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Cryopreservation and storage of embryogenic callus cultures of several Citrus species and cultivars

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Abstract Nucellus-derived embryogenic callus cultures of Salustiana sweet orange were subjected to cryoconservation assays. Cryoprotection with 10%(vol/vol) dimethylsulfoxide, freezing by slow cooling and thawing by fast warming was suitable to recover viable growing cultures and whole plants through embryogenesis. Evaluation of liquid phase R_1 and solid phase R_2 cooling rates using a programmable freezing unit indicated that 100% of embryogenic cultures survived when frozen using a range of cooling rates (R_1 not above 0.5°C min^{-1} and R_2 not above 1°C min ^{1}) and thawed by fast warming. Storage up to 2 years in liquid nitrogen did not affect the growth of the cryopreserved cultures and the recovery of whole plants. Cultures of four cultivars of sweet orange (*C. sinensis* Osb.), three cultivars of grapefruit (*C. paradisi* Macf.), and one cultivar each of lemon [*C. limon* (L.) Burm. f.], Cleopatra mandarin (*C. reshni* Hort. *ex* Tan.), sour orange (C. *aurantium* L.) and Mexican lime [*C. aurantifolia* (Christm.) Swing.] have been successfully cryopreserved. Problems using a viability assessment using fluorescein diacetate staining are discussed.

Key words Genetic conservation · Germplasm conservation · Cryoconservation · Freezing and thawing · Embryogenesis

Abbreviations *BM* Basal medium · DMSO Dimethylsulfoxide · *FDA* Fluorescein diacetate · *LN* Liquid nitrogen · *MS* Murashige and Skoog

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Introduction

Conservation of citrus genetic resources is subject to the limitations experienced with most woody perennials. Most citrus collections are conserved in field plantings which experience losses from biological and climatic hazards. As an alternative, a few collections of selected cultivars are conserved as potted plants in screen houses where they are, nevertheless, still vulnerable to a number of potential accidents; in addition, such conservation is costly, which limits the number of specimens that can be conserved. Today, cryoconservation techniques are considered the most promising alternative to the traditional conservation of live collections (Withers and King 1980).

In Spain, the citrus industry has great economic importance and a citrus germplasm bank of about 400 accessions is being maintained as virus-free plants at the Instituto Valenciano de Investigaciones Agrarias (IVIA). The plants are conserved as potted plants in screened houses and in field collections. This germplasm collection is the source of plant material for genetic studies and for rootstock and cultivar improvement programs based on conventional breeding, somatic hybridization, and genetic transformation.

Survival of citrus tissues subjected to freezing-thawing treatments has been reported for seeds (Mumford and Grout 1979), ovules (Bajaj 1984), embryos (Marín and Duran-Vila 1988; Marín et al. 1993), and embryonic axes (Radhamani and Chandel 1992) from a limited number of species and cultivars. More recently, it has been demonstrated that embryogenic callus and cell cultures survived after being subjected to a number of cryoconservation treatments including conventional cryoprotection with dimethylsulfoxide (DMSO) followed by slow cooling (Kobayashi et al. 1990, Aguilar et al. 1993), vitrification followed by fast cooling (Sakai et al. 1990; 1991a), as well as other simplified procedures (Sakai et al. 1991b; Engelmann et al. 1994).

The application of biotechnologies based on protoplast isolation and fusion for citrus breeding relies on the avail-

ability of embryogenic cell lines (Vardi and Galun 1989; Gmitter et al. 1992) which are also suitable for genetic transformation (Hidaka et al. 1990; L. Peña, personal communication). These cell lines are obtained by induction of embryogenic callus from nucellar tissues of polyembryonic cultivars. The process is time consuming and can only be initiated when ovules at the desirable stage are available. Routine maintenance of valuable embryogenic cells and callus by periodic subculturing is also time consuming and has the risk of potential losses due to human errors. In addition, the embryogenic potential decreases and somaclonal variation increases with long-term subculturing, and the cultures may no longer be suitable for biotechnological applications. Therefore, cryoconservation of embryogenic cultures is a most valuable tool not only for the conservation of genetic resources, but to preserve plant materials in a form ready for further manipulations such as genetic transformation.

Here we report the successful cryopreservation and storage of embryogenic cultures of several citrus species and cultivars.

Materials and methods

Source of embryogenic callus lines

Embryogenic cultures from nucellar tissues of several citrus cultivars were used for cryopreservation assays. The species assayed were: Sweet orange (*Citrus sinensis* Osb.) Salustiana, Washington Navel, Pineapple and Succari; grapefruit (*Citrus paradisi* Macf.) Star Ruby, White and Red Marsh; lemon [*C. limon* (L.) Burm. f.] Lac; Cleopatra mandarin (*C. reshni* Hort. ex Tan.); sour orange (C. *aurantium* L.); and Mexican lime [*C. aurantifolia* (Christm.) Swing.].

