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## High efficiency somatic embryogenesis and plant germination in grapevine cultivars Chardonnay and Brachetto a grappolo lungo

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### Summary

**A highly efficient, reproducible method for somatic embryogenesis induction, plant recovery and embryogenic culture preservation has been developed for cvs Chardonnay and Brachetto a grappolo lungo (*Vitis vinifera*), starting from immature anthers and ovaries. Embryogenic induction efficiency was 2 % and 17 % in anthers for Chardonnay and Brachetto g.l., respectively, and 14 % in ovaries for both cultivars. Embryogenic cultures of both genotypes are still propagating 3.5 years after the initial induction and are still morphogenic. Embryo conversion into plantlets occurred at suitable efficiencies during a 100 d culture for both Chardonnay (37 % and 15 %) and Brachetto g.l. (30 % and 29 %), in the two media tested. Organogenesis was also obtained from cotyledonary leaves of Chardonnay.**

**Key words:** Brachetto, Chardonnay, floral explants, organogenesis, somatic embryogenesis.

### Introduction

High efficient strategies for induction of grape somatic embryogenesis and plant regeneration are essential in view of their application in different breeding perspectives. Somatic embryogenesis proved to be one of the most appropriate tools for *in vitro* manipulation of the genus *Vitis*, e.g. for achieving gene transfer (MARTINELLI 1997; GÖLLES *et al.* 2000; HARST *et al.* 2000; MARTINELLI *et al.* 2000; MARTINELLI and MANDOLINO 2001), induction of somaclonal variation (FALLOT *et al.* 1990; DELOIRE and MAURO 1991; KUKSOVA *et al.* 1997), virus elimination (GOUSSARD *et al.* 1991; GOUSSARD and WIID 1992; SCHAEFERS *et al.* 1994), and germplasm conservation (GRAY and COMPTON 1993).

Moreover, genomic projects have recently been developed for the genus *Vitis* (ABLETT *et al.* 2000; FISCHER *et al.* 2000). They will soon require suitable *in vitro* strategies for studying gene expression in homologous systems; embryo tissues, indeed, proved to be the best cell source for gene transfer to grape (MARTINELLI 1997; MARTINELLI and MANDOLINO 2001).

In a somatic embryogenesis system, the acquisition of embryogenic competence as well as the expression of the

embryogenic program in a callus produced by the plated explant, the efficient embryo conversion into plants and the preservation of the embryogenic ability of the cultures are crucial developmental phases for a successful overall protocol.

Since the 1970s, when first attempts were carried out for obtaining regeneration in the genus *Vitis* (GRESSHOFF and DOY 1974; HIRABAYASHI *et al.* 1976; RAJASEKARAN and MULLINS 1979), the range of grape genotypes that have been successfully subjected to somatic embryogenesis induction has widened, particularly in *Vitis vinifera* (MARTINELLI and GRIBAUDO 2001). However, the technique still needs to be improved for most viticulturally important grape cultivars. In addition, the nature of explants successfully adopted for embryogenesis induction could be extended: anthers proved to be the most responsive explant type (MARTINELLI and GRIBAUDO 2001); ovaries were underestimated explants (BESSIS and LABROCHE 1985; GRAY and MORTENSEN 1987; NAKANO *et al.* 1997).

Here we describe a method developed with cvs Chardonnay and Brachetto a grappolo lungo for a successful management of all protocol steps, starting from anthers and ovaries.

### Material and Methods

**Plant material and somatic embryogenesis induction:** Somatic embryogenesis was obtained from immature anther and ovary cultures. Inflorescences of *Vitis vinifera* cvs Chardonnay and Brachetto a grappolo lungo (Brachetto g.l.) were collected from the vineyard 10 to 14 d before bloom, when anthers became translucent yellow (Fig. 1). The mother plants were grown at Grugliasco (TO) in the didactic grape collection of the Department of Arboriculture and Pomology, University of Turin.

