

## Cryopreservation of embryogenic cell suspensions of the Spanish grapevine cultivars ‘Albariño’ and ‘Tempranillo’

M. E. GONZÁLEZ-BENITO<sup>1)</sup>, C. MARTÍN<sup>1)</sup> and J. R. VIDAL<sup>2)</sup>

<sup>1)</sup>Department of Plant Biology, Universidad Politécnica de Madrid, E.U.I.T Agrícola, Ciudad Universitaria, Madrid, Spain

<sup>2)</sup>Department of Plant Physiology, Facultad de Biología, Universidad de Santiago de Compostela, Santiago de Compostela, Spain

### Summary

**Embryogenic cell suspensions of two elite Spanish grapevine cultivars (‘Albariño’ and ‘Tempranillo’) were successfully cryopreserved by encapsulation-dehydration. The method implied the encapsulation of cells in alginate beads, subsequent culture in liquid medium with 1 M sucrose for 4 days, and desiccation for 2–4 h in the flow of a laminar-flow bench, before immersing in liquid nitrogen. With this simple method, up to 50 % of cell viability in the cryopreserved beads was measured (using the triphenyl tetrazolium method), which corresponded to vigorous growth of 100 % of beads after culture on semi-solid medium. The cryopreserved encapsulated cells were successfully used for initiation of new cell suspensions and their embryogenic capacity was studied. This cryopreservation method is an advance to store ready-to-use competent embryogenic tissue for grapevine genetic transformation projects.**

**Key words:** Cell cultures, desiccation, somatic embryogenesis, encapsulation, liquid nitrogen, *Vitis vinifera*.

### Introduction

Grapevine (*Vitis* sp.), the most important fruit crop grown worldwide, has been genetically transformed using biological (*Agrobacterium*) and physical (Biolistics) methods, and regenerated thereafter (MARTINELLI and MANDOLINO 1994, PERL *et al.* 1996, KIKKERT *et al.* 1996, VIDAL *et al.* 2003). Besides the breeding purpose for improving agronomic traits such as disease resistance (MAURO *et al.* 1995, YAMAMOTO *et al.* 2000, VIDAL *et al.* 2006), the genetic transformation of grapevine is nowadays also an indispensable tool to study gene function. In grapevine, the main plant material used as a target for genetic transformation and regeneration are somatic embryogenic cultures due to their high capacity to develop into plants (GRAY 1995). High regeneration frequency is important because stable transformation of grapevine cells occurs at relatively low frequency (KIKKERT *et al.* 1996, IOCCO *et al.* 2001). After the establishment of grapevine embryogenic suspensions from embryogenic callus (COUTOS-THEVENOT *et al.* 1992), cell suspensions were shown to be the most suitable plant material for grapevine genetic transformation (HEBERT *et al.* 1993, BORNHOF and HARST 2000). How-

ever, the establishment and maintenance of embryogenic cell suspensions in woody species, including grapevine, is particularly difficult (JAYASANKAR *et al.* 1999). The induction of embryogenic callus, subsequent production of cell suspensions and development of somatic embryos has been recently described for several Spanish cultivars (Vidal *et al.* 2009 a). Grapevine cell suspensions must be weekly subcultured, increasing risks of contamination and losing their morphogenic potential with time (KIKKERT *et al.* 2005). Therefore, the development of a protocol for cryopreservation of grapevine embryogenic cell suspensions would allow the possibility of keeping in storage embryogenic material to initiate cell suspensions at any time. The first report on grapevine cell suspension cryopreservation was on rootstock ‘41 B’ (*Vitis vinifera* ‘Chasselas’ x *Vitis berlandieri*) and up to 60 % viability was obtained using controlled cooling (0.5–1 °C/min; DUSSERT *et al.* 1991). More recently, embryogenic cell suspensions of *V. vinifera* cultivars have been cryopreserved: ‘Red Globe’ was cryopreserved following the encapsulation-dehydration method obtaining approximately 70 % viability (WANG *et al.* 2002), and other four cultivars, cryopreserved following the encapsulation-vitrification method, were evaluated with viability percentages ranging from 46 to 82 % after cryopreservation (WANG *et al.* 2004).

