Scientia Horticulturae 224 (2017) 302–305

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Short communication

Cryopreservation of Hamilin sweet orange [(*Citrus sinensis* (L.) Osbeck)] embryogenic calli using a modified aluminum cryo-plate technique

Fernanda Vidigal Duarte Souza[a,b,*], Ergun Kaya[a,c], Lívia de Jesus Vieira[a,d], Antônio da Silva Souza[b], Mariane de Jesus da Silva Carvalho[e], Emanuela Barbosa Santos[e], Alfredo Augusto Cunha Alves[a,b], David Ellis[a,f]

[a] *USDA-ARS National Center for Genetic Resources Preservation, Fort Collins, CO 80526, United States*
[b] *Embrapa Cassava & Fruits, Caixa Postal 007, Cruz das Almas, BA 44380-000, Brazil*
[c] *Mugla Sitki Kocman University, Faculty of Science, Molecular Biology and Genetics Department, 48000, Kotekli, Mugla, Turkey*
[d] *State University of Feira de Santana, 44036-900, Feira de Santana, BA, Brazil*
[e] *Federal University of Recôncavo da Bahia, 44380-000, Cruz das Almas, BA, Brazil*
[f] *Centro Internacional de la Papa, Lima, Peru*

ARTICLE INFO

Keywords:
Cryopreservation
Cryo-plate method
Ex situ conservation
Navel orange
Somatic embryogenesis

ABSTRACT

A modified aluminum cryo-plate technique was applied for cryopreservation of embryogenic citrus calli using aluminum cryo-plate. After encapsulation in a medium with 3% alginate, the beads were placed directly on aluminum foil troughs for exposure to plant vitrification solution (PVS2) incubated on ice for three intervals (30, 45 and 60 min). After rewarming, the calli were incubated under light (16 h/8 h) or dark at 27 ± 2 °C. The survival rates ranged from 59.2% following 45 min of PVS2 exposure to 88.7% after 30 min of exposure. The 30-min PVS2 treatment yielded the highest callus growth (1.4) and percentage of calli with embryos regenerated (43.57%). The highest percentage of embryos regenerated was observed in dark condition (75.2%). The method described using aluminum foil troughs is efficient for cryopreservation of embryogenic citrus calli.

1. Introduction

Citrus is the third most important fruit crop in the world after apples and bananas, and the total area cultivated with the various citrus cultivars covers over 7.2 million hectares, yielding total annual production of approximately 100 million metric tons of fruit (FAO, 2012). Traditionally, citrus germplasm is preserved in clonal orchards, where it is susceptible to pests, diseases and climatic catastrophes (Duran-Vila, 1995).

Cryopreservation of embryogenic calli at ultra-low temperatures (–196 °C) in liquid nitrogen (LN2) is an excellent mean to overcome the challenges inherent to maintaining embryogenic materials and to provide long-term conservation of valuable embryogenic lines (Gonzales-Arnao et al., 2008). There are many studies on Citrus cryopreservation using very different materials. Efficient vitrification- and dehydration-based cooling procedures have been reported for various citrus organs and tissues, including shoot tips (Wang and Deng, 2004), seeds (Kaya et al., 2016), embryonic axes (Cho et al., 2002), somatic embryos (Marin and Duran-Vila, 1988), ovules (Gonzales-Arnao et al., 2003), embryogenic calli (Pérez et al., 1997; Olivares-Fuster et al., 2000) and nucellar cells (Sakai et al., 1990). The aluminum cryo-plate technique for cryopreservation combines encapsulation-dehydration and droplet-vitrification. In this method, shoot tips covered by a thin sodium alginate layer are adhered to an aluminum cryo-plate, loaded (osmoprotection), treated with plant vitrification solution (PVS2) and then cooled by direct immersion of the cryo-plates in liquid nitrogen (Yamamoto et al., 2011, 2012). The proposed changes in this work are the modification of osmoprotection step as well as a simple, cheap, and easy method to mount the aluminum foil troughs to hold encapsulated calluses and make direct immersion in LN2.