The floral clusters were cold-treated at 4 °C for 4–6 d, then sterilized by immersion for 15 min in a sodium hypochlorite solution (2 % active Cl) with a few drops of surfactant. One hundred anthers and 20 ovaries from each cultivar were isolated and placed on a NITSCH and NITSCH (1969) (NN)-based medium with 20 g l<sup>-1</sup> sucrose added, 3 g l<sup>-1</sup> Phytagel (Sigma), 9 µM 2,4-D and 4.4 µM BA (callus induction medium). The pH was adjusted to 5.8; this pH value was adopted

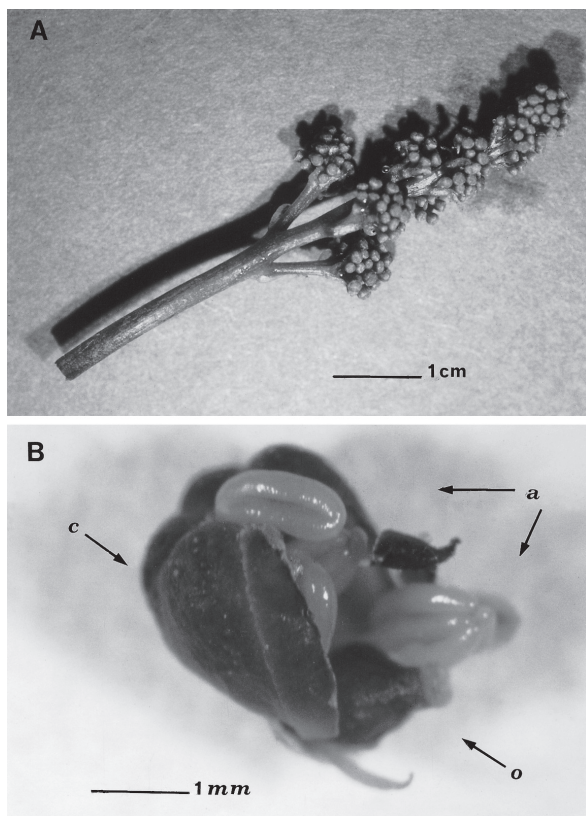


Fig. 1: Floral clusters (A) and a isolated flower (B) with immature anthers (a), the ovary (o) and the dissected calyptra (c). Inflorescences were collected from the vineyard 10 - 14 d before bloom.

for all the media described in this paper unless otherwise specified. In plastic Petri dishes of 90 mm diameter, 30 ml of medium were dispensed and 20 anthers or 5 ovaries were cultured. The cultures were maintained at 26 °C in the dark.

Somatic embryo production was induced by sub-culturing the explant-derived calli on a medium containing NN macroelements and iron, MURASHIGE and SKOOG (1962) microelements, GAMBORG *et al.* (1968) (B5) vitamins, 60 g l<sup>-1</sup>

sucrose, 10 µM NOA, 1 µM BAP, 20 µM IAA, 10 g l<sup>-1</sup> Bacto-Agar and 2.5 g l<sup>-1</sup> activated charcoal (embryo differentiation medium). The pH was adjusted to 6.2.

For long-term maintenance of embryogenic cultures, subcultures were performed monthly according to the method of FRANKS *et al.* (1998). The above cited embryo differentiation medium was alternated every two months with a callus propagation medium; the latter had the same basal composition of the embryo differentiation medium but was supplemented with 4.5 µM 2,4-D, 8.9 µM BA and 10 g l<sup>-1</sup> Bacto-Agar. The pH was adjusted to 6.2.

**Somatic embryo cultures and plant germination:** For elongation, multiplication and individualization, clusters (100 mg fresh weight) of embryos (length: 1-2 mm) were separated from the embryogenic callus at the end of a two-month culture period on the embryo differentiation medium, and were placed into 125 ml flasks containing 60 ml of NN-based liquid medium with 30 g l<sup>-1</sup> sucrose added and 0.5 µM IBA. Cultures were incubated at 25 °C with a 16-h photoperiod (70 µmol m<sup>-2</sup> s<sup>-1</sup> cool white light) and shaken continuously at 90 rpm. Medium was renewed every three weeks.