The goal of the present work was firstly to develop a user-friendly protocol for the successful cryopreservation of embryogenic cell suspensions of two Spanish grapevine cultivars, ‘Albariño’ (white grapes) and ‘Tempranillo’ (red grapes); and secondly, to study the initiation capability of suspensions and embryo development from cryopreserved cells.

### Material and Methods

**Plant material:** Embryogenic calli were obtained from anthers or ovaries of *Vitis vinifera* L. ‘Albariño’ and ‘Tempranillo’, cultured on semi-solid medium MS (MURASHIGE and SKOOG 1962) supplemented with 4.52 µM 2,4-D + 4.52 µM BAP and incubated in the dark at 25 °C (VIDAL *et al.* 2009 a). Cell suspensions were initiated by transferring approx. 200 mg of embryogenic callus to liquid medium GM+NOA (MS salts and vitamins + 4.6 g·l<sup>-1</sup> glycerol + 18 g·l<sup>-1</sup> maltose + 5 µM β-naphthoxyacetic acid -NOA, pH 5.8) and incubated at 25 °C in the dark in a shaker at 120 rpm. Media were refreshed weekly (VIDAL

*et al.* 2009 a) and filtered through a 1.0 mm<sup>2</sup> pore size mesh three days prior to initiate the cryopreservation procedure. Similar medium, used during the procedure, but without the growth regulator (NOA), will be referred as GM. In all semi-solid media subsequently used, pH was adjusted before adding the gelling agent (Phytigel) and autoclaving; and media were dispensed in sterile 9 cm diameter Petri dishes.

**E m b r y o g e n i c c e l l s u s p e n s i o n s c r y o p r e s e r v a t i o n :** Cell suspensions of cultivars ‘Albariño’ and ‘Tempranillo’ were used in the cryopreservation experiments three days after refreshment of liquid medium. The suspensions were sieved through a 70 µm mesh and cells were encapsulated in alginate beads as follows: cells were suspended in GM liquid medium containing 0.4 M sucrose and 2.5 % (w/v) Na-alginate (proportion of 1 g fresh weight of cells: 3 ml alginate solution). The mixture was dripped onto GM medium with 0.4 M sucrose and 100 mM CaCl<sub>2</sub>, and left 30 min to form beads. Fifty-five to 65 beads were formed per gram of embryogenic cells. The beads were then cultured in GM+NOA liquid media with increasing sucrose concentration (0.25, 0.5, 0.75, 1 M, one day on each step), with approximately 150 beads in 50 ml of medium, incubated in the dark in a gyratory shaker (120 rpm). The effect of culture for 1 or 4 d in liquid GM+NOA medium supplemented with 1 M sucrose was also studied in ‘Tempranillo’. After preculture in sucrose-rich medium, beads were surface dried with sterile filter paper and dehydrated in the air flow of a laminar-flow cabinet on top of open Petri dishes, at room temperature, for different periods. In the cryopreserved treatments (+LN), beads were then included in cryovials (10 beads/ 2 ml cryovial) and plunged into liquid nitrogen (LN) for at least one hour. Rewarming took place in a water bath at 40 °C for 2 min. Beads were then placed on semi-solid GM+NOA medium supplemented with 3 g·l<sup>-1</sup> Phytigel and 2.5 g·l<sup>-1</sup> activated charcoal (AC), and incubated in the dark at 25 °C. In the different experiments, three replicates of ten beads each were used per treatment and cultivar. Viability was determined 4 d after culture with the triphenyltetrazolium chloride test (TTC) and expressed as the percentage of reduction of TTC over control beads (cultured for 4 days immediately after encapsulation). For the TTC test, the method of STEPONKUS and LAPHEAR (1967) was followed with some modifications: 4 ml of 0.6 % (w/v) TTC solution in 0.05 M Tris buffer (pH = 7.5) were added to 10 beads and incubated at 30 °C in darkness for 24 h; after removing the solution and washing the beads with distilled water, 5 mL 95 % ethanol were added; formazan was extracted in a boiling water bath for 4 min; after letting cool, 95% ethanol was added to make up to 10 mL volume and mixed; tubes were centrifuged at 1000 rpm for 5 min; finally, supernatant was taken and absorbance measured at λ = 530 nm. Water content of beads was measured by the gravimetric method, drying the beads in an oven at 85 °C for 3 d, and expressed in a fresh weight basis (fwb; three repetitions of 15 beads each).

