

## Embryogenic responses of *Vitis* spp.: Effects of genotype and polyvinylpyrrolidone

by

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**S u m m a r y :** Somatic embryos were obtained from leaf discs excised from cv. Seyve Villard 5276 (*Vitis* sp.) and from a Brazilian interspecific hybrid derived from Madalena (*Vitis* sp.) and Magnolia (*V. rotundifolia*). Experimental procedures were conducted according to HARST (1995) except that phenylalanine was replaced by polyvinylpyrrolidone which improved the embryogenic response of cultivar SV-5276. Standard somatic embryogenesis was confirmed by histological and ultrastructural analysis which also revealed teratological embryonic development. Secondary embryos were observed and documented by scanning microscopy.

**Key words :** Somatic embryogenesis, *Vitis*, histology, DL-phenylalanine, polyvinylpyrrolidone.

### Introduction

Grape is very heterozygous and exhibits pronounced inbreeding depression. In its life cycle the juvenile period is remarkably long, thus *in vitro* genetic manipulation of somatic and/or zygotic cells may contribute to shorten the time for genetic improvement either by supplementing and enhancing existing breeding techniques or by circumventing methods altogether (GRAY and MEREDITH 1992). Although both embryogenic and organogenic pathways of regeneration are well documented, morphogenesis is highly species dependent and plant recovery from somatic embryos remains limited to a few responsive genotypes in the genus *Vitis* (STAMP and MEREDITH 1988; FAURE 1990; GOEBEL-TOURAND *et al.* 1993; FAURE *et al.* 1996 a, b). Several media have been tested in order to enhance regeneration efficiency in grape: MAURO *et al.* (1986) reported beneficial effects of hydrolyzed casein in the induction stage of somatic embryogenesis and higher percentages of anther-derived embryos and plantlets after adding glutamine, phenylalanine and adenine; HARST (1995) observed superior embryogenic responses from leaf discs, due to the influence of 2.5 mM DL-phenylalanine (PHE). In addition, polyvinylpyrrolidone (PVP, MW 40,000) in protoplast cultures was shown to avoid the accumulation of phenolics thereby increasing microcalli formation (REUSTLE and NATTER 1994).

The aim of this work was to verify the morphogenetic responses of three genotypes: *Vitis vinifera* cv. Italia, a French hybrid (cv. Seyve Villard 5276) and a Brazilian hybrid. As cv. SV-5276 has been successfully used as a progenitor to produce wine grapes in South Brazil we tested the protocol described by HARST (1995) to improve its ability to morphogenesis. In addition the effects of PVP were evaluated.

### Material and Methods

**Plant material :** Dormant *Vitis* shoots were exposed to a chilling treatment (2 °C) for 2 months to induce bud burst. Shoot apices were collected and surface-sterilized by immersion in 70 % ethanol for 5 min followed by immersion in 1.2 % NaOCl for 20 min. Explants were rinsed 4 times for 10 min each in sterile water and in a citric acid solution (25 mg·l<sup>-1</sup>, 10 min) and placed on a 0.5 x MS medium (MURASHIGE and SKOOG 1962) supplemented by 2.5 µM benzyl aminopurine (BAP). Vigorous apices were selected and monthly transferred to the same medium devoid of hormones. Leaf discs (7 mm diameter) were excised from *in vitro*-grown grapevine plants, cv. Italia, cv. SV-5276 and from a Brazilian hybrid derived from Madalena (Seibel 11342 x Moscatel Branco, a *Vitis vinifera* cultivar) and Magnolia (*Vitis rotundifolia*).

Leaf discs were placed with their adaxial side in contact with the induction medium, denoted M-1, in Petri dishes (90 mm x 20 mm, 10 explants per plate) containing 20 ml of basal NN (NITSCH and NITSCH 1969) supplemented with naphthoxyacetic acid (NOA, 4.0 mg·l<sup>-1</sup>), thidiazuron (TDZ, 0.9 mg·l<sup>-1</sup>), according to HARST (1995) and Phytigel solidified (Sigma, 1.8 g·l<sup>-1</sup>). PVP was added at 5 g·l<sup>-1</sup> to M-1 medium. Cultures were maintained at 25 ± 3 °C in the dark. Every 4 weeks explants were transferred to fresh NN basal medium. The number of replicates (plates) varied due to differences in the availability of plant material and were two (Italia), 4 (Brazilian hybrid) and 5 (SV-5276).