The development of a rapid, efficient and reproducible system for the cryopreservation of agronomically important citrus cultivars is critical for genetic conservation efforts and would be a vital adjunct to citrus breeding programs. This paper describes, for the first time, an efficient and reproducible modified aluminum cryo-plate technique applied successfully for the cryopreservation of navel orange

* Corresponding author at: Embrapa Cassava and Fruits, Caixa Postal 007, Cruz das Almas, BA 44380-000, Brazil.
E-mail addresses: fernanda.souza@embrapa.br, fernanda-vidigal@hotmail.com, fernanda-vidigal@hotmail.com (F.V.D. Souza), kayaer19@gmail.com (E. Kaya), liviabiol@gmail.com (L.d.J. Vieira), assouza@cnpmf.embrapa.br (A.d.S. Souza), marianejs@yahoo.com.br (M.d.J.d.S. Carvalho), emanuela_bs@hotmail.com (E.B. Santos), alfredo.alves@embrapa.br (A.A.C. Alves), d.ellis@cgiar.org (D. Ellis).

http://dx.doi.org/10.1016/j.scienta.2017.06.042
Received 2 October 2015; Received in revised form 9 June 2017; Accepted 15 June 2017
Available online 11 July 2017
0304-4238/

embryogenic calli.

2. Materials and methods

2.1. Embryogenic calli

The friable, yellow-brown embryogenic calli from Hamilin sweet orange were induced from unfertilized ovules cultivated in modified MT medium (Murashige and Tucker, 1969) supplemented with 500 mg L^{-1} of malt extract (EME medium; Tusal et al., 1990), 50 g L^{-1} of sucrose and 7 g L^{-1} of agar, with the pH adjusted to 5.8. The calli were cultivated in the dark at 27 °C and maintained in subculture at four-week intervals. The calli were used for cryopreservation experiments four weeks after the last subculture.

2.2. Encapsulation and vitrification using aluminum cryo-plates

To encapsulate the callus cells, approximately 200 mg of dissociated callus cells was added to 5 mL of a 3% sodium alginate solution (low viscosity) in MS maintenance medium (Murashige and Skoog, 1962). A pipette was used to transfer a drop of alginate solution containing the callus clusters into liquid MS medium supplemented with 100 mM of $CaCl_2$. The alginate beads containing the callus clusters were then removed from the $CaCl_2$ solution and placed in MS medium containing 0.625 M of sucrose for 24 h. After that, the beads (5 beads/aluminum foil trough) were placed on the bottom of the open aluminum foil trough, and were covered with 80 µL of PVS2 (30% glycerol, 15% ethylene glycol, 15% DMSO and 0.4 M sucrose in MS maintenance medium) and incubated on ice for 30, 45 or 60 min. Following PVS2 exposure, the double aluminum foil troughs containing beads with calli in PVS2 were sealed by folding the top side over. They were then immersed directly in LN2, transferred to 1.5 mL cryo-vials and stored in LN2 (Fig. 1). Control beads were exposed to PVS2 for the same time intervals as the LN-treated material; however, following PVS2 exposure, the control beads were not subjected to LN treatment but were instead placed immediately into washing solution for 20 min.

After 24 h of exposure to LN2, the beads were rewarmed by rapidly removing the frozen aluminum foil troughs from the cryo-vials and immersing them in unloading solution (liquid MS medium containing 1 M of sucrose) for 20 min at room temperature (27 °C ± 2 °C). The LN2-treated calli were maintained on EME medium in the dark at 27 °C ± 2 °C and the survival rate (% beads showing callus growth) and the callus growth were evaluated after 60 days. To measure the development of each embryogenic callus, the callus growth was scored: 4 (growth over the entire bead surface), 3 (growth on 3/4 of bead surface), 2 (growth on 1/2 of bead surface), 1 (growth on less than 1/2 of bead surface) and 0 (no growth) (Fig. 2).

