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Research Note

A simple and efficient method to obtain stable embryogenic cultures from anthers of *Vitis vinifera* L.

L. Torregrosa

Since the first report of somatic embryogenesis (Mullins and Srinivasan 1976) and adventitious organogenesis (Rajasekaran and Mullins 1981), the regeneration of grapevine has been extensively studied (review: Torregrosa 1995). To achieve genetic engineering of grapevine, the regeneration of whole plants from somatic cells or tissues is an imperative pre-requisite. Among the two pathways of regeneration, organogenesis has been found unsuitable for genetic transformation and regeneration of non-chimaeric plants (Colby *et al.* 1991) while embryogenesis has been used successfully several times for transformation since the first report of Mullins *et al.* (1990). Despite the important work carried out on embryogenesis, *Vitis vinifera*, like many other woody species, still appears to be a recalcitrant plant for the initiation and the maintenance of embryogenic tissue culture. Here we report an improvement of the general procedure of embryogenic callus induction from anthers and long-term maintenance of stable undifferentiated embryogenic cultures.

Materials and methods: Woody canes of *V. vinifera* (cvs Portan and Danuta) were kindly provided by Alain Bouquet from the Institut National de la Recherche Agronomique, Villeneuve-les-Maguelonne, France and by Serge Grenan from the Etablissement National pour l'Amélioration de la Viticulture, Grau-du-Roi, France (cvs Syrah cl. 174 and Ugni blanc cl. 384). They were stored in plastic bags at 4 °C until use. Canes were cut into two-node cuttings, rehydrated for 24 h in water, their upper side covered by paraffin and cultured into perlite under cool fluorescent day light (15 h photoperiod at 30 μmol m^{-2} s^{-1} light intensity). The cuttings were first cultured at 28 ± 2 °C at 100 % humidity during 2 weeks and then exposed to 24 ± 2 °C at 50-70 % humidity. Immature inflorescences were obtained within 6-8 weeks using the method described by Mullins (1966). Protocols used to sterilise the flowers and select the explants have been previously described (Torregrosa 1994). The basal medium (C) consisted of half strength MS macroelements (Murashige and Skoog 1962), MS microelements, vitamins and amino acids as described by Torregrosa (1994), casein hydrolysate (1 g l^{-1}) and 1 μM BAP. Different auxin-like compounds were added: 5 μM 2,4-D (C_1), 5 μM NOA (C_2) or 2.5 μM 2,4-D and 2.5 μM NOA ($C_{1/2}$) occasionally combined with 2.5 % of Sigma® polyvinylpolypyrrolidone (CI). The pH values of the media were adjusted to 5.8 with KOH after incorporating 0.8 % w/v Prolabo® agar-agar (C^A) or 0.6 % w/v Sigma® Phytagel (C^P). All induction media were sterilised for 30 min at 110 °C and distributed in 55 x 10 mm Petri dishes (10 ml per plate).

Once isolated the anthers were briefly placed with their abaxial side on the induction medium and individually spaced. Plates were sealed with scellofrais® (polyethylene film) and incubated at 26 ± 1 °C in darkness. During the induction period, the explants were maintained on the same medium without transfer. The experimental unit was represented by one plate containing 30 anthers. Each elementary treatment was represented by 8 plates (240 anthers). Necrosis, callogenesis and embryogenesis of the cultures were registrated. Two independent experiments were carried out using a completely randomized design.

To recover plants, both undivided primary and secondary embryogenic calluses were transferred onto C_2^P medium for 1-2 months (same conditions as above). Single embryos (2-4 mm length, torpedo-heart stage) were carefully excised and plated horizontally onto 100x10 mm Petri dishes containing 25 ml of the MS/2 medium free of plant regulators. After 10-15 d of incubation in darkness, the plates were exposed to light (15 h photoperiod at 15 μmol m^{-2} s^{-1} light intensity) for 7-10 d. At this stage, the well-conformed embryos (2 unlinked green cotyledons, 1-3 mm Ø and 5-10 mm long white hypocotyls) often had an emerging epicotyl. Once transferred into culture tubes containing MS/2 medium and exposed to room culture conditions (15 h photoperiod at 15 μmol m^{-2} s^{-1} light intensity, 26 ± 1 °C, 70 ± 10 % humidity), the somatic embryos developed into normal looking plants.

