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BRIEF COMMUNICATION

Improved cryopreservation procedure for long term storage of synchronised culture of grapevine

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Abstract

Anther-derived pre-embryogenic masses (PEMs) of callus, established *via* suspension cultures, were encapsulated to form synthetic seeds suitable for cryopreservation. The synchronised suspension culture proliferation necessitated the optimisation of plant growth regulators for different cultivars. The growth phase and density of the culture were also important as well as the exposure of cells to vitrification solution containing 0.75 M sucrose with 0.1 M $CaCl_2$ and 2.0 % sodium alginate (pH 5.7). Pre-treatment of the encapsulated cells for 2 d with Nitsch and Nitsch (NN) medium containing 0.75 M sucrose solution followed by dehydration for 4 h in a laminar flow box provided maximum cell viability, which varied from 0 to 40 %. The embryo proliferation from the cryopreserved beads involved warming them and then transfer to NN medium containing glutamine (50 mg dm^{-3}) and activated charcoal (2.5 %). The maximum number of embryos obtained was 31 - 53 per bead. Subculture into the same medium induced secondary embryogenesis, which was initiated from the meristematic region, radicle, and root cap. Proliferation and maturation of secondary embryos was faster than of primary embryos. No phenotypic variation or abnormal structures compared to the control were observed in the regenerated plantlets.

Additional key words: encapsulated cells, glutamine, somatic embryogenesis, sucrose, suspension culture, vitrification.

Genetic improvement of grapevine cultivars by conventional breeding is severely limited due to their high heterozygosity and long juvenile period (Wang *et al.* 2002). Genetic transformation is an alternative method to improve grapevine, but rely on specialised procedures, such as the development and maintenance of somatic embryogenic cell cultures. Browning of cell suspension cultures can result in a total loss of the embryogenic cells in a liquid medium (Bornhoff and Harst 2000). The establishment of synchronised somatic embryo development in embryogenic suspension cultures is a crucial step to obtaining high production potential and provides a means to investigate the somatic embryogenesis process. However, there is still only limited information available for grapevines (Jayasankar *et al.* 2003). In addition, retention of morphogenetic potential by embryogenic cell suspensions is low (Jayasankar *et al.* 1999). Furthermore, embryos produced by liquid culture often yield abnormal plants (Srinivasan and Mullins 1980, Bornhoff and Harst 2000).

Cryopreservation in liquid nitrogen offers great potential for long-term preservation of plant material (Sakai *et al.* 1990). Furthermore, the storage of dehydrated somatic embryos is a simple and inexpensive method of clonal germplasm preservation (Jayasankar *et al.* 2005). However, embryogenic suspension cultures of grapevine have not been extensively tested and optimised for cryopreservation. Therefore, the aim of this study was the development of an effective long-term storage method for embryogenic cell-lines from various grapevine cultivars.

Somatic embryogenic callus was initiated from anthers from the grapevine (*Vitis vinifera* L.) cultivars Sultana, Redglobe, Merlot, Roobernet, Dauphine and

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Abbreviations: BA - benzyladenine; MES - (N-morpholino)ethanesulfonic acid; MS - Murashige and Skoog's medium; NN - Nitsch and Nitsch medium; NOA - 2-naphthoxyacetic acid; PEMs - pre-embryogenic masses.

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Chardonnay according to Franks *et al.* (1998) on modified medium (Vasanth *et al.* 2010). Pre-embryogenic masses (PEMs) were established using a modified Nitsch and Nitsch (1969, NN) liquid medium containing 0.5 g dm^{-3} 2-(N-morpholino) ethanesulfonic acid (MES), 18.6 g dm^{-3} maltose and 4.6 g dm^{-3} glycerol (Coutos-Thévenot *et al.* 1992). The medium was supplemented with 2-naphthoxyacetic acid (NOA; 1.0 mg dm^{-3}) and benzyl-adenine (BA; 0.50 mg dm^{-3}). For the initiation of suspension cell culture, 0.25 - 1.0 g dm^{-3} of embryogenic callus (3-week-old) was transferred into a 500 cm^3 Erlenmeyer flask containing 100 cm^3 of medium. Suspensions were incubated in the dark on an orbital rotary shaker (90 rpm) at 24 °C, and maintained by weekly exchange of 50 % of the old medium with fresh medium (Ben Amar *et al.* 2007). When the cell density increased, the suspensions were divided into two 500 cm^3 flasks (100 cm^3 of suspension plus 30 cm^3 of fresh medium) or brought to a final volume of 200 cm^3 in a 500 cm^3 Erlenmeyer flask by adding 100 cm^3 of fresh medium.

