Chapter 4

In Vitro Cryopreservation of Date Palm Caulogenic Meristems

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Abstract

Cryopreservation is the technology of choice not only for plant genetic resource preservation but also for virus eradication and for the efficient management of large-scale micropropagation. In this chapter, we describe three cryopreservation protocols (standard vitrification, droplet vitrification, and encapsulation vitrification) for date palm highly proliferating meristems that are initiated from vitro-cultures using plant growth regulator-free MS medium. The positive impact of sucrose preculture and cold hardening treatments on survival rates is significant. Regeneration rates obtained with standard vitrification, encapsulation-vitrification, and droplet-vitrification protocols can reach 30, 40, and 70%, respectively. All regenerated plants from non-cryopreserved or cryopreserved explants don't show morphological variation by maintaining genetic integrity without adverse effect of cryogenic treatment. Cryopreservation of date palm vitro-cultures enables commercial tissue culture laboratories to move to large-scale propagation from cryopreserved cell lines producing true-to-type plants after clonal field-testing trials. When comparing the cost of cryostorage and in-field conservation of date palm cultivars, tissue cryopreservation is the most cost-effective. Moreover, many of the risks linked to field conservation like erosion due to climatic, edaphic, and phytopathologic constraints are circumvented.

Key words Cryopreservation, Caulogenic meristems, Encapsulation, Vitrification

1 Introduction

Biotechnology has been used to propagate, improve, and preserve plant genetic resources [1–4]. In date palm (*Phoenix dactylifera* L.), biotechnological techniques have already been employed for in vitro propagation [5, 6]. However, very few commercial tissue culture laboratories are now able to provide suitable quantities of date palm vitro-plants because it is recalcitrant to in vitro regeneration. In fact, date palm tissue cultures grow very slowly: the initiation phase may require more than 24 months, especially when low amounts of plant growth regulators are used to avoid potential somaclonal variation [7]. The presence of uncontrollable

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endophytic bacteria is another serious constraint hampering the large-scale micropropagation of date palm [8].

Cryopreservation refers to the long-term storage of living tissues at an ultra-low temperature (−196 °C) so that it can be revived without loss of regeneration capacity and genetic fidelity. Various technical approaches exist in order to establish cryopreservation techniques. Some are based on a slow freezing and others are based on a rapid-freezing process known as vitrification which enables hydrated living cells to be cooled to cryogenic temperatures without ice formation [1, 2]. All living cells suffer from severe osmotic stress and/or ice crystal damage during the freezing and thawing processes. The most effective ways to minimize such lethal effects are: (a) to use cryoprotective compounds in the culture medium prior to material freezing and (b) to control the transient cooling and warming rates during preservation. Cryopreservation circumvents problems related to the regular re-initiation of in vitro cultures and to the long-term maintenance of proliferating cultures that include risks of contamination, somaclonal variation, or loss of regeneration competency. Several studies have been published on date palm cryopreservation although research is still needed before routine use [9–11]. The successful cryopreservation of proliferating tissue can be of great interest for the development of commercial large-scale micropropagation strategies. Indeed, the availability as a safe backup of cryopreserved germplasm enables the sequential rejuvenation of cultures under production, thus circumventing unwanted drifts linked to long-term proliferation such as hormone habituation, loss of regeneration capacity, or somaclonal variation.

This chapter describes three cryopreservation protocols for date palm highly proliferating caulogenic meristems: standard vitrification, droplet-vitrification, and encapsulation-vitrification protocols.

2 Materials

2.1 Plant Material

Adventitious caulogenic meristems (dedifferentiating cell aggregates) of date palm Deglet Noor cv. from in vitro cultures at an exponential proliferation phase, produced in RITA bioreactors (Fig. 1; *see* **Note 1**).

2.2 Culture Medium and Cryopreservation Solutions

1. Basal culture medium: Murashige and Skoog (MS) medium [12] (Table 1).
2. Preculture medium (PM): Hormone-free MS medium containing 0.52 M (180 g/L) sucrose and 8 g/L agar.
3. Bioreactor medium (BM): Hormone-free liquid MS medium containing 0.2 M (70 g/L) sucrose.

