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Reliable encapsulation-based cryopreservation protocol for safe storage and recovery of grapevine embryogenic cell cultures

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ABSTRACT

An efficient and reliable cryopreservation protocol by dehydration–encapsulation technique was developed for several grapevine cell suspensions. Three embryogenic lines of grapevine (*Vitis* sp. rootstock 110 Richter as well as *Vitis vinifera* cv. Tempranillo and cv. Riesling) have been successfully cryopreserved with direct immersion in liquid nitrogen using one-step freezing procedure. Cryoprotection consisted of pretreatment into liquid culture with increasing sucrose concentration followed by loading with sucrose/glycerol solution. Alginate-encapsulated beads were rewarmed and placed on recovery medium which led to regrowth of frozen embryogenic tissues within 6–8 weeks culture. Factors affecting survival of cryopreserved tissues were investigated. Recovery rates varied among genotypes (from 43% to 78%). Following regrowth, beads transferred to liquid medium generated new proliferating cell suspensions showing active multiplication and higher morphogenetic competence as revealed by RNA yield and quality. The cryogenic procedure adopted here allowed high embryo survival after exposure to liquid nitrogen with successful plantlet regeneration. This high throughput protocol is useful for the conservation of a large collection of embryogenic tissues from endangered *Vitis* species.

1. Introduction

The grapevine is one of the most important fruit crops worldwide (Kikkert et al., 1996). Suspension cultures of isolated plant cells are an invaluable tool for providing the material for high-throughput studies and experimentation on physiologically homogenous population of cells (Boisson et al., 2012). However, grapevine embryogenic cell suspensions are usually susceptible to genetic mutations and loss of embryogenic potential by frequent subculture, as well as the increased risk of microbial contamination (Perl et al., 1995; Dixit, 2005; Ben-Amar et al., 2007; Reustle and Buchholz, 2009). Cryopreservation appears as a safe and cost-effective option for maintenance of these cultures ideally for indefinite periods (Mustafa et al., 2011). It provides the establishment of an *in vitro* collection of plant tissue under liquid nitrogen (LN) for conservation of higher plant germplasm. The storage of these cell lines remains a priority towards long-term maintenance of embryogenic tissues suitable for routine laboratory use and often proposed as the target material in genetic transformation studies (Wang et al., 2005).

Fabre and Dereuddre (1990) were the first to establish the encapsulation–dehydration approach for cryopreservation of potato. Since then, cultured plant cells have been cryopreserved by encapsulation in combination with vitrification (Wang and Perl, 2006) or dehydration methods (Engelmann et al., 2008; Wang et al., 2000). Depending on the cooling treatment used to reach extremely low temperatures, several freezing protocols have been used: (i) slow freezing with controlled-rate cooling or (ii) rapid cooling by plunging the samples into LN. In the rapid cooling protocols, the cells are prepared by osmotic dehydration at optimal level, using cryoprotectant solution, before direct immersion into LN. Intensive success has been achieved with both techniques (Kobayashi et al., 2005; Ishikawa et al., 2006; Wang and Perl, 2006; Peredo et al., 2008).

The first attempt to cryopreserve grape embryogenic suspensions was made using a two-step cooling procedure, but no survival was found with fast freezing (Dussert et al., 1991, 1992). During the last years, cryopreservation of *in vitro*-grown shoot tips of grapevine (LN33-hybrid and cv. Superior) has been developed (Wang et al., 2000, Matsumoto and Sakai, 2003). Others have studied the cryopreservation of *Vitis vinifera* cell suspensions (Wang et al., 2004; Wang and Perl, 2006) as well as embryogenic callus by vitrification. However, the technical reliability of the cryogenic

Abbreviations: 2,4-D, 2,4 Dichlorophenoxyacetic acid; BAP, Benzyl aminopurine; cv, cultivar; LEA, Late embryogenesis abundant; EFα1, Elongation factor; LN, liquid nitrogen; NOA, Naphthoxy-acetic acid; PCV, Packet cell volume; PEMs, pro-embryogenic masses.

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procedure to several grapevine genotypes is a tremendous challenge. Our investigation focuses on the establishment of an improved and simple protocol to efficiently cryopreserve several embryogenic cell lines from *Vitis* species.

Successful cryopreservation is dependent to the target tissue. Embryogenic masses were often used because of their small cytoplasmic-rich meristematic cells which allow a high cryotolerance compared to large vacuolated cells. It is well known that grapevine embryogenic suspensions represent the best target for transformation experiments (Kikkert et al., 1996). We established, in previous work, highly proliferating grapevine cell suspensions using an improved procedure based on conditioned medium and involving secreted extracellular proteins (Ben-Amar et al., 2007; Ben-Amar and Reustle, in press). Cell suspensions seem to be very sensitive to freezing including osmotic dehydration and exposure to low temperatures (Grout, 2007; Engelmann et al., 2008) which could affect the viability of pro-embryogenic masses (PEMs). For this reason, the effect of dehydration on the survival of embryogenic tissues was investigated. Here, we describe a simple method involving encapsulation/dehydration procedure aiming at cryopreserving grapevine embryogenic suspension lines available in our laboratory, keeping them homogenous, highly totipotent and suitable for further gene transfer manipulations.