The approach of Wang *et al.* (2002) was followed to obtain a synchronised suspension cultures in linear growth phase. Cultures were allowed to settle to the

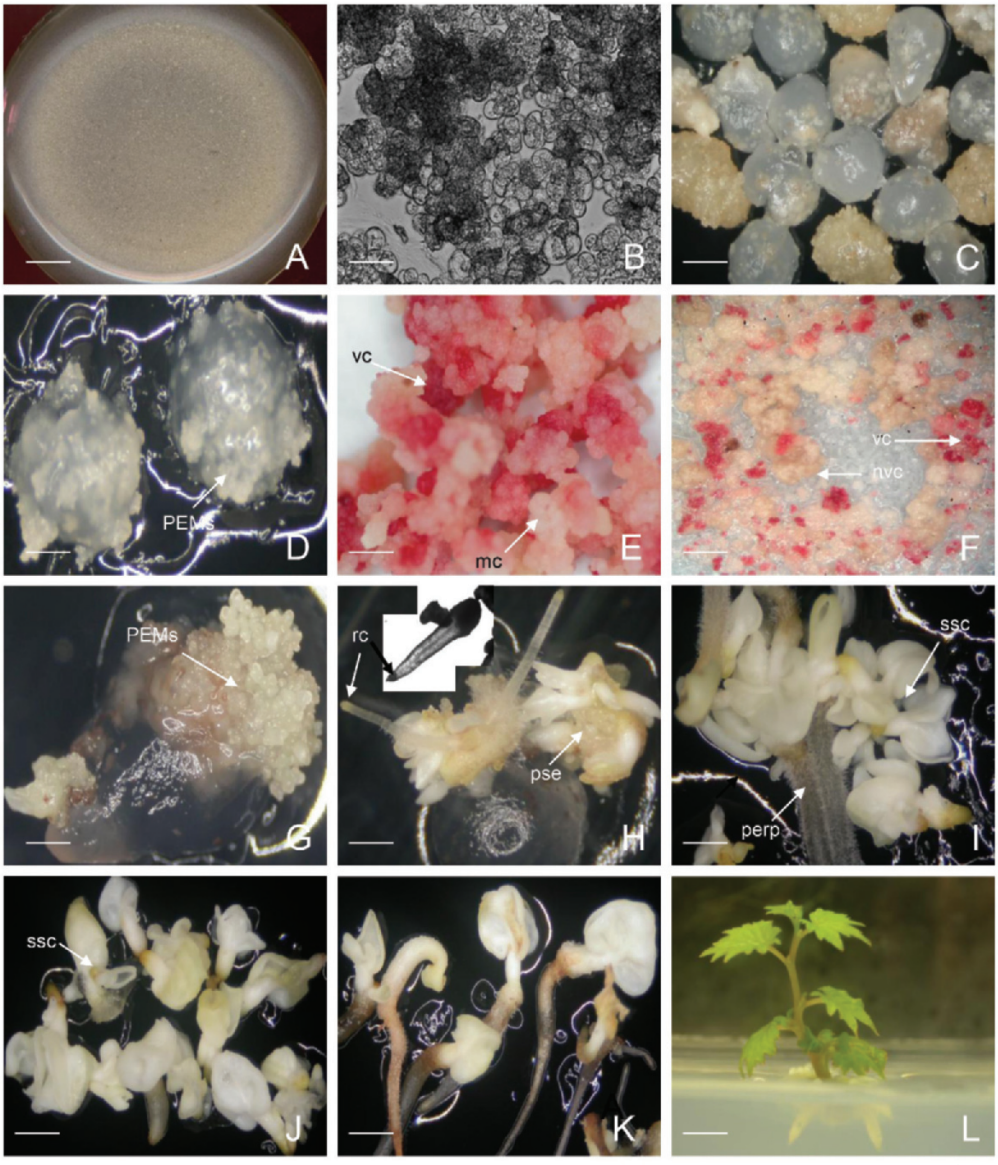

Fig. 1. Somatic embryogenesis after cryopreservation. *A* - Synchronised suspension culture. *B* - Synchronised culture growth. *C* - Sodium alginate encapsulated beads (control). *D* - Cryopreserved beads after re-warming showing somatic embryo initiation. *E* - Control bead viability. *F* - Cryopreserved bead viability. *G* - PEM initiation from beads at the third week of sub-culture. *H* - Primary embryogenesis. *I* - Secondary somatic embryos from the radical portion. *J* - Embryo maturation and regeneration. *K* - Germinated embryo with shoot and roots. *L* - Plantlet formation (mc - mature cells, nvc - non-viable cells, PEMs - pre-embryogenic masses, perp - primary embryo root, pse - primary embryogenesis, rc - root cap, ssc - secondary somatic embryos, vc - viable cells).

bottom of the Erlenmeyer flasks, then the entire liquid medium was removed and the culture medium replaced with post culture media containing various concentrations of sucrose (0.25, 0.5, 0.75 and 1.0 M), with subsequent culturing for 12 h. The cell density was adjusted by removing surplus medium. Finally 1.0 cm^3 of the precultured cells was suspended in 2 cm^3 of 2.0 % (m/v) Na-alginate, 0.75 M sucrose and 2 M glycerol at pH 5.7. The solution was then dropped slowly using a sterile 10 cm^3 pipette into 0.1 M $CaCl_2$ solution containing 0.75 M sucrose and 2 M glycerol (20 cm^3 solution) in an Erlenmeyer flask at room temperature and left for 5 min, to form beads (about 4 mm in diameter). When the above method was followed, each bead contained about 25 % cells according to Bachiri *et al.* (1995). The beads were then rapidly surface dried by blotting on cellulose tissue and directly dehydrated with plant vitrification solution (PVS_2, Sakai *et al.