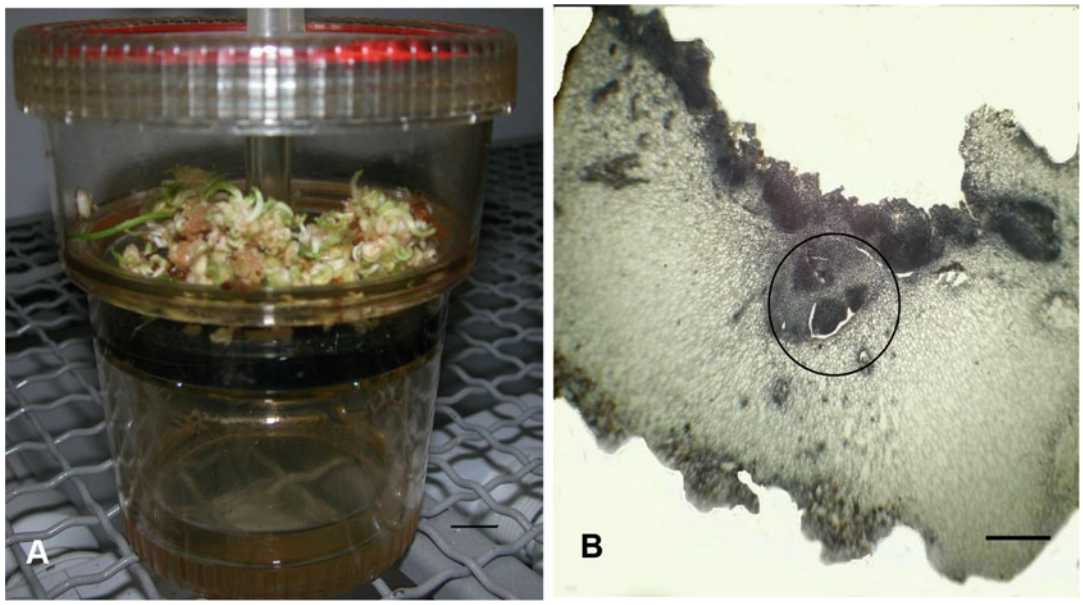

Fig. 1 (**a**) Caulogenic cultures growing in a RITA bioreactor, scale bar: 0.5 cm, (**b**) histological section showing the explants (caulogenic meristems) used for cryopreservation, scale bar: 0.5 mm

4. Loading solution (LS): Hormone-free liquid MS medium containing 2 M (145.36 mL/L) glycerol and 0.4 M (136.9 g/L) sucrose.
5. Vitrification solution (VS2) [13]: Hormone-free liquid MS medium containing 3.26 M (236.93 mL/L) glycerol, 2.42 M (136.66 mL/L) ethylene glycol, 1.9 M (135.09 mL/L) dimethyl sulfoxide (DMSO), and 0.4 M (136.9 g/L) sucrose.
6. Recovery solution (RS): Hormone-free liquid MS medium containing 1.2 M (410.7 g/L) sucrose and 22 μM (0.1 mg/L) 2,4-dichlorophenoxyacetic acid (2,4-D).
7. Posttreatment medium (PTM): Hormone-free liquid MS medium containing 0.52 M (180 g/L) sucrose.
8. Regeneration medium (RM): MS medium supplemented with 0.14 M (50 g/L) sucrose, 22 μM (0.1 mg/L) 2,4-D and 8 g/L agar.
9. Elongation medium (EM): Hormone-free liquid MS medium supplemented with 0.14 M (50 g/L) sucrose.
10. Rooting medium (RoM): Liquid MS medium supplemented with 0.14 M (50 g/L) sucrose and 4 mg/L Indole-3-butyric acid (IBA).
11. Solutions to adjust pH: 0.5 N NaOH and 0.5 N HCl.

Table 1
Composition of MS medium [12]

Medium composition	Concentration (mg/L)
Major inorganic nutrients	
$Mg\ SO_4.7H_2O$	370
KH_2PO_4	170
KNO_3	1900
$CaCl_2.\ 2H_2O$	440
NH_4NO_3	1650
Minor inorganic nutrients	
$MnSO_4.\ 4H_2O$	22.3
$ZnSO_4.\ 7H_2O$	8.6
$H_3\ BO_3$	6.2
KI	0.83
$Na_2\ MoO_4.\ 2H_2O$	0.25
$CuSO_4.\ 5H_2O$	0.025
$CoCl_2.\ 6H_2O$	0.025
Iron source	
$Fe\ SO_4.7H_2O$	27.84
Na_2 EDTA	37.24
Vitamins and amino acids	
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	0.1
Myo-inositol	100
Glycine	2
Glutamine	200

2.3 Reagents

1. Liquid nitrogen.
2. Fixative solution (FS): Chromic acid 5 g/L, glacial acetic acid 50 mL/L formaldehyde 150 mL/L, and ethanol 50 mL/L.
3. Hematoxylin Solution (HS): 100 mg/L hematoxylin in ethanol.
4. Staining solution (SS): 100 mL/L hematoxylin and 100 mL/L glycerol.