Nucellar callus lines were initiated as described elsewhere (Duran-Vila 1995; Galiana, 1995). Callus lines of White grapefruit, Red Marsh grapefruit, Lac lemon and Succari sweet orange were kindly donated by J. W. Grosser (Citrus Research and Education Center, University of Florida). All the cultures used had been originally maintained by monthly subculture to fresh basal medium (BM) medium containing Murashige and Skoog (1962) (MS) salts, 100 mgl^{-1} myoinositol, 1 mgl^{-1} pyridoxine-HCl, 1 mgl^{-1} nicotinic acid, 0.2 mgl^{-1} thiamine-HCl, 500 mgl^{-1} malt extract, 50 gl^{-1} sucrose and 1% Bacto agar. The medium was adjusted to pH 5.7 prior to autoclaving at 121°C for 20 min and dispensed as 25-ml aliquots into 9-cm-diameter disposable petri dishes. The cultures were kept at 27±1°C, under 16 h day illumination with a photosynthetic photon flux of 43 µmol m^{-2} s^{-1}.

Pretreatment and cryoprotection

Cells (150–200 mg of loose cells from callus) were transferred to 2-ml cryotubes (screw-cap polypropylene ampoules) containing 1.8 ml of liquid medium (BM without agar) supplemented with 10% (vol/vol) DMSO. The cells were resuspended in the medium and maintained at 4°C for 30 min. In each assay at least five cryotubes were employed.

Freezing procedures

In preliminary assays using Salustiana sweet orange, three cooling conditions were assayed. For fast cooling, the cryotubes were immersed directly in liquid nitrogen (LN). For step-wise cooling, the cryotubes were placed in a freezer at –20°C for 2 h, transferred at –70°C for 18 h and finally immersed in LN. For slow cooling, the cryotubes were placed into a cooling bath (Heto CB 10) containing pure methanol as coolant, and an estimated cooling rate of about 0.5°C min^{-1} down to –40°C was consistently achieved. Cryotubes containing the samples were then immersed in LN.

In the rest of the assays, controlled cooling was achieved by a programmable freezing unit (Minicool LC 40) which controls the automatic injection of LN into a cooling chamber. The freezing program is defined by several parameters: (a) starting temperature T_1 at which the chamber is held before introducing the samples; (b) freezing point T_2 of the cryoprotectant solution which must be determined for each cryoprotectant solution; (c) liquid phase cooling rate R_1 between T_1 and T_2; (d) cooling power W needed to overcome the latent heat of fusion, which is applied as soon as the temperature of the liquid phase sample falls below T_2; (e) time during which the cooling power W is applied; (f) temperature T_3 at which the cooling rate can be changed; (g) solid-phase cooling rate R_2 between T_2 and T_3; (h) cooling rate R_3 between T_3 and the final temperature T_4.

The freezing program used was defined as follows: T_1=4°C, T_2=–6°C, T_3=–40°C and T_4=–150°C. The cooling power set at W=5 and applied for 40 s provided a smooth cooling curve. Several cooling rates R_1 (0.1, 0.2, 0.5 and 1.0°C min^{-1}) and R_2 (0.1, 0.2, 0.5, 1.0 and 5.0°C min^{-1}) were assessed and the final cooling R_3 was set at 20°C min^{-1}. In all the assays, the cooling curve was confirmed with a temperature sensor in a test sample.

After reaching the final temperature provided by the cooling system chosen, the cryotubes were placed into a LN container. In routine assays, the cells were kept in LN overnight.

Thawing procedures

In preliminary assays using Salustiana sweet orange, two thawing conditions were tested. For fast warming, the cryotubes were immersed for 5 min in a water bath kept at 37°C. Slow warming was performed by leaving the cryotubes for 15 min at room temperature. In the rest of the assays, the samples were thawed by fast warming.

Storage

In the storage assays, the cryotubes containing frozen cells at –196°C were stored for several periods (1 day, 1 week, 1 month, 3 months, 1 year, and 2 years) under different storage conditions: –196°C (in LN), –150, –80, and –20°C (electrical freezers).

Regrowth of callus and recovery of whole plants

After thawing, the cryoprotection solution was removed from the cryotubes and the cells were washed three times with 1.8 ml of liquid BM. The cells were transferred to fresh solid BM and kept in the same environmental conditions as described above. For our purposes, growth recovery of the cultures after freezing and thawing was considered the most desirable viability test. The embryos produced by the growing cultures were individually transferred to tubes containing 25 ml fresh BM for germination. After 8 weeks, the young plantlets were transplanted to soil and gradually conditioned to greenhouse conditions.

Cell survival assessment

Samples of cultures subjected to freezing, storage and thawing were subjected to viability tests by staining with fluorescein diacetate (FDA) (Widholm 1972). The conditions were those described by Nadel (1989).

Results and discussion

In the preliminary assays with Salustiana sweet orange, six cooling/thawing treatments were visually evaluated (Table 1). Cultures subjected to slow cooling and fast thawing started growing 2–6 weeks after the freezing/thawing treatment. All of the growing cultures produced embryos and had the same appearance as the unfrozen controls with and without cryoprotection. No growth was observed in cultures subjected to the other freezing/thawing treatments.

