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Research Note

An easy and convenient method for maintenance of embryogenic cultures of *Vitis vinifera*

P. Croce, C. Vannini and M. Bracale

DASS Dipartimento di Ambiente, Salute e Sicurezza, Università dell'Insubria, Varese, Italy

K e y   w o r d s : *in vitro* culture, embryo.

Introduction: Regeneration of *Vitis vinifera* cultivars is generally achieved *via* somatic embryogenesis. Since this process is genotype- and explant type-dependent there is no general protocol for regeneration to grape. Various explants including anthers, ovaries (Martinelli *et al.* 2001 a), leaves (Scorza *et al.* 1996), petioles and tendrils (Salunkhe *et al.* 1997) have been tested to establish an embryogenic culture.

Grapevine embryo cultures have been used as a tool for the selection of toxin resistant strains (Jayasankar *et al.* 2000) and for transformation, where the availability of a homogeneous and constant supply of embryogenic explants is an important prerequisite (Scorza *et al.* 1996).

The maintenance of embryogenic competence is genotype dependent, too. Embryogenic callus cultures of cvs Chardonnay and Brachetto have been maintained for more than three years, alternating a 2,4-D containing medium (non-differentiation) and a differentiation medium every two months (Martinelli *et al.* 2001 a). Perennial embryo cultures of *Thompson seedless* have been maintained by subculturing the proembryogenic masses produced by the culture itself on a hormone-free medium (Scorza *et al.* 1996, Li *et al.* 2001), or by recurrent cycles of induction of secondary embryogenesis in *Vitis rupestris* (Martinelli *et al.* 2001b). The embryogenic competence of the primary explants can also be preserved by culturing the embryogenic material obtained from the primary explants (Perrin *et al.* 2001).

In this paper, a convenient method for the cyclic induction and proliferation of embryogenic cell culture is presented.

Material and Methods: Inflorescences from *Vitis vinifera* cvs Trebbiano d'Abruzzo, Trebbiano toscano 3D, Trebbiano toscano R4, Sangiovese, Bombino bianco, Regina and Montepulciano were harvested two weeks prior to bloom.

The anthers were immature, green or only partially yellow in colour. Inflorescences were sterilized with a diluted commercial sodium hypochlorite solution (2 % active chlorine) and 0.05 % v/v Tween 20 for 15 min. Flowers were excised from the inflorescence and dissected in order to separate anthers from ovaries.

Sterile anthers and ovaries were placed on initiation medium (Martinelli *et al.* 2001 a). Briefly, initiation medium consisted of NN salts (Nitsch 1969) with 20 $g \cdot l^{-1}$ sucrose, 2.5 g Gelrite (Duchefa), 9 µM 2,4-D, and 4.4 µM BAP. The pH was adjusted to 5.8 with KOH before autoclaving .

Petri dishes were incubated at 24 °C in the dark. Cultures were visually screened for the formation of embryogenic callus after 30 and 50 d.

Embryogenic calli were identified by visual screening (Perrin *et al.* 2004) and transferred after 30 or 50 d from the initiation to the proliferation medium consisting of modified GS1CA (Franks *et al.* 1998) containing NN salt and vitamins, with the exception of ammonium nitrate 60 $g \cdot l^{-1}$ sucrose. 1 µM BAP was added before autoclaving, while 20 µM IAA and 10 µM NOA were added after autoclaving. The medium was solidified with 2.5 $g \cdot l^{-1}$ Gelrite (Duchefa) and pH was adjusted to 5.8 with KOH before autoclaving.

Culture maintenance is based on a two-step cyclic procedure. The first step involves the induction of embryogenesis from somatic embryos: single somatic embryos were plated on the induction medium, modified PIV (Franks *et al.* 1998), (NN salt and vitamins, 60 $g \cdot l^{-1}$ sucrose, 8.9 µM BAP, 4.5 µM 2,4-D, 2,5 $g \cdot l^{-1}$ Gelrite, pH adjusted to 5.8 before autoclaving), and incubated for 30 d in the dark at 24 °C. The second step involves the proliferation of newly formed and synchronised somatic embryos: calli produced by the somatic embryos cultured on the induction medium were transferred onto the proliferation medium (composition as before, but with addition of 720 $mg \cdot l^{-1}$ ammonium nitrate) for 30 d in order to produce secondary embryos. Embryos produced on proliferation medium were suitable material for the initiation of another inductive cycle if transferred onto the induction medium.