2.4 Equipment

1. Horizontal laminar flow hood.
2. RITA Bioreactors.
3. Aluminum foil.
4. Micropipette.
5. Cryotubes.
6. Microfilters.
7. Rotary microtome.
8. Autoclave.

3 Methods

3.1 Culture Media Preparation

1. Refer to Subheading 2.2 and Table 1 for the composition of culture media, mix components, and adjust the pH to 5.8.
2. Sterilize all media for 15 min using a standard autoclave.

3.2 Caulogenic Cultures Maintenance

1. For caulogenic vitro-cultures multiplication, use RITA bioreactors for the temporary immersion of cultures in liquid medium. The RITA vessel is made of two compartments: the explants are cultivated in the upper compartment, and the lower one holds the liquid medium (Fig. 1a).
2. Cultivate 6-bud clusters per bioreactor using 200 mL BM. The immersion cycle is 15 min every 24 h, and the culture medium is renewed once every 4 months (*see* **Note 2**).
3. Incubate cultures in a growth chamber set at 28 °C and 16-h photoperiod (30 μmol/m^2/s).

3.3 Histology

1. Put the explant in the fixative solution for 24 h.
2. Dehydrate gradually the explant with ethanol solutions (50, 60, 70, 80, 90, and100%) for 1 h each.
3. Embed the explant in paraffin.
4. Perform serial sections (10 μm) with a rotary microtome.
5. Mount the sections on glass slides and stain with SS solution for 4 h.

3.4 Cryopreservation Solutions Preparation

1. Refer to Subheading 2.2 for the composition of cryopreservation solutions, mix components of each solution, and adjust the pH to 5.8.
2. Sterilize all solutions using 0.22 μm membrane filters, and keep them at −20 °C.

3.5 Cryopreservation Protocols

1. Excise the caulogenic meristems from tissue showing bud initiation (Fig. 1b; *see* **Note 3**).
2. Culture tissue-bearing caulogenic meristems on preculture medium (PM) or incubate them at 4 °C (cold hardening treatment) for 2 days (*see* **Notes 3** and **4**).

3.5.1 Standard Vitrification Protocol

1. Transfer the caulogenic meristems (explants) to a Petri dish containing 15 mL loading solution (LS), and incubate at room temperature for 20 min (Fig. 2).