**I n i t i a t i o n o f c e l l s u s p e n s i o n s a n d e m b r y o p r o d u c t i o n f r o m c r y o p r e s e r v e d c e l l s :** Beads of ‘Albariño’ and ‘Tem-

pranillo’ cells were prepared and cultured for 4 d on 1 M sucrose GM + NOA liquid medium, as stated previously. Three sequential steps of the cryopreservation protocol were studied. Control beads (after the sucrose culture), desiccated beads (after 4 h desiccation) and cryopreserved beads (after similar pretreatments and stored in LN for at least 1 h) were used to study their capability as starting material for “de novo” suspensions re-growth and later embryo development. After the sequential treatments, beads were first cultured for 1 wk on semi-solid GM+NOA+AC medium, and then for 7 additional weeks on semi-solid GM + NOA medium. Beads were then studied for cell suspension production capability: 1 bead was cultured in 15 ml of liquid GM+NOA medium (3 repetitions per treatment and cultivar), in a shaker at 120 rpm with incubation at 25 °C in the dark (day 0). Medium was refreshed weekly (removing half of the volume and adding the same volume of fresh medium) twice. On the third week, medium was added to double the initial volume (30 ml). One week later, the suspensions were filtered through a 70 µm mesh and the total fresh weight was measured. Regrowth was determined by calculating the increase of fresh weight per gram of bead over 28 d culture.

Simultaneously, to study the capability of encapsulated cells (beads) to allow the development of somatic embryos, two beads were cultured per Petri dish, at day 0, on embryo development medium (MS½ + AC: MS half strength salts and full strength vitamins, with 3 g·l<sup>-1</sup> AC, 30 g·l<sup>-1</sup> sucrose and 2.5 g·l<sup>-1</sup> Phytigel). Three repetitions were used per treatment and cultivar. Cultures were incubated at 25 °C in darkness. After four weeks, well formed embryos of at least 1 cm length were counted, transferred to the same fresh medium (approx. 20 embryos per 9 cm Petri dish), and incubated at 25 °C with indirect 10 µmol·m<sup>-2</sup>·s<sup>-1</sup> irradiance from cool white fluorescent tubes and 16 h photoperiod. Four weeks later, the number of germinated embryos (with expanded cotyledons) was counted.

## Results

**E m b r y o g e n i c c e l l s u s p e n s i o n s c r y o p r e s e r v a t i o n :** The effect of a step-wise preculture in increasing sucrose concentration (0.25, 0.5, 0.75, 1 M, one day on each step) was explored studying the viability of cells after cryopreservation when the final sucrose concentration was 0.75 M or 1 M followed by 2 to 6 h desiccation (Fig. 1). The detrimental effect of the increasing sucrose concentration steps was observed as viability was ca. 43 % of that of control beads (no treatment) for ‘Tempranillo’ (both 0.75 and 1.0 M), and 67-38 % for ‘Albariño’ (0.75 and 1.0 M, respectively). In both cultivars, low viability percentages after cooling were observed at all desiccation treatments studied (10-30 %) but not significantly different from non-cooling treatments (Fig. 1). There was not a significant difference of the viability of cryopreserved beads between using a final concentration of 0.75 M and 1 M sucrose, in both cultivars.

A longer preculture period on 1M sucrose liquid medium (4 d vs. 1 d) was studied for ‘Tempranillo’ without the

