Chapter 12
Grapevine (*Vitis* spp.)

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12.1 Introduction

Somatic embryogenesis, i.e. the initiation of embryos from plant somatic tissues, is employed as a multiplication method for several plant species. Conversely, with regard to grapevine somatic embryogenesis is mostly used in breeding programs, embryo tissues being the best sources for regeneration of genetically modified plants (Franks et al. 1998; Reustle and Buchholz 2009; Gambino and Gribaudo 2012; Gray et al. 2014). In addition, somatic embryogenesis has been proposed as a strategy aimed at inducing somaclonal variation (Kuksova et al. 1997) and as a tool for additional aims such as the separation of periclinal chimeras (Franks et al. 2002), while improvements in setting up grapevine cryopreservation protocols have been obtained (González-Benito et al. 2009; Vasanth and Vivier 2011). Its interest as a tool in functional genomics studies has recently increased (Gambino et al. 2014) thanks to the availability of the reference genome in grapevine (Jaillon et al. 2007) and the consequent need of characterization of the putative genes identified in silico. Somatic embryogenesis is also a precious tool for studying embryo development as somatic embryos follow a developmental pathway very similar to that of their zygotic counterparts (Dodeman et al. 1997).

In this context, a reliable technique for the production of somatic embryos and regenerated plantlets from an ample number of *Vitis* genotypes is an essential pre-requisite for any application of somatic embryogenesis. The large genetic variability occurring in the genus *Vitis* may strongly influence the results. During the years, a broad number of protocols and media composition has been proposed. Details can be found in reviews such as those of Martinelli and Gribaudo (2009), and more recently Dhekney et al. (2016). From the available literature some

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common, main developmental phases can be identified: (a) Induction of callogenesis and embryogenic competence in cultured explants; (b) Culture of embryogenic calli and expression of the embryogenic program; (c) Long term culture of embryogenic callus and preservation of the embryogenic ability of the cultures or re-initiation of embryogenic calli from somatic embryos; (d) Development of somatic embryos into plantlets.

12.2 Materials

a. Grapevine inflorescences
b. Laminar-flow hood, forceps, scalpels, glass bead sterilizer, sterile cutting pads
c. Autoclave, pH meter, balances
d. Tissue culture chambers
e. Stereomicroscope
f. Sodium hypochlorite, wetting agent, sterile distilled or deionized water
g. Culture media (see Table 12.1), also available on the market as products for micropropagation

Table 12.1 Basal composition of culture media

Chemicals (μM)	Basal medium NN	Basal medium MS
$CaCl_2$	1500	1500
KH_2PO_4	500	625
KNO_3	9400	9400
NH_4NO_3	9000	10305
$MgSO_4$	750	750
$ZnSO_4{\cdot}7H_2O$	34.8	14.95
$Na_2MoO_4{\cdot}2H_2O$	1.03	0.51
$CuSO_4{\cdot}5H_2O$	0.1	0.05
$MnSO_4{\cdot}H_2O$	112	50
H_3BO_3	162	50
$CoCl_2{\cdot}6H_2O$	–	0.05
KI	–	2.5
FeNaEDTA	100	50
Myo-inositol	555	555
Nicotinic acid	4	4
Pyridoxin HCl	2.43	2.43
Thiamine HCl	0.3	0.3
Glycine	26.64	26.64

Basal medium NN has macro and microelements from Nitsch and Nitsch (1969), and vitamins from Murashige and Skoog (1962). Basal medium MS is from Murashige and Skoog (1962), with mineral salts at half concentration

h. Plant growth regulators (PGRs): 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzyladenine (BA), naphtoxy acetic acid (NOA), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA)
i. Tissue culture agar, gellan gum (Gelrite™, Phytagel™, etc.), sucrose
j. Generic laboratory glassware and plasticware (beakers, Erlenmeyer flasks, cylinders, glass autoclavable bottles)
k. Sterile Petri dishes (for media A, B, C and G: see below), Parafilm®
l. Single-use or re-usable containers for plant tissue cultures (for media D, E and F: see below)
m. Peat pellets (Jiffy7®)

Basic media composition is listed in Table 12.1. Required composition and modifications for different culture stages are listed in Table 12.2.

12.3 Method

The regeneration procedure includes:

(a) Embryogenic culture initiation (acquisition of embryogenic competence): culture of initial explants and induction of callogenesis
(b) Embryo differentiation (expression of the embryogenic program)
(c) Embryo germination and development into plantlets
(d) Long term culture of embryogenic callus and preservation of the embryogenic ability
(e) Micropropagation and acclimatization of the resulting plantlets.