Two additional experiments were carried out with cv. SV-5276. In the first, PVP (10 g·l<sup>-1</sup>) was added to the M-1 medium of plates with 10 leaf discs each. The trial was replicated 12 times. Ten weeks later, the number of somatic embryos per explant and plate was determined. The devel-

opmental stage of embryos was evaluated according to Faure's classification, *i.e.* globular, heart, torpedo and T+ (FAURE *et al.* 1996 a, b). Embryos were induced to germinate in NN basal medium below fluorescent tubes (16 h.d<sup>-1</sup>, 30  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). In the second experiment, 4 treatments were used, all with 5 replications and 10 leaf discs per plot: M-1 medium (control); M-1 + PHE (2.5 mM); M-1 + PVP (5 g.l<sup>-1</sup>); M-1 + PHE (2.5 mM) + PVP (5 g.l<sup>-1</sup>). Embryos produced in these cultures were transferred to half-strength MS medium, under light.

For histological analysis, somatic embryos (type T+ and giant ones) and callus samples from SV-5276 were collected after 10 weeks of culture, at the time of transference to fresh medium. Samples were fixed in Karnovsky solution (KARNOVSKY 1965), dehydrated in ethanol and glycol methacrylate (Reichert-Jung) was infiltrated. Once polymerized, sections of 5  $\mu\text{m}$  thickness were obtained with a rotary microtome (Leica) and stained in toluidine blue. For scanning electron microscopy, somatic embryos were dehydrated in ethanol, dried to a critical point (CO<sub>2</sub>) and attached to aluminium stubs. Specimen were coated with gold (30-40 nm layer) and examined in a SEM (Zeiss, DSM940A, 5 kV).

## Results

On the 28th day of culture, the Brazilian hybrid showed conspicuous callus formation (100 % frequency) at the surface of leaf discs. This response was also observed for Italia but the calli were smaller and oxidation occurred in the medium. SV-5276 exhibited a tendency to develop small brownish callus from the wounded area that evolved for a typical embryogenic culture as described by STAMP and MEREDITH (1988) after 8 weeks. Somatic embryos were observed 15 d later. Alternatively, this response was not observed in the other two genotypes.

Concerning SV-5276, the mean number of somatic embryos produced per leaf disc placed on M-1 containing PVP at 10 g.l<sup>-1</sup> was  $95.5 \pm 13.4$ . Five percent of these embryos were characterized as T+ and giants. Most of them presented teratological morphology and failed to germinate. Twenty percent of the embryos transferred to the light regime developed into entire plants after 80 d.

In the control treatments, PVP (5 g.l<sup>-1</sup>) and PHE (2.5 mM) + PVP (5 g.l<sup>-1</sup>) the first somatic embryos were observed after 8 weeks. Approximately 6 % of the embryos produced in media containing PVP, developed into entire plants. Embryo formation was stimulated by PVP and occurred at high frequency (82 %) compared to control (40 %). PHE inhibited the embryogenic response even in the presence of PVP (16 %).

Longitudinal sections of the white embryo-like structures visible at the surface of cultures were similar to globular and heart stage of grape zygotic embryos, those at an advanced stage presenting suspensors.

The process of embryo formation was not synchronous. Some were bipolar showing a small and flattened shoot apex while some presented hypocotyledonary apex and expanded cotyledons exhibiting a dome-like meristematic region. Most of the T+ developing embryos had more than two cotyledons (Fig. 1 A, B). The large size of T+ was due to an increase in the number of cell layers of the fundamental meristem (Fig. 1 B). These embryos had a poorly defined protodermis since some sectors still presented dividing cells (Fig. 1 C, arrow). Arrows in Fig. 1 C and D indicate surface accumulation of phenolic substances and tracheary element differentiation in the hypocotyledonary axis, respectively. T+ embryos continued to germinate showing development of roots and hypocotyls, assuming a giant shape (Fig. 2 A). These embryos presented a multicotyledonary crown and shoot meristem was absent. Several secondary embryos were formed from the joined portion between hypocotyl and root (Fig. 2 B, arrow, C and D).