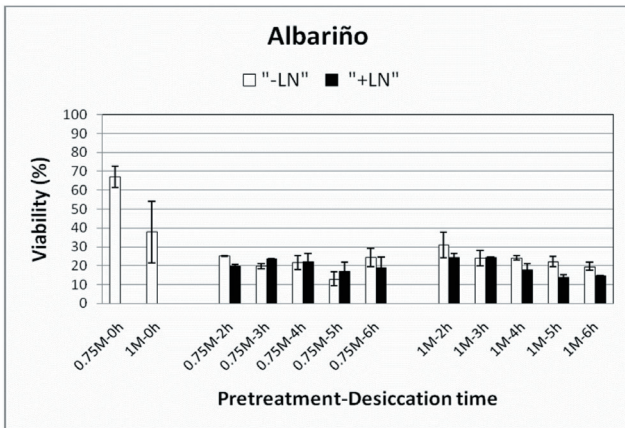
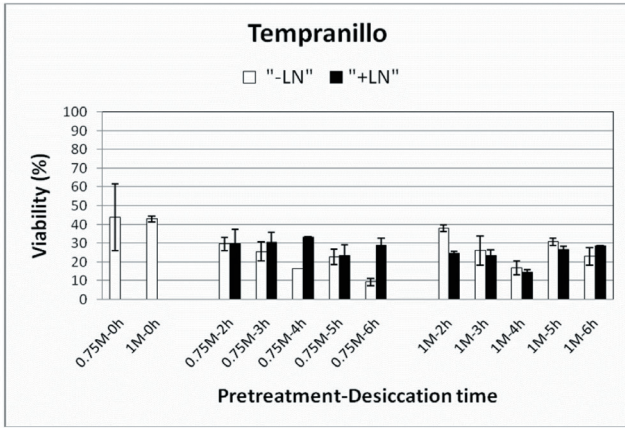


Fig. 1: Viability (measured by TTC test as percentages of the values of control beads) of encapsulated embryogenic cells of cultivars ‘Tempranillo’ and ‘Albariño’, precultured in increasing sucrose concentration media (0.25, 0.5, 0.75, 1 M, one d on each step, up to 0.75M or 1 M), subsequently desiccated in the laminar flow bench for different periods (2 to 6 h) and cryopreserved (+ LN) or not (- LN). Bars = standard deviation.

step-wise increase of sucrose concentration (Fig. 2). The longer preculture period improved viability after cooling for desiccation periods of 4 h or longer, which corresponded to water contents of approximately 20 % fwb (Fig. 2 inset). The effect of 4 d preculture in 1 M sucrose was further studied in ‘Tempranillo’ and ‘Albariño’ with 2 and 4 h desiccation. There was not a significant difference in the viability obtained after cooling with the two desiccation periods in both cultivars (Fig. 3); however, ‘Albariño’ was more affected by desiccation than ‘Tempranillo’. Although viability, determined by the TTC test, after cryopreservation was between 36 % and 50 %, all cryopreserved beads (100 %) of both cultivars showed vigorous growth after eight weeks in culture (Fig. 4).

**Initiation of cell suspensions and embryo development from cryopreserved cells:** The initial fresh weight of the beads (just after encapsulation) was  $29.0 \pm 1.5$  mg with no significant difference between cultivars (data not shown). After 8 week culture on semi-solid medium, following the three protocol steps studied (4 days in 1 M sucrose culture, 4 h-desiccation, and cryopreservation), re-growth was higher in ‘Tempranillo’ than in ‘Albariño’ (Tab. 1). Further-

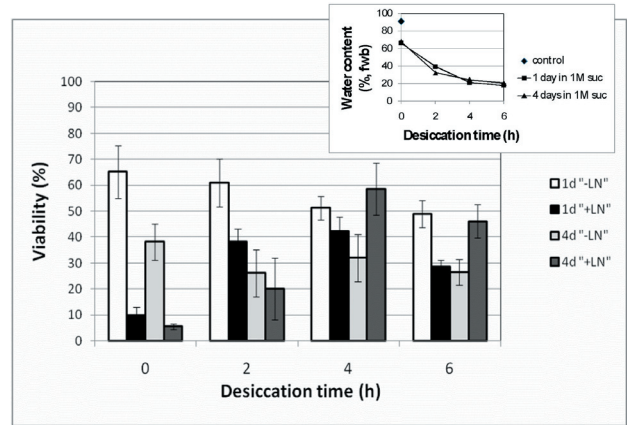


Fig. 2: Viability (measured by TTC test as percentages of the values of control beads) of encapsulated embryogenic cells of ‘Tempranillo’, after 1 or 4 d culture in 1 M sucrose, subsequent desiccation in the laminar flow bench for different periods and cryopreserved (+ LN) or not (- LN). Inset: water content of beads (fwb). (Bars = standard deviation).