Mature somatic embryos were isolated from liquid cultures and placed with the radicals downward on NN-based media containing 15 g l<sup>-1</sup> sucrose and 9 g l<sup>-1</sup> Bacto-Agar. In plastic Petri dishes of 90 mm diameter, 30 ml of medium was dispensed. Two media were compared: the first was hormone-free, while the second contained 4.4 µM BA and 0.5 µM IBA. On the first medium 330 and 216 somatic embryos of the cultivars Chardonnay and Brachetto g.l., respectively, were planted, and on the second medium 355 and 181, respectively. In each dish, 10-12 somatic embryos were planted. Cultures were incubated at 25 °C with a 16-h photoperiod (70 µmol m<sup>-2</sup> s<sup>-1</sup> cool white light) and media were renewed monthly. Germination was defined as the first internode emerging (Fig. 2, b), and its efficiency was calculated as the percentage of germinating somatic embryos related to the total of plated embryos (Table).

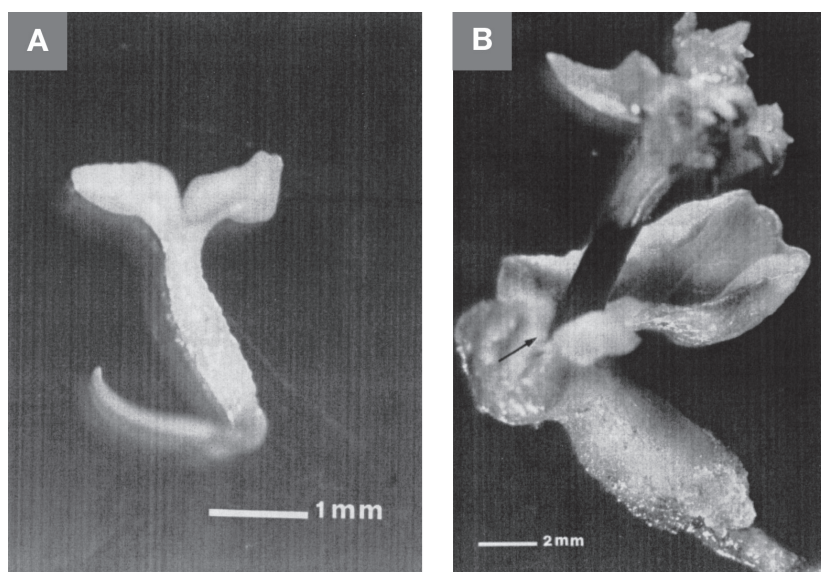


Fig. 2: Mature somatic embryo derived from Chardonnay anthers isolated from in-liquid culture (A). Somatic embryo derived from Brachetto a grappolo lungo ovary during germination (B): the emergence of apex from the cotyledonary leaves is clearly visible (arrow). Embryo shape and development are typical.

Table

Germination efficiency of cvs Chardonnay and Brachetto a grappolo lungo in a 100-d culture, in the presence or absence of growth regulators, respectively. Efficiency is expressed as the proportion of germinated somatic embryos related to the total number of embryos (Chardonnay: 355 and 330 embryos, respectively; Brachetto g.l.: 181 and 216 embryos, respectively)

Cultivar	Days	Germination (%)	
		with growth regulators	without growth regulators
Chardonnay	20	11	0
	30	19	4
	40	27	7
	50	32	9
	60	35	11
	70	36	12
	80	37	12
	90	37	14
	100	37	15
	Brachetto g.l.	20	5
30		14	2
40		22	3
50		25	5
60		27	15
70		29	16
80		29	18
90		29	21
100		30	29

For further plant elongation and micropropagation, apical and axillary buds were planted on NN-based medium containing 15 g l<sup>-1</sup> sucrose and 9 g l<sup>-1</sup> agarose in Magenta boxes containing 75 ml of medium, under the same cultural conditions.

**Organogenesis from somatic embryos of Chardonnay:** For induction of shoot organogenesis, a sample of 228 somatic embryos of Chardonnay were deeply planted with the radicals downward into a NN-based medium containing 15 g l<sup>-1</sup> sucrose and 9 g l<sup>-1</sup> Bacto-Agar, 4.4 µM BA and 0.5 µM IBA. Two weeks later, cotyledonary leaves were dissected from the embryos, placed on the same medium, and incubated at 25 °C with a 16-h photoperiod (70 µmol m<sup>-2</sup> s<sup>-1</sup> cool white light). For further plant elongation and micropropagation, shoots were transferred to the same medium but without growth regulators under identical cultural conditions.