For the production of plants, embryos were placed on WPM medium with 20 $g \cdot l^{-1}$ sucrose, 2,5 µM BAP, 3 $g \cdot l^{-1}$ Gelrite, pH 5,8 at 24 °C, photoperiod 16 h light, until germination and the emission of 3 leaves. Plantlets were then transferred to pots and acclimated in the greenhouse.

Results and Discussion: E m b r y o g e n e s i s   i n d u c t i o n : Most of the explants produced callus after 30 d of culture on the initiation medium. Embryogenic calli were white to yellow and were friable and compact, while non-embryogenic calli were white, soft and spongy or else yellow to brown and wet. All of the tested cultivars formed embryogenic callus at least from the ovaries.

The rate of formation of the proembryogenic callus was highly dependant on the type of the explants and the cultivars tested (Table).

The percentage of embryogenic callus induction was higher for the ovaries and lower for the anthers, confirming the high susceptibility of ovaries in producing these particular cells (Martinelli *et al.* 2001 a).

E m b r y o   p r o d u c t i o n : All of the embryogenic calli produced in the induction phase were transferred onto the proliferation medium. In 4 cultivars out of the 7 tested, the callus produced somatic embryos that enlarged, turned green and emitted the radicle. In these cases, the green embryos formed new embryos at the root-shoot transition zone that could be sub-cultured on the same medium for an indefinite number of cycles. The embryos produced were asynchronous, heterogeneous and small in quantity making this pro-

Correspondence to: Dr. P. Croce, DASS Dipartimento di Ambiente, Salute e Sicurezza, Università dell'Insubria, Via Peschiera 20, 21100 Varese, Italy. E-mail: p.croce@infinito.it

Table 1

Number of explants cultured for each cultivar and percentage of the explant producing proembryonic callus

Cultivars tested	Explant type and number*		Efficiency of embryogenic callus induction (%)[a]	
	Anthers	Ovaries	Anthers	Ovaries
Trebbiano d'Abruzzo	600	118	0	14.40 ± 10.0
Trebbiano Toscano 3D	450	96	0	5.20 ± 0.88
Trebbiano Toscano R4	350	70	0	12.86 ± 3.53
Sangiovese	900	112	2.70 ± 0.8	5.36 ± 2.33
Bombino blanco	850	130	0.24 ± 1.4	6.92 ± 5.4
Regina	550	120	1.82 ± 1.7	7.50 ± 5.3
Montepulciano	400	70	1 ± 1.13	34.29 ± 19.44

*Sum of the explant cultured in three different repetitions.
[a]Data from all experimental repetitions were used for means and standard deviation calculation.

cedure not suitable to produce embryos amenable for transformation.

Culture maintenance: In order to establish a synchronous embryo culture, avoiding embryo germination, we decided to establish a two-step procedure. The first step was the subculture of single somatic embryos on a 2,4-D containing medium in order to promote the formation of embryogenic material. The synthetic auxin 2,4-D is known to inhibit embryo germination, stimulating somatic embryogenesis.

When cultured on the induction medium, the embryos formed calli that, in some cases, were of the embryogenic type. Alternatively Regina and Sangiovese also produced masses of translucent proembryos able to convert into somatic embryos once placed on the proliferation medium.

The second step was the subculture of the calli on the proliferation medium. When cultured on the differentiation medium, the embryogenic-type calli were able to generate large amounts of somatic embryos with a synchronous pattern of growth and which did not germinate on the medium (Figure). Once on the proliferation medium, all of the embryogenic calli produced in the induction medium proliferated into somatic embryos but, in contrast to the report of MARTINELLI *et al.* (2001a) for Chardonnay and Brachetto, no formation of callus of any sort was observed. This confirms the difficulty of establishing a general method for the maintenance of perennial embryogenic cultures of *Vitis* sp.

White somatic embryos produced on the proliferation medium were used to induce somatic embryogenesis for a potentially indefinite number of cycles. Embryogenic cultures have been maintained with this method for more than two years. Interestingly, the embryos produced by secondary embryogenesis were more susceptible to producing morphogenetic material than primary embryos. A side from being synchronised, the formed embryos also had the advantage of a massive production of embryos amenable to transformation.

Figure: Embryos produced on the proliferation medium after one month from subculture.

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