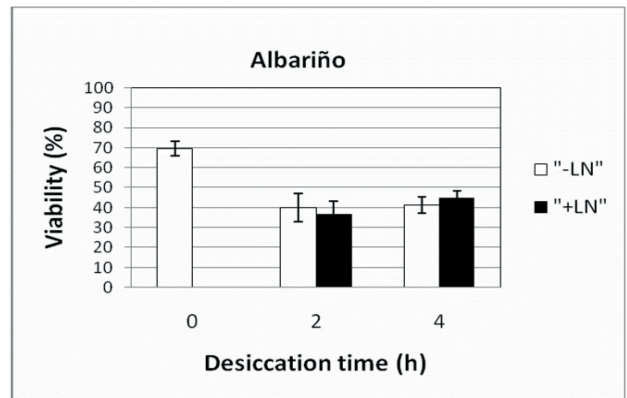
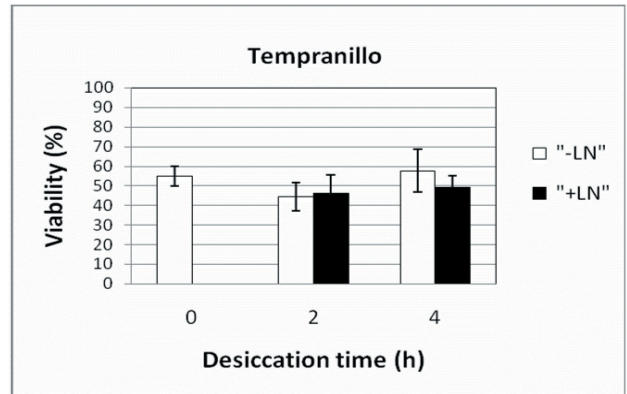


Fig. 3: Effect of desiccation time (2 and 4 h) on survival of encapsulated cells of ‘Tempranillo’ and ‘Albariño’ without or after cryopreservation (beads had been cultured 4 d in 1 M sucrose previously to desiccation). Bars = standard deviation.

more, desiccated and cryopreserved beads showed a more intensive growth than beads only cultured in sucrose-rich medium. This growth trend was maintained after further 4 week culture in liquid medium, which strongly favoured the growth of cryopreserved cells (Tab. 1).

The number of emerged embryos per gram of bead 4 weeks post culture of beads on embryo development medi-

Table 1

Re-growth of encapsulated embryogenic cell cultures of ‘Tempranillo’ and ‘Albariño’, which had been subjected to culture for 4 d on 1 M sucrose medium (0 h), followed by 4 h desiccation (4 h) and (when cryopreserved) immersion in liquid nitrogen (4 h + LN), after 8 week culture on semisolid medium, and after further 4 weeks in liquid GM medium. Re-growth: fresh weight increase (g) per g of fresh weight of bead. For each cultivar and regrowth medium, means with the same letter are not significantly different according to Duncan’s multiple test,  $p < 0.05$

	Regrowth after 8 weeks on semi-solid medium (mean $\pm$ SD)			Regrowth after further 4 weeks in liquid medium (mean $\pm$ SD)		
	0 h	4 h	4 h + LN	0 h	4 h	4 h + LN
	Tempranillo	3.36 $\pm$ 0.36 c	4.70 $\pm$ 0.42 b	6.39 $\pm$ 0.29 a	8.24 $\pm$ 1.67 b	17.45 $\pm$ 3.38 b
Albariño	2.16 $\pm$ 0.52 b	3.17 $\pm$ 0.37 a	3.62 $\pm$ 0.51 a	6.72 $\pm$ 1.60 b	14.38 $\pm$ 2.39 a	18.75 $\pm$ 2.81 a