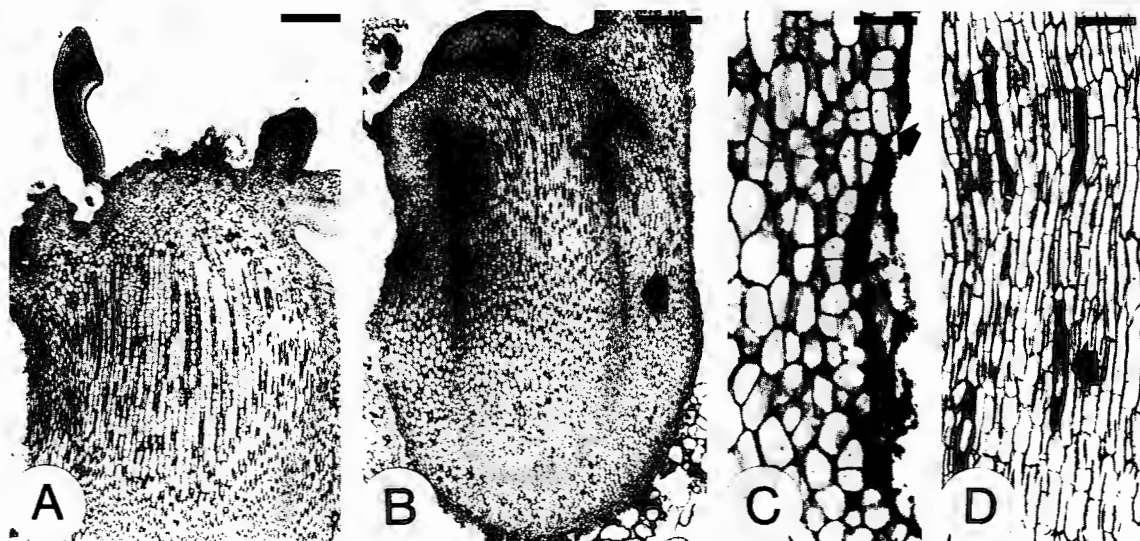


Fig. 1: Two longitudinal serial sections of the same T+ somatic embryo (Seyve Villard 5276). A. Presence of more than two cotyledonary leaves and the absence of shoot meristem (bar = 540  $\mu\text{m}$ ). B. Hypocotyl expansion, partial protodermic layer disorganization and precocious differentiation (arrow) of the tracheary elements (bar = 540  $\mu\text{m}$ ). C. Detail of the protodermis exhibiting cell divisions (arrow, bar = 52  $\mu\text{m}$ ); D. Differentiation of the tracheary elements (arrow, bar = 100  $\mu\text{m}$ ).

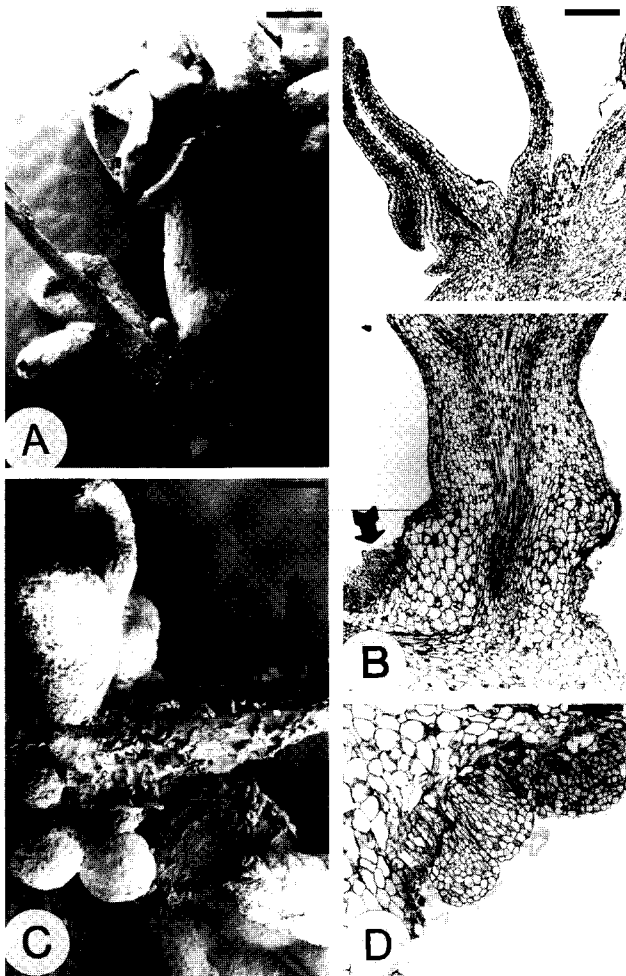


Fig. 2: A. Scanning electron micrograph of a giant somatic embryo (cv. Seyve Villard 5276) exhibiting a multicotyledonary crown and secondary embryos emerging from the region between hypocotyl and root (bar = 2mm). B. Longitudinal section of the giant embryo showing several cotyledons, absence of shoot meristem and two globular secondary embryos (arrow, bar = 540  $\mu$ m). C and D. Scanning electron micrograph (bar = 1 mm) and longitudinal section (bar = 200  $\mu$ m) of globular secondary embryos.

## Discussion

A common problem in inducing grapevine somatic embryogenesis and conversion into plants is the strong dependence on genotype (STAMP and MEREDITH 1988). According to HARST (1995) Seyval blanc shows a high frequency of embryogenic explants (75 %) while this frequency can decrease to 15 % for Chancellor and to 25 % for other species, e.g. *Vitis thumbergii*.

The favorable effect of PVP on the embryogenic response of SV-5276 was firstly demonstrated in this work.