### Results and Discussion

**Induction of somatic embryogenesis:** Somatic embryogenesis was obtained from both anther and ovary cultures, with different efficiencies depending on genotype and explant source. After a 4-month induction, 2 % of Chardonnay and 17 % of Brachetto g.l. anther cultures became embryogenic, while 14 % of the ovary cultures of both cultivars gave embryos.

Anthers are the most widely used organs for grapevine somatic embryogenesis initiation, and supposed to be the most suitable explant. Conversely, ovaries are less adopted, even though immature ovule cultures were attempted in one of the first papers on grape somatic embryogenesis (MULLINS and SRINIVASAN 1976). In further studies primary explants from ovules or ovaries were rarely applied (BESSIS and LABROCHE 1985; GRAY and MORTENSEN 1987; NAKANO *et al.* 1997). According to our experience, on the other hand, ovaries proved to be suitable material for establishing somatic embryogenesis for Chardonnay as well as for other cultivars, *e.g.* Barbera, Moscato bianco, Nebbiolo (GRIBAUDO 2001); in most cases they proved to give better results than anthers. Similar results are reported by NAKANO *et al.* (1997), who compared several *V. vinifera* cvs, hybrids of *V. vinifera* x *V. labruscana* and a cultivar of *V. rupestris*. Five genotypes regenerated adventitious embryos from ovary explants, with efficiencies generally higher than from anthers and leaves, ranging from 1.3 to 12.9 %. GRAY and MORTENSEN (1987) obtained a 10 % regeneration efficiency using ovaries for inducing somatic embryogenesis in *V. longii* 'Microsperma'.

**Culture maintenance:** The indefinite maintenance of embryogenic cultures is a crucial part of a somatic embryogenesis protocol, particularly for genotypes with low embryogenic induction efficiency from primary explants. Thus, several methods have been attempted to avoid a progressive total loss of cultures associated with embryo germination and maturation, as well as culture aging within the subcultures (MARTINELLI and GRIBAUDO 2001; MARTINELLI *et al.* 2001).

Our strategy, based on the protocol of FRANKS *et al.* (1998), is a two-step cycle that could be indefinitely repeated. In the first step, callus proliferation is obtained on the callus propagation medium within a two-month culture while, in the second step, development of embryogenic callus and somatic embryos are induced within an additional two-month culture on the embryo differentiation medium. The cited callus propagation medium also proved to efficiently induce embryogenic callus formation from primary explants (FRANKS *et al.* 1998; GRIBAUDO 2001).

The gradual morphogenic organization becomes visible during the second step, when compact, white structures (embryogenic nuclei) start to organize; from these, embryogenic callus and somatic embryo clumps will be regenerated. The subsequent transfer of embryogenic callus to the first medium produces a gradual disorganization and massive callus propagation. A new induction cycle can be started at this point, ensuring that morphogenic competence has been preserved in the cultures.

No difference was noted in any aspect between embryogenic cultures originating from ovaries or from anthers. Chardonnay and Brachetto g.l. showed a similar behavior, however, in the latter cultivar, callus was less brownish and presented more embryogenic nuclei though the amount of embryo clumps were less numerous at the end of the regeneration phase.

Three and a half years after induction from both, ovary and anther explants embryogenic cultures of both genotypes are still propagating; they continue to be morphogenic.

**Somatic embryo cultures:** In liquid culture, somatic embryos were induced to grow, mature and individualize. From an embryo clump of 100 mg, in a few weeks a relevant amount of embryos could be obtained for both cultivars, Chardonnay developing more profusely and faster. Cultures were not synchronized, and different maturity steps (globular, heart, torpedo, polarized with or without root elongation and open cotyledons) were present in the same culture. Besides a typical shape (Fig. 2), with culture aging various deformities concerning size (giant embryos) and morphology (cauliflower-like embryos and embryos with long hypocotyles, vestigial or multiple cotyledons, and absence of root) as well as culture darkening were observed. Similar teratologies have also been described for other genotypes (GOEBEL-TOURAND *et al.* 1993).