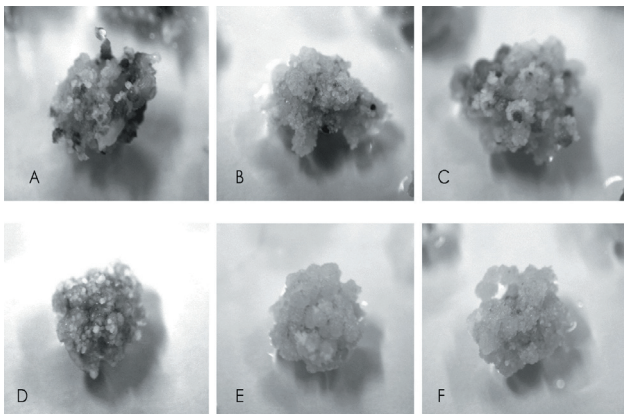


Fig. 4: Cell mass growth from beads of encapsulated embryogenic cells of ‘Tempranillo’ (A-C) and ‘Albariño’ (D-F) 8 weeks after initiation of bead culture, following 1M sucrose culture for 4 d (A, D), subsequent 4 h desiccation (B, E) and immersion in liquid nitrogen for 1 h (C, F).

um was similar in all treatments for ‘Tempranillo’ (Tab. 2). However, in ‘Albariño’, the number of embryos was reduced after desiccation and cryopreservation, although this figure was higher in ‘Albariño’ than in ‘Tempranillo’ (Tab. 2, Fig. 5). In only one month after germination induction a high number of germinated embryos were obtained. The rate of germinated embryos per emerged embryo ranged between 16 and 36 % for ‘Albariño’ and was in the range of 33 % for ‘Tempranillo’.

### Discussion

In the present study, a simple method for cryopreservation of embryogenic cell suspensions of two international cultivars of grapevine has been developed. Among the methods for cryopreservation, those based on the vitrification of extra- and intracellular solutions (ENGELMANN 2004; GONZÁLEZ-BENITO *et al.* 2004) have the advantage of not requiring expensive devices to decrease temperature gradually, as it is the case in the controlled cooling method developed for grapevine cell suspensions (DUSSERT *et al.* 1991). The cryopreservation of grapevine embryogenic cell suspensions has been previously described using two methods based on the vitrification of solutions: encapsulation-dehydration (WANG *et al.* 2002) and encapsulation-vitrification (*sensu stricto*; WANG *et al.* 2004). The experi-

ence in our laboratory using a encapsulation-vitrification method by desiccating beads with PVS2 (“Plant Vitrification Solution 2”; SAKAI *et al.* 1990) showed that the three cultivars tested (‘Tempranillo’, ‘Garnacha’ and ‘Sultani-na’) in a preliminary study proved to be highly sensitive to the vitrification solution (results not reported here). This sensibility was not found in the rootstock ‘110 Richter’, studied by WANG *et al.* (2004). Therefore, the encapsulation-dehydration method was explored in our laboratory. We modified the method developed by WANG *et al.* (2002) in order to obtain high viability of the cell suspensions after cryopreservation and also to make it more “user-friendly”. We confirmed the usefulness of preculture of cell suspensions in high-sucrose liquid medium after the cells were encapsulated (WANG *et al.* 2002) by the other genotypes and treatment durations.

In the present study, similar viability percentages after cryopreservation were obtained with a step-wise increase of sucrose concentration (to final concentration of 1 M) compared to preculture in 1 M sucrose for 1 day. Both ‘Tempranillo’ and ‘Albariño’ embryogenic cells showed sensibility to sucrose treatment as, even with the lower concentration tested (0.25 M) viability was lower than that of the control beads (relative 100 %; data not shown). Previous studies with cell suspensions (BACHIRI *et al.* 1995; WANG *et al.* 2002) had shown that preculture in 1 M sucrose for longer periods increased viability after cooling. Similarly, viability of ‘Tempranillo’ embryogenic cells increased after cooling when 4 days culture in 1M sucrose was used, instead of 1 d. We also found that a sucrose-rich preculture period of 4 d in 1M sucrose allowed nearly 50 % viability after cryopreservation (measured by the TTC test) in both cultivars. This percentage of viability with the TTC test allowed the vigorous growth of all (100 %) cryopreserved beads, which were able to promote cell growth in semi-solid medium.