Results and Discussion: When cultured on induction medium, anthers did not undergo any changes for the first week of culture and then developed as described by Newton and Goussard (1990).

For all genotypes tested, NOA induced higher percentages of anther necrosis than 2,4-D (Fig. 1), particularly when combined with agar-agar. Generally, browning appeared first in the interlocular zone from the connective tissue and then developed into the locules and filament but sometimes necrosis was localized only on the locules, the central zone remaining white.

NOA showed the highest effect of callogenesis induction in terms of the percentage of anthers showing callogenesis but also in terms of callus abundance. Calluses induced by NOA emerged from all the anther tissues and were very soft and disorganized. Calluses induced with 2,4-D emerged from the abaxial side of the anther, mainly from the connective tissue and the filament stub, and were smaller but more compact than those obtained with NOA. Calluses initiated with NOA, although more abundant than with 2,4-D, did not present embryogenic competences, especially with cvs Syrah and Ugni blanc.

Phytagel® was found not only superior to agar for the amount of embryogenic structures formed, as reported by

Correspondence to: Dr. L. Torregrosa, ISVV-M, Agro-Montpellier, UFR de Viticulture, 2 Place Pierre Viala, F-34060 Montpellier Cedex 01, France. Fax: +33-499-612064.
E-mail: torregrosa@ensam.inra.fr

Present address: Dr. L. Torregrosa, CSIRO Plant Industry, Horticulture Unit, PO Box 350, Glen Osmond, SA 5064, Australia. Fax: +61-8-83038601. E-mail: laurent.torregrosa@pi.csiro.au

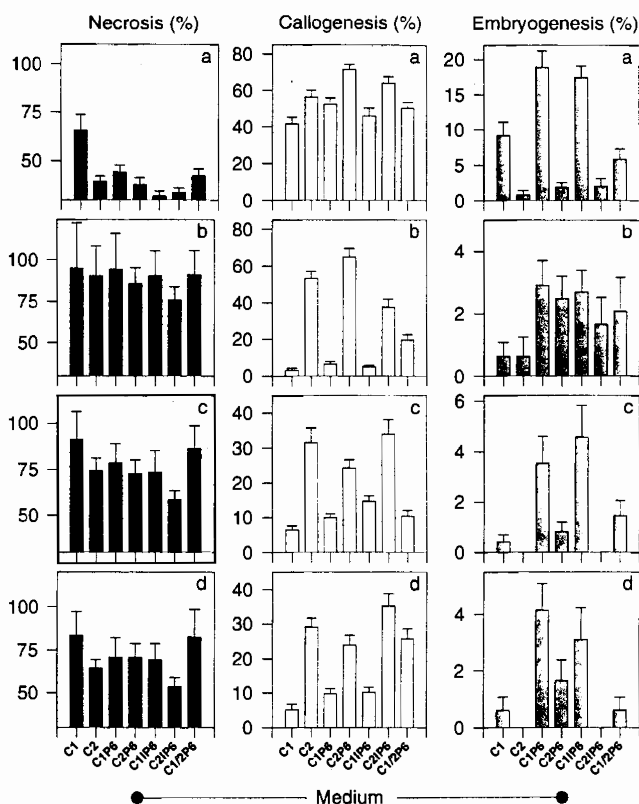

Fig. 1: Effects of the induction medium on the percentage of anthers showing necrosis, callogenesis or embryogenesis for *V. vinifera* cvs Portan (a), Danuta (b), Syrah (c) and Ugni blanc (d) after 60 d in culture (pooled data from two independent experiments with 8 replicates, mean values ± SE).

PERL *et al.* (1995), but also for their earlier induction of embryogenesis. With agar, embryogenic structures did not appear during the first month of culture and, generally, the first subculture was not possible within 120 d. With Phytagel, the first embryogenic structures became visible within 15 d and the first subculture was possible 75 d (sometimes 60 d) after the initiation of the anther culture.