* 1990). Some of the encapsulated cells were osmoprotected with the loading solution containing 2 M glycerol and 0.75 M sucrose at 27 °C for 0 to 120 min before dehydration with PVS_2. After dehydration, the beads were rapidly surface-dried by blotting on cellulose tissue and then 25 beads were transferred into a 10 cm^3 cryotube and immersed directly in liquid nitrogen. Later the solution was removed and the beads were post cultured for 2 d in NN liquid medium containing 0.75 M sucrose. After 2 d, the sucrose solution was removed and the beads were rapidly surface dried by blotting with *Whatman* filter paper and placed in a sterile Petri dish in the laminar flow chamber (LFC). In the above method, the calcium chloride solutions were prepared at various concentrations in liquid NN medium containing the same growth regulators as mentioned before.

The water content of the beads was measured by drying them for 0 - 7 h and the final mass measured. Recoveries were assessed in terms of the percentage of samples that underwent re-growth, and the pre-embryonic mass (PEM) regenerated from the cryo-stored units. Viability was determined using the triphenyltetrazolium chloride (TTC) reduction assay according to Steponkus and Lanphear (1967). Cryo-tubes removed from liquid nitrogen were allowed to thaw rapidly in a water bath at 40 °C for 3 min. Later, 25 beads were post-cultured at 27 °C in the dark in a Petri dish containing 40 cm^3 of solidified (0.3 % *Gelrite*) NN medium with NOA (1.0 mg dm^{-3}), BA (0.50 mg dm^{-3}), glutamine (25 - 75 mg dm^{-3}) and activated charcoal (2.5 g dm^{-3}). The cryopreserved cells were analyzed for viability after storage for 1 h, 1 week, 6 months and 1 year. The beads were also tested for re-growth on 0.6 % (m/v) TTC solution containing 0.75 M sucrose and potassium phosphate buffer (pH 7.5) at 27 °C for 24 h. Viability was measured after post-culture and expressed as the percentage reduction in TTC activity over the control. Re-growth was determined by calculating the number of beads responding and the number of embryos generated over 15-d post-culture on solid medium at 25 ± 2 °C in the dark.