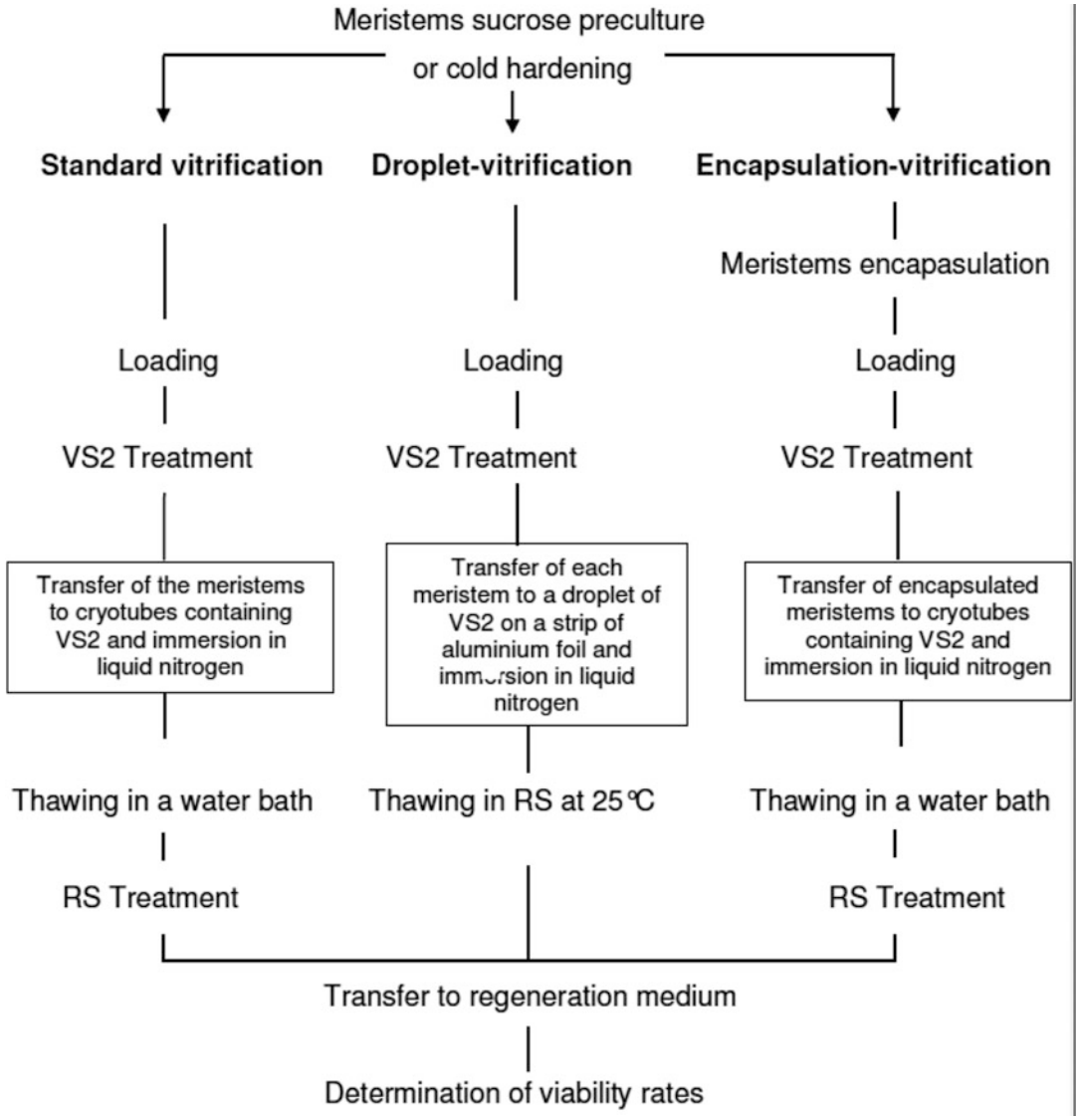

Fig. 2 Description of the cryopreservation protocols. *LS* loading solution; *VS* vitrification solution; *RS* recovery solution

2. Replace the loading solution by ice-cooled VS2 solution.
3. Put the explants in a Petri dish containing VS2 solution for 30 min at 0 °C.
4. Transfer the explants into 2 mL cryotubes containing 0.5 mL VS2, and then plunge them into liquid nitrogen.
5. After 1 h in liquid nitrogen storage, thaw the cryotubes in a water bath at 40 °C for 2 min.
6. Put the explants in a Petri dish containing RS at room temperature for 15 min.
7. Place the explants onto two sterile filter papers on top of PTM.
8. Incubate the explants in the dark at 28 °C.
9. After 2 days, transfer the explants onto RM.

3.5.2 Droplet-Vitrification Protocol

1. Transfer explants (tissue-bearing caulogenic meristems) to 15 mL loading solution (LS) for 20 min.
2. Replace the loading solution by ice-cooled VS2 solution.
3. Place the explants in a Petri dish containing VS2 solution for 30 min at 0 °C.
4. Transfer the explants to a droplet of VS2 on a strip of aluminum foil (3/7 mm), and then plunge them into liquid nitrogen.
5. For permanent cryostorage, transfer frozen foil strips to 2 mL cryotubes filled with liquid nitrogen.
6. After 1 h in LN storage, treat in a Petri dish the explants by RS at room temperature for 15 min.
7. Place the explants onto two sterile filter papers on top of PTM.
8. Incubate the explants in the dark at 28 °C.
9. After 2 days, transfer the explants onto RM (*see* **Note 5**).

3.5.3 Encapsulation-Vitrification Protocol

1. Put the explants bearing caulogenic meristems into previously autoclaved 3% (w/v) sodium alginate, dissolved in MS medium amended with 0.2 M (68.4 g/L) sucrose without $CaCl_2$. Suck them up with a micropipette, and drop them gently into 75 mM $CaCl_2 \cdot 2\ H_2O$ in MS medium supplemented with 0.2 M (68.4 g/L) sucrose (*see* **Note 6**).
2. Transfer the encapsulated plant tissue into the loading solution for 20 min (Fig. 2).
3. Replace the loading solution by ice-cooled VS2 solution.
4. Hold the encapsulated explants with VS2 solution for 60 min at 0 °C.

5. Transfer the encapsulated explants into 2 mL cryotubes containing 0.5 mL VS2, and then plunge them into liquid nitrogen (LN).
6. After 1 h in LN, thaw the cryotubes containing the alginate beads in a water bath at 40 °C for 2 min.
7. Put the beads in a Petri dish containing RS at 25°C for 15 min.
8. Place the alginate beads onto two sterile filter papers on top of PTM.
9. Incubate the beads in the dark at 28 °C.
10. After 2 days, transfer the explants onto RM to get buds (Fig. 3a; *see* **Note 7**).