The medium employed in the present study also proved to be excellent for *V. rupestris* somatic embryo cultures (MARTINELLI *et al.* 1993); conversely, with different *V. vinifera* cultivars (Superior seedless, Red globe and Gamay) better cultures were obtained in hormone-free liquid media (MARTINELLI, unpubl.).

**Plant germination:** The Table presents germination efficiency obtained for the two cultivars during a 100-d culture. For both, Chardonnay and Brachetto g.l., relevant efficiencies were obtained on both media. In the presence of growth regulators, however, a notably better performance was achieved in shorter culture times, and suitable efficiencies were achieved after one months. Conversely, enhancement of root development was observed on the hormone-free medium.

Further micropropagations produced several subclones for both cultivars. Plantlets showed normal morphology, and only in few cases vitrifications were observed.

The protocol presented here proved to be suitable to induce embryo conversion into plants at high efficiencies for two genotypes. This is an important prerequisite for regeneration. Conversely, this has been reported to occur erratically and with variable results among the genus *Vitis*, since acceptable conversion efficiencies have been obtained only for a few species and *V. vinifera* cvs; in *V. rupestris*, germination of petiole-derived somatic embryos occurred at a frequency of 51 % in the presence of IBA (MARTINELLI *et al.* 1993) while, among *V. vinifera* cvs, highest efficiencies were obtained with Chardonnay. In this cultivar, in the absence of growth regulators, GOEBEL-TOURAND *et al.* (1993) observed germination of functional plantlets from anther-derived somatic embryos with an efficiency of 70 %, and JAYASANKAR *et al.* (1999) obtained 88 % of plant regeneration from suspension cultures of anther- and ovary-derived somatic embryos.

**Organogenesis from somatic embryos of Chardonnay:** During germination tests on the medium containing growth regulators, we noticed a relevant shoot differentiation on cotyledonary leaves of Chardonnay if they came casually in contact with the medium; this occurred if somatic embryos were deeply planted. Organogenesis did not prevent embryo germination.

To verify organogenic competence of cotyledons, an assay was performed. Regeneration from somatic embryo

cotyledons could be an interesting system for massive plantlet production after foreign gene transfer.

Out of 228 somatic embryos tested, 18 % regenerated shoots from the central vein of cotyledons within two weeks, each cotyledonary leaf producing many shoots (Fig. 3). Shoot development was completed when leaves were isolated and placed onto a medium with identical composition, and within 50 d 93 % regenerated whole plantlets. In few cases (2.2 %) shoots and/or somatic embryos were obtained from the hypocotyl (not shown).

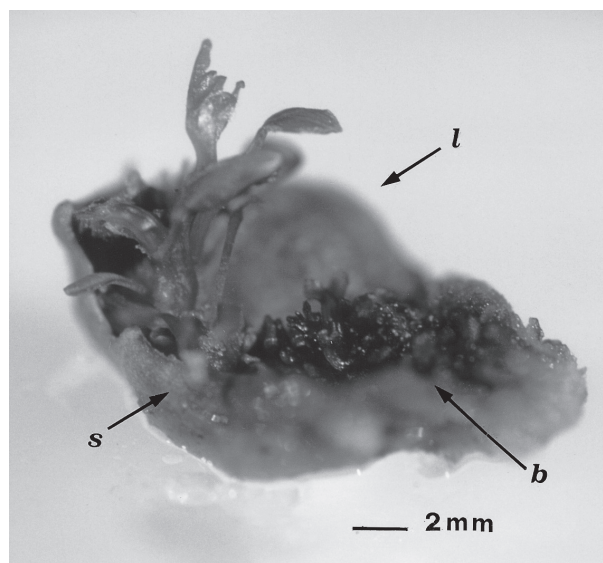


Fig. 3: Organogenesis from somatic embryos of Chardonnay: Buds (b) and shoots (s) emerging from the mid-vein of a cotyledonary leaf (l). Bud overproduction is noticeable.

As an alternative to embryo germination, adventitious bud induction had previously been obtained from hypocotyls and cotyledons of dormant somatic embryos (VILAPLANA and MULLINS 1989), while organogenesis was achieved from embryo tissues after the production of a green disorganized callus (MARTINELLI and MANDOLINO 1994). The regeneration event described here, on the contrary, did prove compatible with embryo germination.

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