When embryogenic callus was maintained in solid culture and subcultured over a long period, the embryogenic potential was reduced, particularly after more than three years and cultures produced large amounts of polyphenols. Furthermore precocious embryo germination and re-callusing was observed (data not shown). The friable and white granular calli, which make up the globular embryos, were chosen for the establishment of embryogenic suspension cultures. PEMs were transferred from solid culture into fresh liquid medium and a suspension culture was established within two months (Fig. 1*A*). The cell density necessary for cell division was reached after 2 weeks (Fig. 1*B*).

Table 1. Changes in the water content of the encapsulated cells after the second-step preculture in 0.75 M sucrose (control - sodium alginate beads without cells) in the laminar flow chamber. Effect of various sucrose concentrations on the viability of encapsulated cells. Cryopreserved beads re-growth in response to different glutamine concentrations. The experiments were conducted thrice with 3 replications per treatment

	Sultana	Redglobe	Merlot
Time [min]	water content [% of control]		
60	100	100	100
120	61.82	57.00	78.68
180	45.45	50.25	65.00
240	41.82	46.11	52.75
300	40.00	41.65	36.40
360	38.18	32.64	28.16
420	38.18	28.24	28.16
480	38.18	28.24	28.16
Sucrose [%]	viability [%]		
0.00	77.83±7.02	90.51±7.71	65.80±8.50
0.25	66.20±8.74	83.08±6.56	59.05±8.89
0.50	31.94±4.04	72.95±7.51	29.60±4.04
0.75	15.97±2.08	37.82±5.28	11.64±4.58
1.00	1.55±1.15	10.13±3.21	0.43±0.00
Glutamine [mg dm^{-3}]	number of embryos [$bead^{-1}$]		
0	28.66±3.06	16.55±2.00	37.66±5.00
25	45.33±5.00	35.66±3.52	46.66±3.09
50	48.22±5.24	43.88±5.08	52.33±6.11
75	41.11±2.16	40.77±2.44	50.44±2.18

An increase in the duration of the post-culture enhanced the viability of the dehydrated and encapsulated beads compared to the control; the viability was 77.83 % for Sultana, 90.51 % for Redglobe, 65.80 % for Merlot and less for Roobernet, Dauphine and Chardonnay (data not shown). The cell viability thus ranged from 83.08 to 0.43 % depending on the cultivar and the sucrose concentration. Using this approach, it was possible to determine the optimum sucrose concentrations for embryo production and cryopreservation for the cultivars Sultana, Redglobe and Merlot (Table 1). It was found that

0.75 M sucrose gave the best embryo survival. If the concentration was higher than 1.0 M, then the cell suspension did not undergo embryo formation. On the other hand, exposure to an insufficient sucrose concentration led to a decrease in viability. The appropriate concentration of sucrose avoids crystallization of water within the cells, which causes injury during freezing. It also stabilizes membranes (Fahy *et al.* 1984). The optimal dehydration treatments for different plant species vary considerably (Engelmann and Takagi 2000, Takagi 2000).

The viability of the cryopreserved beads is further influenced by the post-culturing conditions. We found that stepwise preculture and recovery plating, as well as the use of semi-solid instead of liquid medium, were essential for preserving embryo viability. It was found that the competence of the somatic embryos that had undergone cryoprotection was comparatively higher than those that had not been treated. Encapsulation was carried using 2 % sodium alginate in 0.1 M $CaCl_2 . 2\ H_2O$ and 5 min of polymerization (Fig. 1*C*). If a lower concentration of sodium alginate is used, then the beads dissolved in post culture medium, so a short treatment time of 5 min was used instead of a standard 0.1 M $CaCl_2$ treatment for 30 min.

The initial water content of the beads (expressed as % of the control) was 61.8 % for Sultana, 57 % for Redglobe and 78.6 % for Merlot, on a fresh mass basis. On dehydration, this rapidly decreased to 40.0, 41.6 and 36.4 %, respectively after 4 h and continued to decrease further (Table 1).

Beads were placed in fresh medium for re-hydratation and after 4 h cells started to slowly emerge from beads. Because of a slow initial growth of the cells, all the cultivars needed two subcultures. Sucrose pre-treatment is generally effective in enhancing cell growth after the re-initiation of cultures (Table 1). An inappropriate sucrose concentration, the post culture conditions and the dehydration state of the cryopreserved beads may result in the immediate death of the tissue or complete blackening of the beads. In the vitrification-based cryopreservation, the cells have to be dehydrated by exposure to a high concentrated solution prior to immersion in liquid nitrogen. However, in the present study, no cell re-growth of Redglobe was found using plant vitrification solution, which is similar to the findings of Wang *et al.* (2004), who used cv. Richter 110.