In the present study, the morphogenic potential of cryopreserved grapevine cells either increased (‘Tempranillo’) or decreased (‘Albariño’) compared to non-cryopreserved non-desiccated encapsulated beads. These two types of responses have been previously reported in the literature, *i.e.* no change in the embryogenic potential (SAKAI *et al.* 1991, NISHIZAWA *et al.* 1993) or decrease (GUPTA *et al.* 1987). WANG *et al.* (2002) found that cell suspensions of grapevine ‘Red Globe’ increased their embryogenic potential after cooling to LN temperature. This response has been

Table 2

Number of developed embryos per g of bead after 4 week culture, and percentage of germinated embryos after 4 additional weeks. Beads had been previously subjected to sequential treatments [4 d culture in 1 M sucrose (0 h), 4 h desiccation (4 h + NL)] and cultured for 8 weeks on semi-solid medium. For each cultivar and evaluation type, means with the same letter are not significantly different according to Duncan's multiple test,  $p < 0.05$ .

Nd: no data

	No. developed embryos/grams of beads (mean $\pm$ SD)			% Germinated embryos/developed embryos (mean $\pm$ SD)		
	0 h	4 h	4 h + LN	0 h	4 h	4 h + LN
	Tempranillo	124 $\pm$ 48 a	157 $\pm$ 31 a	104 $\pm$ 19 a	Nd	33 $\pm$ 10 a
Albariño	400 $\pm$ 74 a	216 $\pm$ 26 b	281 $\pm$ 84 b	18 $\pm$ 5 a	36 $\pm$ 24 a	16 $\pm$ 11 a

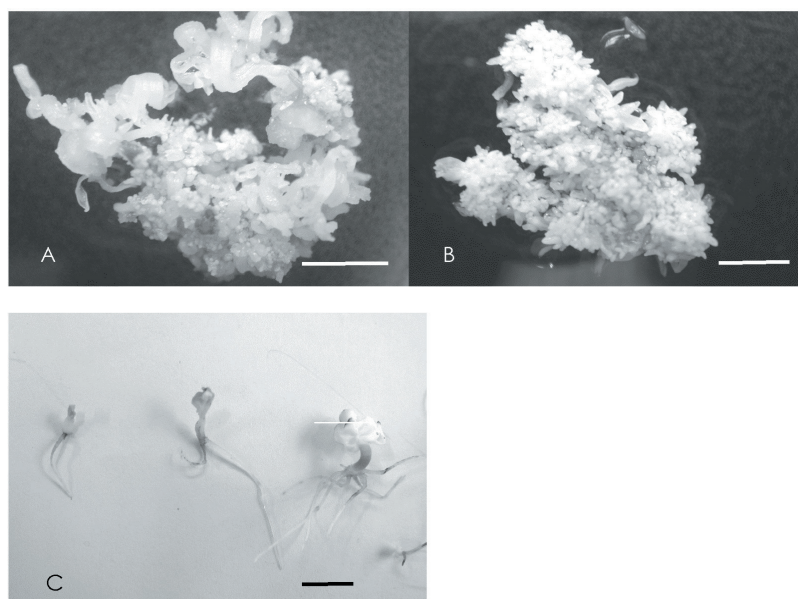


Fig. 5: Emergence of embryos after 4 week culture on development medium of cryopreserved beads of cultivars 'Tempranillo' (A) and 'Albariño' (B). (C) Emerged somatic embryos of 'Albariño' at different development stages. Bars = 0.5 cm.

related to a cell selection during cryopreservation procedure, during which the less embryogenic cells (more differentiated) would be more prone to be damaged, as some histological studies have demonstrated (HAGGMAN *et al.* 1998), thus facilitating the regrowth of cells with embryogenic capacity.

We have developed a simple method for the cryopreservation of cell suspensions of two grapevine cultivars ('Tempranillo' and 'Albariño') using encapsulation-dehydration, with nearly 50% viability measured by the TTC test, which corresponded to a vigorous cell growth in 100 % of the beads after recovery in semi-solid medium. After a culture period of 8 weeks on semi-solid recovery medium, those cryopreserved cells were able to initiate re-growth of cell suspensions and to promote the development of somatic embryos. This user-friendly cryopreservation protocol for embryogenic cell suspensions of grapevine should be very useful for genetic transformation projects, such as molecular breeding purposes or functional genomics studies (VIDAL *et al.* 2009 b). The embryogenic cultures could be initiated and safely stored by cryopreservation until the transformation experiments are performed, without losing their morphogenic capacity.

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