2.3. Regeneration of somatic embryos

The LN-treated calli were placed on MT medium (Murashige and Tucker, 1969), modified with 500 mg L^{-1} of malt extract and supplemented with 27 g L^{-1} of maltose and 2.2 g L^{-1} of Phytagel. The embryonic development was carried out by two different approaches. The LN-treated calli were incubated under 16 h light/8 h dark photoperiod provided by cool daylight fluorescent lamps (50 µmol $^{-1}$ m^{-2} s^{-1}) at 27 ± 2 °C or at the same temperature but in constant darkness. A mass of callus corresponding to that grown from a bead was transferred. At this stage these calli were not weighed. For each combination of PVS2 exposure time versus photoperiod, we inoculated 10 calli per plate for a total of 60 calli (30 calli in the light and 30 in the dark). After 90 days, the percentage of calli that produced developing embryos was recorded, and the average number of embryos/callus/treatment was calculated.

The conversion of somatic embryos to whole plants was carried out by placing the developed embryos on medium containing MS salts supplemented with 1% sucrose, charcoal (10 g L^{-1}), benzyladenine (0.04 mg L^{-1}), gelrite (1.5 g L^{-1}) and agar (4.5 g L^{-1}). The cultures were maintained at 27 ± 2 °C and exposed to a 16 h light/8 h dark photoperiod provided by the same fluorescent lamps.

2.4 Experimental design, data collection and statistical analysis –

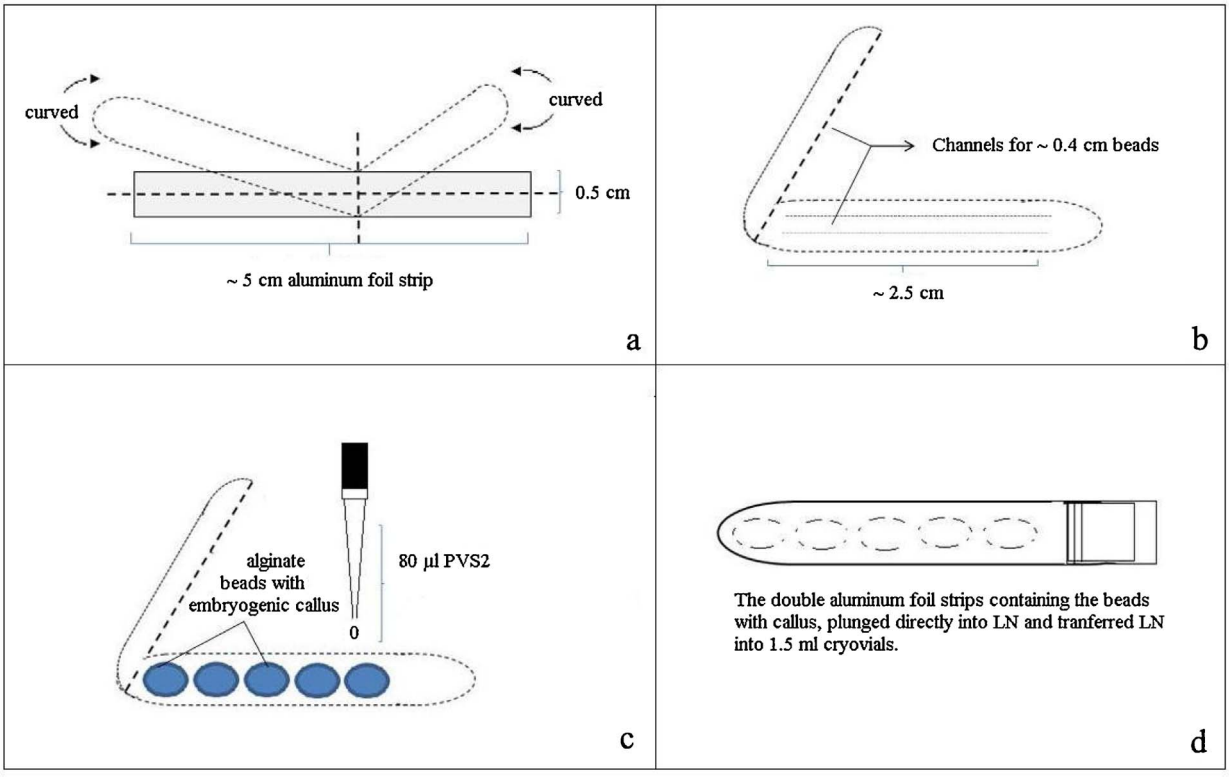

Fig. 1. Illustration of the procedures for preparation of the double aluminum foil troughs.