2. Materials and methods

2.1. Cell culture

Three grapevine varieties were used for this study: the rootstock 110 Richter (*Vitis berliandieri* × *Vitis rupestris*) and two *V. vinifera* cultivars cv. Riesling and cv. Tempranillo. Somatic embryogenesis was induced from anther cultures as described in previous reports (Coutos-Thévenot et al., 1992; Ben-Amar et al., 2007). Anther-derived proembryogenic masses were used as starting material to initiate liquid cultures. Cell suspensions were grown in liquid Nitsch and Nitsch (NN) medium (Nitsch and Nitsch, 1969) containing 20 g l^{-1} maltose; 4.6 g l^{-1} glycerol; 0.5 g l^{-1} MES according to (Coutos-Thévenot et al., 1992; Ben-Amar et al., 2007) supplemented with 1 mg l^{-1} NOA and 0.25 mg l^{-1} BAP as growth regulators. The culture was initiated with 2 g fresh weight of proembryogenic masses (2–3 weeks post subculture) into a 250 ml Erlenmeyer flask containing 50 ml of medium. Suspensions were incubated in the dark on an orbital rotary shaker (100 rpm) at 24 °C, and maintained by weekly subculture as described previously (Ben-Amar et al., 2007). Cells for cryopreservation assays were taken 3 days after subculture from established exponentially growing embryogenic suspensions of grapevine.

2.2. Encapsulation of suspension cells and cryogenic procedure

Two different methods of cryopreservation were compared. Firstly, in the dehydration–encapsulation protocol, embryogenic suspensions were precultured in liquid medium with increasing sucrose concentrations (0.25 M; 0.5 M; 0.75 M and 1.0 M sucrose) for 1 day each in 250 ml flasks under the conditions described before. After that, cells were harvested by removal of the culture medium. The PEMs were resuspended in 2% (w/v) sodium alginate (Sigma–Aldrich, Germany) solution (1 ml of packet cell volume PCV with 2 ml alginate solution) and then immediately dropped into 0.1 M calcium chloride solution for 10–15 min to allow the formation of Ca-alginate beads (5 mm in diameter). Alginate beads were treated for 3 days at 24 °C (80 rpm on rotary shaker) with loading solution containing a combination of 2 M glycerol and 0.4 M sucrose. Following osmoprotection treatment, the beads placed in Petri dishes were subjected to desiccation by air-drying under a sterile laminar flow for 8 h before freezing. Secondly, in the encapsulation–dehydration protocol, the embryogenic material was immobilized first in alginate and then the encapsulated beads were incubated with increased concentrated sucrose solutions (from 0.25 to 1 M sucrose) and loaded in 0.4 M sucrose/2 M glycerol for 3 days prior to air-desiccation.

In both cases, dehydrated beads were placed in 2 ml sterile cryovials (10 beads/cryotube) which were plunged quickly in LN at −196 °C and held for at least 90 min. In the case of slow freezing procedure, cell suspensions were treated similarly (preculture–encapsulation–loading–desiccation) except that the cryovials were incubated first for 30 min on ice and for 45 min in −20 °C then for 45 min in −80 °C before exposure to LN. Each treatment was replicated 6 times with 25 beads/plate for each replicate. After storage, vials were rapidly rewarmed by shaking in a water-bath at 40 °C for 5 min.

2.3. Water content measurement

The water content was determined as the fresh weight of 100 beads in each time point-dried weight/initial fresh weight. The dried weight was achieved by incubating 100 beads in the oven at 60 °C during 48 h according to Wang et al. (2000). Kinetic monitoring of the water content of encapsulated beads from three grapevine genotypes (cv. Riesling, cv. Tempranillo and cv. 110 Richter) was carried out during 8 h of desiccation.

2.4. Viability and regrowth of embryogenic masses

After immersion in LN and thawing, alginate-encapsulated beads were plated (25 beads/petri dish) on recovery Nitsch Nitsch solid medium supplemented with 30 g l^{-1} sucrose, 3 g l^{-1} gelrite, 2.5 g l^{-1} activated charcoal and 1 mg l^{-1} 2,4-D/0.25 mg l^{-1} BAP for regrowth according to Wang and Perl (2006). Viability rate was expressed by the percentage of developing beads showing cell mass growth 2 months post-freezing. Non cryopreserved samples (-LN) were taken as control. Each experiment was done with six replicates for each tested variety.

2.5. Transfer in suspension culture and plantlet recovery

The beads were transferred into liquid culture (25 beads/50 ml medium) in 250 ml flasks. The embedded cells were released into the culture medium from crushed cryopreserved beads. Suspensions were initiated using 25 ml of fresh medium and 25 ml of conditioned medium containing extracellular compounds, obtained from well-established cultured cells harvested at the 8th day after the last subculture, as described in previous reports (Ben-Amar et al., 2007, 2010) in order to enhance cell proliferation. The maintenance of these suspensions was performed under dark with shaking (80 rpm) at 24 °C and subcultured at 1-week interval. Plant regeneration from established embryogenic suspension cultures was performed after 8 weeks of culture according to (Coutos-Thévenot et al., 1992; Jayasankar et al., 1999; Ben-Amar et al., 2007).

2.6. RNA isolation

Total RNA was isolated from cryopreserved and non cryopreserved embryogenic grapevine callus using Trizol® kit (Invitrogen, Karlsruhe, Germany) following the manufacturer's instruction. Removal of contaminating genomic DNA was achieved by a combination of RNase-free DNase treatment followed by a final purification with RNeasy MiniElute Cleanup Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Quantity and quality of RNA samples were examined by spectrophotometry and