12.3.1 Embryogenic Culture Initiation

Stamens (anthers plus filaments) have been the most widely used starting material for culture, and successful initiation of regenerable embryogenic calli has been obtained from stamens of a considerable number of grapes. Immature pistils (ovaries plus styles, stigmas and receptacles) also represent a good starting explant, with the advantage of being collectible easily and simultaneously with anther excision. Below we refer to the described explants simply as anthers and ovaries. In some cultivars the efficiencies obtained from ovaries can be notably higher than compared to anthers, ovaries being more responsive to embryogenic induction (Nakano et al. 1997; Martinelli et al. 2001; Kikkert et al. 2005; Vidal et al. 2009).

Even whole flowers proved to be suitable material for initiating embryogenic cultures for some cultivars: their collection from the inflorescence is easier and faster than excision of anthers and ovaries from the flower itself; it can be done without the use of a stereomicroscope and damage to the explant is unlikely. No morphological difference was noted among embryogenic cultures originated from ovaries, flowers, or anthers (Gambino et al. 2007).

Table 12.2 Formulations of Grapevine Media

Chemicals	A	B	C	D	E	F	G
	Callus induction	Embryo induction	Embryo germination	Shooting	Rooting	Micro propagation	Long-term culture
Basal medium	NN	NN	NN	MS	MS	MS	NN
Sucrose (g/L)	60	60	30	15	20	20	60
2,4-D (μM)	4.5	–	–	–	–	–	5
BA (μM)	8.9	1	–	10	–	–	1
NOA (μM)	–	10	–	–	–	–	–
IAA (μM)	–	20	–	–	–	–	–
IBA (μM)	–	–	–	–	2.5	–	–
NAA (μM)	–	–	–	–	2.5	–	–
Activated charcoal (g/L)	–	2.5	2.5	–	–	–	–
Gellan gum (g/L)	3	–	–	–	–	–	5
Agar (g/L)	–	10	10	9	8	8	–
pH	5.8	5.8	6.2	5.7	5.6	5.6	5.7

The pH is adjusted with 1 N NaOH or 0.5 N HCl prior to autoclaving at 121 °C for 10 min. IAA is filtered and aseptically added to autoclaved medium when the medium temperature has decreased to about 55 °C. Pour 25 ml medium to 90 mm Petri dishes (media A, B, C, G) or an appropriate volume to plant tissue culture containers (media D, E, F)