In subsequent experiments, the media C_1^P proved to be the best media for long-term maintenance of the embryogenic calluses (data not shown). With more than 10 genotypes of *Vitis* (including 7 *V. vinifera* cultivars), we could induce indefinite embryogenic cultures (for more than two years) with high proliferation rates (x 10^{12} per year) and without loss of regenerative ability (usually 50-70 % of the somatic embryos excised and transferred onto the MS/2 medium for germination, developed a normal stem suitable for further micropropagation).

Therefore we use only the C_1^P medium, based on 2,4-D as the auxin-like compound and Phytagel as the gelling agent, to initiate and maintain stable embryogenic cultures of *V. vinifera* (Fig. 2). The method presented here is very simple compared to the protocol generally used for somatic embryogenesis of grapevine and gives very good results.

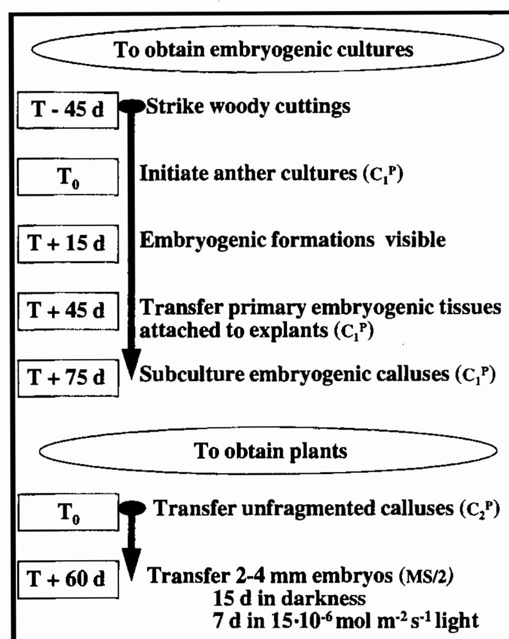

Fig. 2: Initiation and maintenance of embryogenic cultures from anthers of *V. vinifera* and regeneration of whole plants (T_0, day culture initiated; media used in brackets).

COLBY, S. M.; JUNCOSA, A. M.; MEREDITH, C. P.; 1991: Cellular differences in *Agrobacterium* susceptibility and regenerative capacity restrict the development of transgenic grapevines. J. Amer. Soc. Hort. Sci. **116**, 356-361.

MULLINS, M. G.; 1966: Test plants for investigations of the physiology of fruiting in *Vitis vinifera* L. Nature **209**, 419-420.

- -; SRINIVASAN, C.; 1976: Somatic embryos and plantlets from an ancient clone of grapevine (cv. Cabernet-Sauvignon) by apomixis *in vitro*. J. Exp. Bot. **27**, 1022-1030.

- -; TANG, F.C.A.; FACCIOTTI, D.; 1990: *Agrobacterium*-mediated genetic transformation of grapevines: transgenic plants of *Vitis rupestris* Scheele and buds of *Vitis vinifera* L. Bio-Technology **8**, 1041-1045.

MURASHIGE, T.; SKOOG, F.; 1962: A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol. Plant. **15**, 473-497.

NEWTON, D. J.; GOUSSARD, P. G.; 1990: The ontogeny of somatic embryos from *in vitro* cultured grapevine anthers. S. Afr. J. Enol. Viticult. **11**, 70-75.

PERL, A.; SAAD, S.; SAHAR, N.; HOLLAND, D.; 1995: Establishment of long-term embryogenic cultures of seedless *Vitis vinifera* cultivars - a synergistic effect of auxins and the role of abscisic acid. Plant Sci. **104**, 193-200.

RAJASEKARAN, K.; MULLINS, M. G.; 1981: Organogenesis in internode explants of grapevines. Vitis **20**, 218-227.

TORREGROSA, L.; 1994: Culture *in vitro* et transformation génétique de la vigne: Mise au point de protocoles de micropropagation et de régénération par organogenèse et embryogenèse chez les hybrides *Vitis* x *Muscadinia*. Obtention chez deux porte-greffe de racines et de plantes transgéniques produisant la protéine capsidiale du virus de la mosaique chromée de la vigne (GCMV). Thèse Doct. ENSA-M, Montpellier.

- -; 1995: Biotechnologies de la vigne: les techniques de régénération *in vitro* (synthèse). Prog. Agric. Vitic. **112**, 479-489.