When Sultana, Redglobe and Merlot cryopreserved beads were transferred to culture media; embryogenic callus proliferation increased and produced large numbers of embryos within 15 d (Fig. 1*G*). The re-growth medium consisting of NN macroelements, MS microelements and B_5 vitamins and supplemented with NOA (1.0 mg dm^{-3}), BA (0.5 mg dm^{-3}), activated charcoal (2.5 g dm^{-3}) and *Gelrite* (0.3 %) proved to be best and gave the highest frequencies of somatic embryos (Fig. 1*H*). This medium enhanced the production of primary embryos, which were subsequently used for the production of secondary embryos. The liquid media tested reduced viability compared to solid media (data not shown); similar results have been reported by Wang *et al.* (2002).

Embryogenic callus was formed and covered the outside surface of the beads (Fig. 1*D*) and the viability of cells was proved by the TTC method (Fig. 1*E*). High capacities for synchronised somatic embryo development were observed 2 weeks after induction and then the PEMs were transferred to new NN medium. The PEMs showed the ability to produce globular embryos and, in addition, heart, early torpedo and late torpedo stages were observed after about 4 weeks in fresh medium. The mature embryos obtained from cryopreserved clumps produced by all cultivars were cultured NN medium containing activated charcoal (2.5 g dm^{-3}). However, for Merlot, the re-establishment medium contained NOA (0. 5 mg dm^{-3}), BA (0.5 mg dm^{-3}), glutamine (50 mg dm^{-3}) and activated charcoal (2.5 g dm^{-3}). After the second subculture in new medium, secondary somatic embryos were generated after 2 weeks (Fig. 1*I*). There were no morphological differences between the three cultivars and all the cultivars recorded an adequate re-growth percentage. However, somatic embryo induction and conversion efficiency were different (Table 1).

Elongated embryos, when transferred onto fresh medium for germination, started to form green shoot-like structures (Fig. 1*K*) without any intermediary step. Generally, plantlets were obtained within 3 months from all the cultivars tested. Subculturing was done on the same medium as for secondary embryogenesis. The percentage viability of the cryopreserved cells after cryoprotection was higher than that of the controls. In addition, the mean number of embryos that developed from cryopreserved clumps after a 15 d exposure was relatively higher than that of the controls, indicating that cryopreservation improved morphogenic competence.

Established plantlets with expanded primary leaves were cultured on MS medium for the induction of lateral roots (Fig. 1*L*). The survival rate of plantlets was shown to be above 90 % which is similar to what was reported for other species (Ray and Bhattacharya 2010).

In this paper, an improved protocol for the cryopreservation of grapevine was presented. Cultivar-specific optimisation was necessary, especially with regards to the optimum concentration of osmotics when preparing the cryo-beads. The cryopreserved cells retained their potential for re-growth, followed by embryo regeneration and development. Cryopreservation is appropriate for the conservation of individual genotypes of specific importance. The use of these *in vitro* techniques could improve the production processes for elite genotypes of grapevine (Rajasekaran and Mullins, 1979, Srinivasan and Mullins 1980, Faure *et al.* 1996). Synthetic seeds have multiple advantages over organogenesis for propagation, which include better handling, potentially easier long-term storage, a greater scale-up potential and a lower cost of production, specifically when scarce and highly valuable genotypes need to be maintained for long periods..

References

Ben Amar, A., Cobanov, P., Boonrod, K., Krczal, G., Bouzid, S., Ghorbel, A., Reustle, G.M.: Efficient procedure for grapevine embryogenic suspension establishment and plant regeneration: role of conditioned medium for cell proliferation. - Plant Cell Rep. **26**: 1439-1447, 2007.

Bornhoff, B.A., Harst, M.: Establishment of embryo suspension cultures of grapevines (*Vitis* L.). - Vitis **39**: 27-29, 2000.

Bachiri, Y., Gazeau, C., Hansz, J., Morisset, C., Dereuddre, J.: Successful crypreservation of suspension cells by encapsulation-dehydration.- Plant Cell Tissue Organ Cult. **43**: 241-248, 1995.

