



Cryopreservation of grape (*Vitis vinifera* L.) using encapsulation-dehydration technique

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Abstract

This investigation was carried out to optimize a protocol for cryogenic storage of grape variety "Black Matrouh" using Encapsulation-Dehydration technique. Shoot tip explants were encapsulated in Na-alginate, precultured on medium with increasing sucrose concentrations for 4 days at 5°C under dark, dehydrated for 0, 2, 4, 5 and 6h under laminar flow, then immersed into liquid nitrogen for 1h. After thawing, they were post-cultured on recovery medium for one month. Results of this study showed that the optimal dehydration time for encapsulated shoot tip explants before immersion in LN was 5 to 6 h with moisture content ranging from 20.31 to 18.39% as the survival percentage ranged from 26.67 to 33.33%, respectively.

Keywords: Cryopreservation, Grape, Shoot tip, Encapsulation-dehydration.

Introduction

Grape (*Vitis vinifera* L.) is one of the extensively grown fruit crop in the world and the second crop in production after citrus in Egypt (Abdel Gawad *et al.*, 2011). Some of grape varieties are being vanished owing to genetic drift, bad agronomic practices and short of conservation strategies (Tehrim and Sajid, 2011).

Biotechnological strategies, based on concepts of *in vitro* plant, cell, tissue and organ culture, have been developed as an alternative and an ancillary measure in response to the problems related to the conservation of plant germplasm in the field (Scherwinski-Pereira and Costa, 2010).

In recent years, cryopreservation represents the only safe and cost-effective option for long-term conservation of germplasm of non-orthodox seed species and vegetatively propagated species (Engelmann, 2010). Cryopreservation involves conservation of plant material at ultra-low temperature in liquid nitrogen (-196°C); thus ensuring that all metabolic processes stop, so plant material can be conserved for unlimited periods (Panis and Lambardi, 2005). In this way, subcultures are not required and the threat of somaclonal variation is reduced.

Encapsulation-dehydration cryopreservation methods are based on successive osmotic and evaporation dehydration of plant cells (Swan *et al.*, 1999). In this method, developed by Fabre and Dereuddre (1990), explants (usually meristems or embryos) are firstly encapsulated in alginate beads (which can also contain mineral salts and organics),

thus forming "synthetic seeds". Then, the synthetic seeds are precultured in liquid medium with a high sucrose concentration ranging from 0.3 M to 1.5 M for at least one day, dried down to a moisture content of 20-30% (under laminar airflow or using silica gel) and subsequently rapidly frozen in LN (Panis and Lambardi, 2005). Wang *et al.* (2000) successfully cryopreserved *in vitro*-grown shoot tips of the "LN33" hybrid (*Vitis* L.) and cv. "Superior" (*Vitis vinifera* L.) by encapsulation-dehydration. Cultivar "Superior" was much less resistant to dehydration than the "LN33" hybrid with only 20% survival obtained after 10h of dehydration. Zhao *et al.* (2001) reported that axillary buds sampled from *in vitro* plants of four grape varieties could withstand cryopreservation using the encapsulation-dehydration technique. Regrowth percentages ranged between 15 and 40%. Gonzalez-Benito *et al.* (2009) successfully cryopreserved embryogenic cell suspensions of two elite Spanish grape cultivars "Albarino" and "Tempranillo" by encapsulation-dehydration. Although cryopreservation has many advantages, freezing and thawing injuries related to membrane structure and function, which would result in low survival percentages, are still the major limiting factors (Ashmore, 1997). In addition, the inability to put general guidelines for cryopreservation for all plants had made it impossible to state a cryopreservation protocol as every plant has its own unique needs for cryopreservation (Lipavska H and Vreugdenhil, 1996).

Therefore, attempts have been made in this study to develop an efficient protocol for the cryogenic

storage of "Black Matrouh" grape using encapsulation-dehydration method.

Materials and Methods

This study was carried out in the Tissue Culture Laboratory, National Gene Bank, Agricultural Research Center, Egypt, during the period from 2013-2014.

Plant material: *In vitro*-grown plantlets of "Black Matrouh" were cold hardened at 5°C on three-quarter Murashige and Skoog (1962) basal medium plus 3.0% sucrose, 0.7% agar and without growth regulators for 4 weeks under complete darkness. Shoot tips which consist of apical dome with three to five tiny primordial leaves were excised under a binocular microscope and used as explant material.