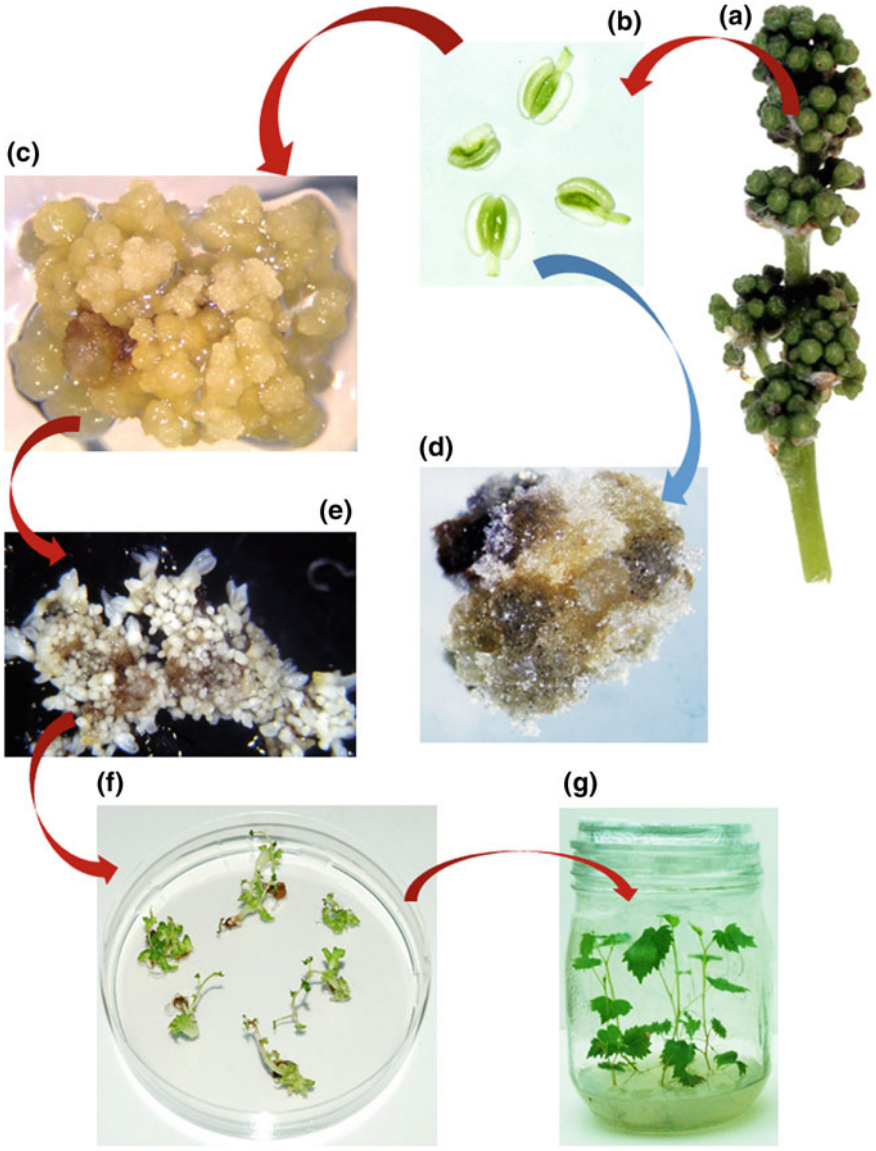

Fig. 12.1 Plant regeneration via somatic embryogenesis in grapevine. **a** Immature inflorescence suitable for explant collection; **b** immature stamens (anthers plus filaments); **c** embryogenic callus; **d** non-embryogenic callus; **e** embryo development from embryogenic callus; **f** embryo germination; **g** micropropagation of plantlets derived from somatic embryos

As for the best developmental stage to initiate anther culture, here we describe the stage that is most often reported best for *Vitis vinifera* (Faure et al. 1996; Gribaudo et al. 2004; Vidal et al. 2009), although in the case of some cultivar or

species of the genus *Vitis* somatic embryogenesis occurred more frequently if explants were collected at later developmental stages (Gribaudo et al. 2004; Bouamama et al. 2007; Prado et al. 2010).

(i) Use immature inflorescences for embryogenic culture initiation. Collect flower in the vineyard 10–14 days before full bloom, when: inflorescence are still compact but flowers gradually separate from each other (Fig. 12.1a), calyptra is dark green and gradually lengthens, anthers are green and translucent (Fig. 12.1b), the pollen mother cells are in late pre-meiotic phase (corresponding to the active synthesis of DNA and proteins needed for meiosis)
(ii) Surface-sterilize the inflorescences for 15 min with a solution of sodium hypochlorite (1.5% available chlorine) containing a few drops of a wetting agent (for instance Tween 20®), and rinse several times with sterile distilled or deionized water.
(iii) Store inflorescences in sterile Petri dishes or other closed container at 4 °C for 2 up to a maximum of 5 days.
(iv) Perform a second surface sterilization before use as described in (ii)
(v) Using a stereomicroscope and under a laminar-flow hood carefully open the flowers, excise intact stamens (anthers plus filaments) and pistils (ovaries plus styles, stigmas and receptacles) by separating them from the calyptra. Place stamens and pistils in Petri dishes containing the medium A.
(vi) Seal Petri dishes with Parafilm® and place in the dark at 26 °C.

12.3.2 Embryo Differentiation

After three months of culture on medium A, most explants have produced callus. However, callus type is seldom homogeneous and can be classified in embryogenic (generally granular white or slightly yellow callus, sometimes associated to dark callus) (Fig. 12.1c) or non-embryogenic (dry and compact or watery and soft callus; colours can vary from yellow to brown) (Fig. 12.1d) (Gambino et al. 2006; Vidal et al. 2009; Prado et al. 2010). Initial embryogenic cultures are generally asynchronous. Transfer the cultures onto medium B is needed to allow embryo differentiation and development (Fig. 12.1e).

(i) Transfer the explants that have produced embryogenic callus onto medium B.
(ii) Seal Petri dishes with Parafilm® and place in the dark at 26 °C.
(iii) Every 4 weeks transfer the cultures onto fresh B medium.

