENANCE**

### *Induction of callus tissue*

On CIM plant explants formed soft and very friable callus, white to yellowish in the case of foliole explants and more yellow to light green from the petioles. For the period of 50 – 60 days 80 % of the explants formed callus tissue. The formation of calli started 15 days of induction, first at the wounded places. To day 60 whole foliole look as a boll of friable callus (Fig. 2a right). The increase of callus originated from petioles is weaker than in leaflets (Fig. 2a left).

The subculture is carried out every 20 days. If the callus tissue is already formed, take the explants carefully by scalpel blade because the tissue is very friable and soft.

### *Embryo induction*

The process started when the explants are transferred on EIM. First green zones on calli appeared 10-15 days. They are around 5-10 per petiole explants and 5-15 for foliole. They grow and became thicker (Fig. 2b). Embryo induction completed for 25-30 days.

Subculture explants with induced embryos on fresh medium once for the above-mentioned period.

This step is a crucial. If the explants with induced embryos are not transfer at the correct moment they easily formed green thick callus, which is not embryogenic.

### *Embryo development*

The process is performed on EDM and the presence of BAP and casein hydrolysate stimulates the formation and further development of embryo structures. Embryos complete their development with the formation of cotyledonary leaves (Fig. 2c). Half of the induced somatic embryos are capable of finishing their development. The duration of this process is 25-30 days. Only well-developed structures should be transferred to ECR medium. Partially developed embryos need to continue on EDM medium.

### *Embryo conversion and rooting*

On ECR medium embryos with formed cotyledonary leaves convert to plantlets. Sometimes they are formed as a cluster and need to be separated in order to form a root (Fig. 2d). The duration of the process depends on the embryos' vigor from 20-40 days. The subculture on this medium continues to the formation of healthy plantlets, which can continue their development on MS basal medium for plant maintenance.

Total optimal period for embryo regeneration following this protocol is 120 days. The efficiency of the procedure is the production of 50 – 60 vigorous plantlets starting from 15

foliole explants. In a case of petiole explants the efficiency is 60-65 plantlets starting from 30 petioles. Regenerated plants possess normal phenotype and set seeds with good fertility. The process of embryo formation is summarized in Table 4.

Both protocols presented in this chapter (1 and 2) are suitable for application of gene transfer method via *Agrobacterium*. Protocol 2 is characterized by induction of callus tissue, but the formed calli could be used for initiation of fine cell suspension culture (see chapter “Cell suspension cultures”).

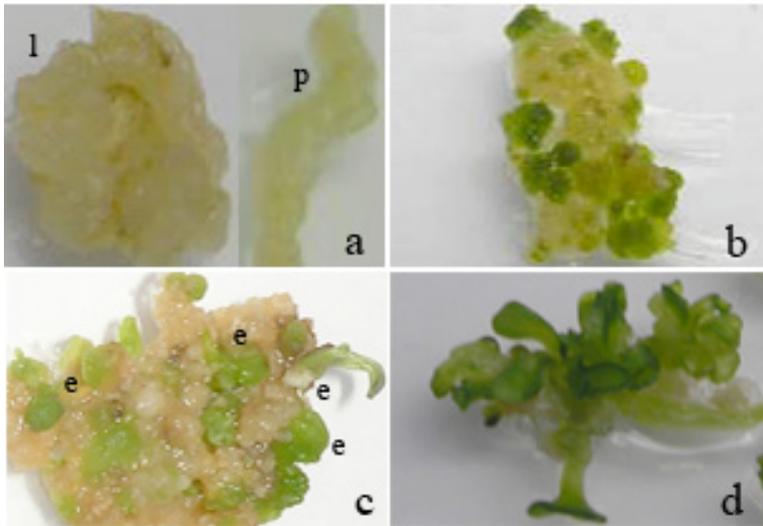
#### **EQUIPMENT REQUIRED:**

Laminar flow cabinet, forceps, scalpel blade, Petri dishes. All chemicals used are ordered from Duchefa Company.

#### **REFERENCES**

- Chabaud M, Larssonneau C, Marmouget C, Huguet T.** 1996. Transformation of barrel medics (*Medicago truncatula* Gaetrn.) by *Agrobacterium tumefaciens* and regeneration via somatic embryogenesis of transgenic plants with the *MtENOD 12* nodulin promoter fused to *gus* reporter gene. *Plant Cell Reports* **15**,305–310
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**Figure 2.** Somatic embryo formation in *M. truncatula* cv. Jemalong 2HA. a-callus formation (l-leaf, p-petiole); b-induction of green embryogenic zones; c-development of somatic embryos (e); d-cluster of plantlets.



**Table 3.** Media composition.

MS medium	CIM medium	EIM medium	EID medium	ECR medium
MS Major Salts	B5 Major Salts	MS Major Salts	MS Major Salts	MS Major Salts
MS Minor Salts Iron EDTA	B5 Minor Salts Iron EDTA	MS Minor Salts Iron EDTA	MS Minor Salts Iron EDTA	MS Minor Salts Iron EDTA
MS Vitamins	B5 Vitamins	MS Vitamins	MS Vitamins	MS Vitamins
Sucrose 3%	Sucrose 3 %	Sucrose 3%	Sucrose 3%	Sucrose 1%
Agar 0.7%	Agar 0.7 %	Agar 0.7 %	Agar 0.7%	Agar 0.7%
	2.4D 1 mg/l	NAA 0.3 mg/l	BAP 0.05 mg/l	NAA 0.1 mg/l
	kinetin 0.2 mg/l	BAP 0.9 mg/l	Casein hydr. 250 mg/l	Casein hydr 250 mg/l
	adenine 1 mg/l			
	<i>myo</i> -Inositol 500 mg/l			
	Casein hydr. 500 mg/l			