No favorable effects of PHE (a precursor of phenols) were detected on the embryogenic response. In the absence of PVP, PHE was not able to stimulate explants to respond, probably due to the increased phenol levels caused by PHE. Our results differ from those of HARST (1995) who used cv. Seyval blanc, supporting the idea of a strong genotype effect on grapevine morphogenic responses.

Histological studies confirmed that the *in vitro* responses correspond to somatic embryogenesis due to an establishment of bipolarity of embryo-like structures which have no vascular connection with maternal tissues. It is supposed that embryogenesis proceeded from single cells since embryoids were attached by a narrow suspensor-like organ. In culture, somatic embryos passed through globular, heart-shaped, torpedo, T+ and some acquired giant shapes as proposed by FAURE *et al.* (1996 a, b).

In addition, torpedo somatic embryos had less differentiated structures and small and flattened shoot apical meristem. Subsequently, the apical meristem showed a dome shape and cotyledons were expanded. These two forms were concomitant. Shoot apex differentiation was noted after the torpedo state and a multicotyledonary crown was recognized in advanced stages. Besides regular embryoids, others showed teratological features as reported by FAURE *et al.* (1996 a, b).

During the passage from torpedo to giant stage some alterations were noted, especially those typical of zygotic precocious germination (FINKELSTEIN and CROUCH 1984): anticipated root development and interruption in meristematic activity at the shoot apex. The block of somatic embryo conversion could be due to precocious germination leading to abnormal structure and activity of the shoot meristem.

Moreover, heterogeneity of form and growth of SV-5276 somatic embryos were observed in cultures developed from young leaves of clone CH76 of *V. vinifera* cv. Chardonnay, as reported by GOEBEL-TOURAND *et al.* (1993). Some teratological aspects previously described in grapevine cultures, such as precocious differentiation of tracheary elements and the absence of a well-defined protodermic layer were also observed. This study describes the formation of secondary and tertiary embryos over long periods, directly from the epidermis of the hypocotyl region. Similar results were obtained by PASSOS (1991) using different genotypes and culture conditions. It appears that direct embryogenesis of unicellular origin is a valuable prerequisite for grapevine genetic transformation.

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## References

- FAURE, O.; 1990: Embryons somatiques de *Vitis rupestris* et embryons zygotiques de *Vitis* sp.: Morphologie, histologie, histochimie et développement. *Canad. J. Bot.* **68**, 2035-2315.
- ; AARROUF, J.; NOUGAREDE, A.; 1996 a: Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L.): Proembryogenesis. *Ann. Bot.* **78**, 23-28.

- -; - -; - -; 1996 b: Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L.): Embryonic organogenesis. *Ann. Bot.* **78**, 29-37.
- FINKELSTEIN, R. R.; CROUCH, M. L.; 1984: Precociously germinating rapeseed embryos retain characteristics of embryogeny. *Planta* **162**, 125-131.
- GOEBEL-TOURAND, I.; MAURO, M. C.; SOSSOUNTZOU, L.; MIGINIAC, E.; DELOIRE, A.; 1993: Arrest of somatic embryo development in grapevine: Histological characterization and the effect of ABA, BAP and zeatin in stimulating plantlet development. *Plant Cell Tiss. Org. Cult.* **33**, 91-103.
- GRAY, D. J.; MEREDITH, C. P.; 1992: Grape. In: F. A. HAMMERSCHLAG, R. E. LITZ (Eds.): *Biotechnology of Perennial Fruit Crops*, 229-262. CAB. International, Wallingford.
- HARST, M.; 1995: Development of a regeneration protocol for high frequency somatic embryogenesis from explants of grapevines (*Vitis* spp.). *Vitis* **34**, 27-29.
- KARNOVSKY, M. J.; 1965: A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27**, 137-138.
- MAURO, M.-C.; NEF, C.; FALLOT, J.; 1986: Stimulation of somatic embryogenesis and plant regeneration from anther culture of *Vitis vinifera* cv. Cabernet-Sauvignon. *Plant Cell Rep.* **5**, 337-380.
- MURASHIGE, T.; SKOOG, F.; 1962: A revised medium for rapid growth bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473-479.
- NITSCH, J. P.; NITSCH, C.; 1969: Haploid plants from pollen grains. *Science* **163**, 85-87.
- PASSOS, I. R. S.; 1991: Desenvolvimento de metodologia para a obtenção de embriogênese somática em videira. Master Thesis. Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo.
- REUSTLE, G.; NATTER, I.; 1994: Effect of polyvinylpyrrolidone and activated charcoal on formation of microcallus from grapevine protoplasts (*Vitis* sp.). *Vitis* **33**, 117-121.
- STAMP, J. A.; MEREDITH, C. P.; 1988: Somatic embryogenesis from leaves and anthers of grapevine. *Sci. Hortic.* **35**, 235-250.

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