Coutos-Thévenot, P., Goebel-Tourand, I., Mauro, M.C., Jouanneau, J.P., Boulay, M., Deloire, A., Guern J.: Somatic embryogenesis from grapevine cells. I. Improvement of embryo development by changes in culture conditions. - Plant Cell Tissue Organ Cult. **29**: 125-133, 1992.

Engelmann, F., Takagi, H. (ed.): Cryopreservation of Tropical Plant Germplasm. - JIRCAS, International Agricultural Series, Tsukuba 2000.

Fahy, G.M., MacFarlene, D.R., Angell, C.A., Meryman, H.T.: Vitrification as an approach to cryopreservation. - Cryobiology **21**: 407-426, 1984.

Franks, T., He, D.G., Thomas, M.: Regeneration of transgenic *Vitis vinifera* L. Sultana plants: a genotypic and phenotypic analysis. - Mol. Breed. **4**: 321-333, 1998.

Faure, O., Aarrouf, J., Nougarède, A.: Ontogenesis, differentiation and precocious germination in anther-derived somatic embryos of grapevine (*Vitis vinifera* L.): proembryogenesis. - Ann. Bot. **78**: 23-28, 1996.

Jayasankar, S., Bonbada, B.R., Li, Z., Gray, D.J.: Comparative anatomy and morphology of *Vitis vinifera* (*Vitaceae*) somatic embryos from solid- and liquid-culture-derived proembryogenic masses. - Amer. J. Bot. **90**: 973-979, 2003.

Jayasankar, S., Gray, D.J., Litz, R.E.: High-efficiency somatic embryogenesis and plant regeneration from suspension culture of grapevine. - Plant Cell Rep. **18**: 533-537, 1999.

Jayasankar, S., Van Aman, M., Cordts, J., Li, Z., Dhekney, S., Gray, D.J.: Long term storage of suspension culture-derived grapevine somatic embryos and regeneration of plants. - *In Vitro* cell. dev. Biol. Plant **41**: 752-756, 2005.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nitsch, J.P., Nitsch, C.: Haploid plants from pollen grains. - Science **163**: 85-87, 1969.

Rajasekaran, K., Mullins, M.G.: Embryos and plantlets from cultured anthers of hybrid grapevines. - J. exp. Bot. **30**: 399-407, 1979.

Ray, A., Bhattacharya, S.: Storage and conversion of *Eclipta alba* synseeds and RAPD analysis of converted plantlets. - Biol. Plant. **54**: 547-550, 2010.

Sakai, A., Kobayashi, S., Oiyama, I.: Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var.*brasiliensis* Tanaka) by vitrification. - Plant Cell Rep. **9**: 30-33, 1990.

Steponkus, P.L., Lanphear, F.O.: Refinement of the triphenyl tetrazolium chloride method of determining cold injury. - Plant Physiol. **42**: 1423-1426, 1967.

Srinivasan, C., Mullins, M.G.: High-frequency somatic embryo production from unfertilized ovules of grapes. - Sci. Hort. **13**: 245-252, 1980.

Takagi, H.: Recent developments in cryopreservation of shoot apices of tropical species. - In: F. Engelmann, H., Takagi, H. (ed.): Cryopreservation of Tropical Plant Germplasm – Current Research, Progress and Application. Pp. 178-193. IPGRI, Rome 2000.

Vasanth, K., Korkie, M., Lerm, T., Vivier, MA.: Somatic embryogenesis from immature grapevine anther explants: towards development of an efficient protocol for *Vitis vinifera* L. (submitted) 2010.

Wang, Q., Gafny. R., Sahar, N., Sela, I., Mawassi, M., Tanne, E., Perl, A.: Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions by encapsulation-dehydration and subsequent plant regeneration. - Plant Sci. **162**: 551-558, 2002.

Wang, Q., Mawassi, M., Sahar, N., Li, P., Violeta, C.T., Gafny, R., Sela, I., Tanne, E., Perl, A.: Cryopreservation of grapevine (*Vitis* spp.) embryogenic cell suspension by encapsulation-vitrification. - Plant Cell Tissue Organ Cult. **77**: 267-275, 2004.