Encapsulation-dehydration and cryopreservation: Excised explants were suspended in liquid half strength MS basal medium supplemented with 3.0% (w/v) sodium-alginate (Na-alginate), 2.0 M glycerol and 0.4 M sucrose. The mixture, with the shoot tip explants, was dispensed with a sterile pipette into 0.1 M calcium chloride (CaCl₂) solution containing 2.0 M glycerol and 0.4 M sucrose at room temperature for 30 min, to form beads (about 5 mm in diameter), each bead containing one shoot tip explant. After encapsulation, excess CaCl₂ solution was poured off and the beads were then stepwise precultured on half strength MS basal medium solidified with 0.25% phytigel and enriched with increasing sucrose concentrations of 0.25, 0.50, 0.75 and 1.00 M for 4 days, with 1 day for each step at 5°C under complete darkness. The precultured beads were placed on sterilized filter paper in 9-cm Petri dishes and dehydrated by air drying in a laminar flow hood cabinet at room temperature and humidity for 0, 2, 4, 5 and 6h. For each dehydration period, 15 shoot tip explants were placed in 2.0 ml cryotubes and rapidly immersed in liquid nitrogen for 1h.

Determination of moisture content (MC): Beads

moisture content was measured during dehydration but prior to freezing in LN. Moisture content (MC%) was calculated according to Gupta and Reed (Gupta and Reed, 2006).

$MC\% = [(FW-DW)/FW] \times 100$, presented as percentage (%)

Fresh weight (FW) represents the weight of beads after each dehydration time (0, 2, 4, 5 and 6h), while dry weight (DW) represents the weight of beads after oven drying at 80°C until the weight stabilizes.

Thawing and post-culture: The cryotubes which had immersed in LN were rapidly thawed in a water bath at 40°C for 3 min and then washed by liquid MS medium. Encapsulated explants subjected to dehydration alone (non-cryopreserved) or to dehydration and LN immersion (cryopreserved) were post-cultured on three-quarter strength MS medium plus 3.0% sucrose, 0.25% phytigel and supplemented with 0.75 mg l⁻¹ BAP and 0.50 mg l⁻¹ IBA (Al-Mousa et al., 2015), maintained in the dark at 25°C for 7 days, then transferred to normal conditions (16h day with light intensity of 3000 lux).

Statistical analysis: The experiment was arranged in a completely randomized design with three replicates, 15 shoot tip explants for each replicate. Survival percentage (%) was determined as the percentage of total number of shoot tip that turned green and formed shoots within one month after post-culture.

Results and Discussion

A typical curve of changes in moisture content (MC) of precultured beads, during dehydration under laminar air flow, is illustrated in Fig (1). The alginate beads initially contained MC of about 76.39%. Within 2h of dehydration, MC declined sharply to 44.86%, and then gradually dropped to 25.27, 20.31 and 18.39% with increasing dehydration time to 4, 5 and 6h, respectively.

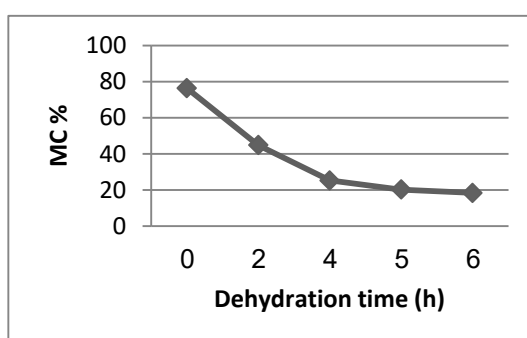


Fig 1. Effect of dehydration time on moisture content of encapsulated shoot tips of "Black Matrouh".

For non-cryopreserved (-LN) encapsulated explants, results in Table (1) showed that the survival percentage decreased in line with increasing dehydration time and decreasing bead moisture content. Also, the cryopreserved (+LN) encapsulated explants gave significantly lower values compared to non-cryopreserved (-LN) encapsulated explants. This confirms that the damage of ice crystal occurs in the cells. However, increasing dehydration time could improve the survival percentage. Therefore, the dehydration step was necessary to avoid intracellular ice crystals; even though increasing dehydration time led to damage of encapsulated explants.

The survival of encapsulated shoot tip explants after plunging in liquid nitrogen was detected only after 5h of dehydration when moisture content of beads reached 20.31%. The greatest survival percentage for cryopreserved (+LN) encapsulated explants was obtained after 6h of dehydration when the beads attained 18.39% moisture content. Whereas, a dehydration time lower than 5h was not effective in inducing cell dehydration and promoting subsequent shoot regeneration as all encapsulated shoot tip explants desiccated less than 5h failed completely to survive after plunging in liquid nitrogen.

Table 1. Effect of dehydration time on moisture content (%) and survival percentage of encapsulated shoot tip explants of "Black Matrouh" non-cryopreserved (-LN) and cryopreserved (+LN) in liquid nitrogen

Dehydration time (h)	Moisture content (%)	Survival (%)	
		-LN	+LN
0	76.39	93.33 a	0.00 e
2	44.86	80.00ab	0.00 e
4	25.27	66.67bc	0.00 e
5	20.31	60.00 c	26.67 d
6	18.39	53.33 c	33.33 d
Mean		70.67 A	12.00 B

Means followed by a letter in common in the same column are not significantly different at 0.05 level of probability.