3.6 Plant Regeneration Protocol

1. To generate multiple bud clusters, subculture the obtained buds onto RM medium once every 2 months for many times.
2. For shoot elongation, transfer bud clusters onto a thin film of liquid RM medium once every 2 months for many times (Fig. 3b).
3. For shoots rooting, transfer shootlets (5–10 cm height) onto RoM medium.

4 Notes

1. A caulogenic meristem is a tissue that can produce an adventitious bud. It is important to point out that this protocol is also effective for two other tested date palm cultivars, Barhee and Khenaizi.
2. Cultivation of date palm shoots in RITA vessels proliferates efficiently by using liquid medium and the control of the gaseous environment of in vitro cultures [14].
3. In this protocol, only small explants (<2 mm) are able to tolerate cryo-treatments [15].
4. The beneficial effect of both sucrose preculture and cold hardening on post-thaw regeneration is clearly demonstrated [16, 17]. Sucrose treatments increase the osmolarity of intracellular solutes, thus reducing the detrimental effect of VS2 and the formation of intracellular ice upon subsequent immersion of the explants in liquid nitrogen. Both sucrose and cold acclimation treatments increase proline content in date palm tissue.
5. The droplet vitrification is the most efficient strategy for the cryo-banking of date palm tissue. The highest regeneration rates obtained with the standard vitrification, encapsulation-vitrification, and droplet-vitrification protocols are 30, 40, and 70%, respectively [1].

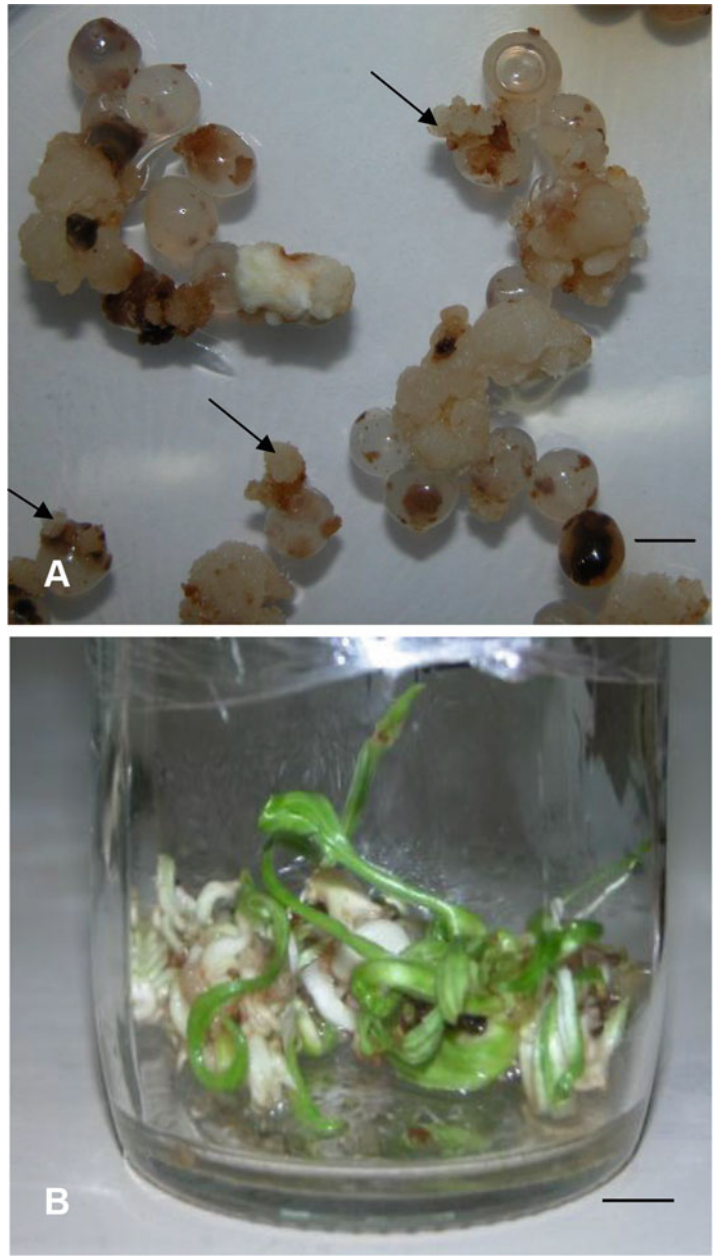

Fig. 3 Regrowth (*see arrows*) of date palm caulogenic meristems after cryopreservation using encapsulation-vitrification protocol. (**a**) 2 months after thawing, (**b**) 6 months after thawing, scale bar: 0.5 cm

6. Alginate is nontoxic to date palm meristems and protects efficiently against cryodamages [1].
7. We have to wait for 3–5 months to produce buds from survived meristems [18].

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