12.3.3 Embryo Germination and Development into Plantlets

From about 5 months after culture initiation onwards, single large embryos can be isolated from the embryogenic callus (Fig. 12.1e) and transferred to medium C, at 24 °C under light (photoperiod 16 h). Conversion of somatic embryos into plants is accompanied by development of the primary root, greening of hypocotyls and cotyledons and formation of the shoot apex with one or two foliar primordia (Redenbaugh et al. 1986) (Fig. 12.1f).

However, as germination and plant recovery can vary considerably depending on the genotype and the embryo morphological and physiological state, various dormancy-breaking treatments have been proposed (Martinelli and Gribaudo 2009; Larrouy et al. 2017) and can be applied in case of germination failure. The following simple strategy is based on the one proposed by Franks et al. (1998):

(i) Remove the basal part of the germinating embryo cutting it at the hypocotyl
(ii) Subculture the cut upper part of the embryo on medium D for shoot growth
(iii) Culture the resulting shoot for 2 weeks on medium E for root induction
(iv) Transfer to medium F for further growth.

All the steps are at 24 °C under light (photoperiod 16 h).

12.3.4 Long-Term Culture of Embryogenic Callus and Preservation of the Embryogenic Ability

During the subsequent subcultures, the embryogenic competence of the callus can be maintained through subcultures performed monthly, alternating every two months medium A and medium B (Franks et al. 1998; Gambino et al. 2005).

However, this strategy is not always advisable for a long-term culture as several grapevine cultivars tend to reduce their embryogenic ability if subjected to many cycles of callogenesis/differentiation. In addition, in some cultivars those culture conditions may impair the successive germination of developed embryos. In these cases, the embryogenic callus obtained from floral explant cultured on medium A can be transferred onto medium G and subcultured every month on the same medium. It is important to subculture only the pro-embryogenic masses, carefully selecting the finely granular, friable callus and discarding the black parts and the developed embryos that can be present.

12.3.5 Micropropagation and Acclimatization

(i) Micropropagate plantlets derived either from direct embryo germination or from the embryo germination protocol above described by periodically

culturing apical cuttings (3–4 cm long) on the PGRs-free medium F (Fig. 12.1g) in suitable plant tissue culture containers

(ii) When transfer to *ex-vitro* conditions is required, collect apical cuttings (4–5 cm long) from the micropropagated plants and place them in soaked, autoclaved peat pellets (Jiffy7®) for 2–4 weeks

(iii) For acclimatization, replace the container lid with plastic film and gradually remove the film within the space of 8–10 days
All the previous steps are at 24 °C under light (photoperiod 16 h).

(iv) Transfer the acclimatized plantlets to greenhouse and, after a suitable period of growth, to soil in the field.

12.4 Steps Requiring Further Protocol Refinements

The protocol for plant regeneration via somatic embryogenesis in grapevine described in this chapter proved efficient for many cultivars during a twenty-year experience in our laboratory (Gribaudo et al. 2017). However, further protocol refinements or modifications will be useful, particularly concerning the embryo conversion into plant and the extension of the range of regenerating genotypes.

Germination step is particularly troublesome, as it can impair the success of the whole procedure. In principle, germination may occur in white, well-shaped, well-polarized embryos with root and shoot axes, a hypocotyl and two cotyledons. Germination is hindered by physiological anomalies such as endodormancy, and/or by morphological abnormalities. Abnormal, missing or non-functional apexes have been observed as well as other embryo teratologies (Goebel-Tourand et al. 1993; Faure et al. 1998; Larrouy et al. 2017). The pronounced anomalous behavior where the embryo exhibits continuous growth leading to abnormal structure and function of the shoot meristem has been connected with the 'precocious germination', an event already described for zygotic embryos during in vitro culture (Finkelstein and Crouch 1984). Indeed the morphological and physiological state of embryos and the culture conditions are crucial aspects in promoting embryo conversion and ensuring the success of the culture. A number of strategies and protocols have been proposed (Martinelli and Gribaudo 2009) but their efficiency must be tested in specific culture conditions.

The strong genotype influence on the performance of in vitro cultures in general, and of grapevine embryogenic culture in particular, makes difficult to set up a unique protocol optimal or even efficient for all cultivars and species of the genus *Vitis* (Oláh et al. 2009; Vidal et al. 2009; Gribaudo et al. 2017). Undeniably the embryogenic response in a grapevine cultivar involves a complex interaction of the genotype with explants, culture medium and culture conditions (Dhekney et al. 2016). Therefore, cultivar or species not yet tested for their embryogenic capacity or having a low response to the described protocol may be evaluated on alternative induction media, gleaning suggestions from the substantial available literature.

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