The survival percentage of cryopreserved shoot tip explants was low (12.00%) compared to non-cryopreserved shoot tip explants (70.67%). In this concern, Benelli *et al.* (2003) reported that encapsulated and cryopreserved explants of grape rootstock "Kober 5 BB" have shown unsatisfactorily low survival and regrowth percentages. Dehydration before immersion in liquid nitrogen was the major deciding factor for obtaining survival from cryopreserved materials. Present results demonstrated that the optimal dehydration time for encapsulated explants before immersion in LN was 5-6h with moisture content ranging from 20.31 to 18.39% as the survival percentage ranged from 26.67 to 33.33%, respectively. Similarly, Wang *et al.* [8] reported that survival percentage of cryopreserved shoot tip explants of grape hybrid "LN33" and grape cultivar "Superior" was detected only when moisture content of precultured beads reached 22.5%. For the "LN33" hybrid, the highest survival (60%) was obtained at 15.6% water content, while 17.6% water content gave the highest survival (40%) for cv. "Superior". Wang *et al.* (2002) found that the highest viability (78%) was recorded with 20.6% water content after 6h of dehydration.

High dehydration tolerance is prerequisite for successful cryopreservation by encapsulation-dehydration because protoplasm must have low

water content to vitrify, thus avoiding ice crystal formation when exposed to liquid nitrogen (Benson, 1999). However, lower water content may cause dehydration damage to the cell, often in the form of membrane alternation. Scanning differential Calorimetric studies showed that alginate beads dried to 20-23% moisture vitrify on exposure to LN and form stable glasses that do not form ice crystals on rewarming (Dumet *et al.*, 2002). This stability is reflected in the high regrowth of viable shoot tips in beads dried to approximately 20-23% moisture.

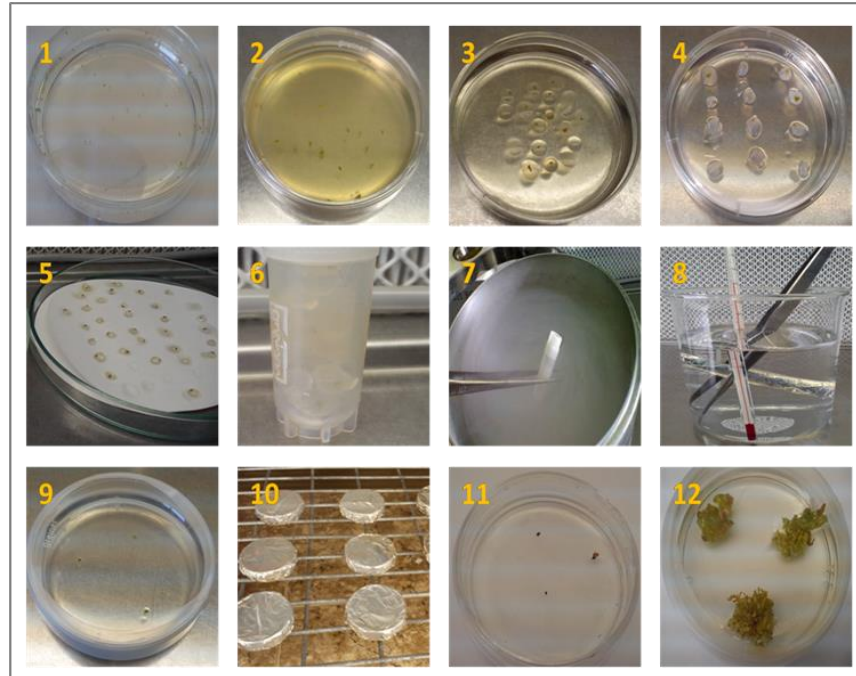


Fig 2. Stages of cryopreservation of "Black Matrouh" shoot tip explants by encapsulation-dehydration.

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|-------------------------------------------------------------------------|-----------------------------------------------------------------------------|
| 1-Dissection of shoot tip explants | 2-Imbedding shoot tips in Na-alginate |
| 3-Formation of beads in 0.1M CaCl ₂ | 4-Preculturing in MS with increasing sucrose concentration |
| 5-Desiccation under laminar air flow | 6-Encapsulated shoot tip explants after desiccation in cryotube |
| 7-Cryotube in container of liquid nitrogen | 8-Thawing of encapsulated shoot tip explants in a water bath at 40°C |
| 9-Reculturing on recovery medium | 10-Incubation under dark for 7 days |
| 11-Cryopreserved encapsulated shoot tip explants survival after 4 weeks | 12-Non-cryopreserved encapsulated shoot tip explants survival after 4 weeks |

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