Further assays were performed to evaluate the effect of the cooling rate using a programmable freezing unit. The cultures were subjected to ten different treatments with different values of liquid-phase and solid-phase cooling rates, R_1 and R_2, respectively. Values of R_1 ranging from 0.1 to 0.5°C min^{-1} and R_2 ranging from 0.1 to 1.0°C min^{-1} did not affect the survival of the cultures (Table 2), but none of the cultures subjected to R_1=1.0°C min^{-1} or R_2=5.0°C min^{-1} grew after freezing and thawing. Cultures started growing 2–6 weeks after freezing and thawing regardless of the cooling rates R_1 and R_2, and had the same appearance as the unfrozen controls (Fig. 1A,B). The growing cultures produced embryos without any induction treatment. Embryos grew normally into heart-shape, torpedo, and cotyledonary stages. Samples of 20 cotyledonary-stage embryos were selected from each of the eight treatments that allowed survival and were independently transferred to fresh medium for germination. After 8–12 weeks, plantlets were transplanted to soil and grew normally, showing the same morphology as the plants recovered from the unfrozen controls.

To evaluate the effect of storage, cultures of Salustiana sweet orange were frozen by controlled cooling (R_1=0.5°C min^{-1}; R_2=0.5°C min^{-1}) and, after reaching the final temperature (T_4=–150°C), were placed in LN and transferred to four different storage conditions. All of the cryopreserved cultures stored at –150°C (electrical freezer) and –196°C (LN) presented the same growth patterns as the unfrozen controls and produced embryos (Fig. 1C, Table 3). Samples of 20 embryos from cryopreserved cultures stored for 1 year in LN were transferred to fresh medium and produced whole plants after 8–12 weeks (Fig. 1D). The plants had the same morphology as the unfrozen controls (Fig. 1E). Storage at –80°C and –20°C impaired further growth of cryopreserved cultures (Table 3). Cryopreserved cultures stored for 2 years in LN also produced embryos which are following the same pattern of growth as the embryos from cryopreserved cultures stored for 1 year. Immediately after thawing, samples of cryopreserved cultures stored at –20°C were subjected to cell survival assessment tests. Cell survival was comparable to that of control cultures stored in LN overnight. However when the same test was used after a week in culture, it became apparent that none of the cells from cryopreserved cultures stored at –20°C survived whereas the control cultures still presented very high survival rates.

The cryopreservation protocol developed for Salustiana sweet orange was assayed for cultivars of sweet orange, grapefruit, lemon, Cleopatra mandarin, sour orange, and Mexican lime. The cultures were subjected to controlled cooling (R_1=0.5°C min^{-1}; R_2=0.5°C min^{-1}) and after reaching the final temperature (T_4=–150°C) were stored in LN overnight and thawed by fast warming. As illustrated in Table 4, the protocol was successfully used for cryoconservation of all the cultivars assayed. All of the cultures presented the same appearance and growth rate as the un-

Table 1 Survival and growth of Salustiana sweet orange callus cultures subjected to freezing/thawing treatments

Treatment			Percent viable (number of cultures)	Embryogenesis
Cryoprotection	Freezing	Thawing		
+	–	–	100 (14)	+
–	–	–	100 (9)	+
+	Fast	Slow	0 (20)	–
+	Fast	Fast	0 (20)	–
+	Step-wise	Slow	0 (30)	–
+	Step-wise	Fast	0 (30)	–
+	Slow	Slow	0 (32)	–
+	Slow	Fast	100 (26)	+

Table 2 Effect of liquid-phase and solid-phase cooling rates on the survival and growth of callus cultures of Salustiana sweet orange. Liquid phase coolig rate R_1 from 4°C to –6°C. Solid-phase cooling rate R_2 from –6°C to –40°C. Control cultures were subjected to cryprotection treatments but were not frozen

Cooling rates	Percent viable (number of cultures)	
R_1–R_2 (°C min^{-1})	Control	Frozen
0.1–0.1	95 (19)	96 (57)
0.1–0.2	100 (7)	100 (20)
0.1–0.5	100 (5)	100 (24)
0.1–1.0	100 (9)	100 (26)
0.1–5.0	100 (20)	0 (19)
0.2–0.5	100 (9)	100 (19)
0.2–1.0	100 (3)	100 (21)
0.5–0.5	100 (10)	100 (35)
0.5–1.0	100 (7)	100 (18)
1.0–0.5	100 (10)	0 (24)

Table 3 Survival and growth of Salustiana sweet orange cultures after storage. Survival was estimated as the percentage of cultures that grew after freezing and thawing. At least 20 cultures were used in each treatment (*NT* not tested; storage at –150°C was only tested for up to 3 months)

Storage	Temperature			
	–20°C	–80°C	–150°C	–196°C
1 day	0	60	100	100
1 week	0	0	100	100
1 month	0	0	100	100
3 months	0	0	100	100
1 year	0	0	NT	100
2 years	0	0	NT	100

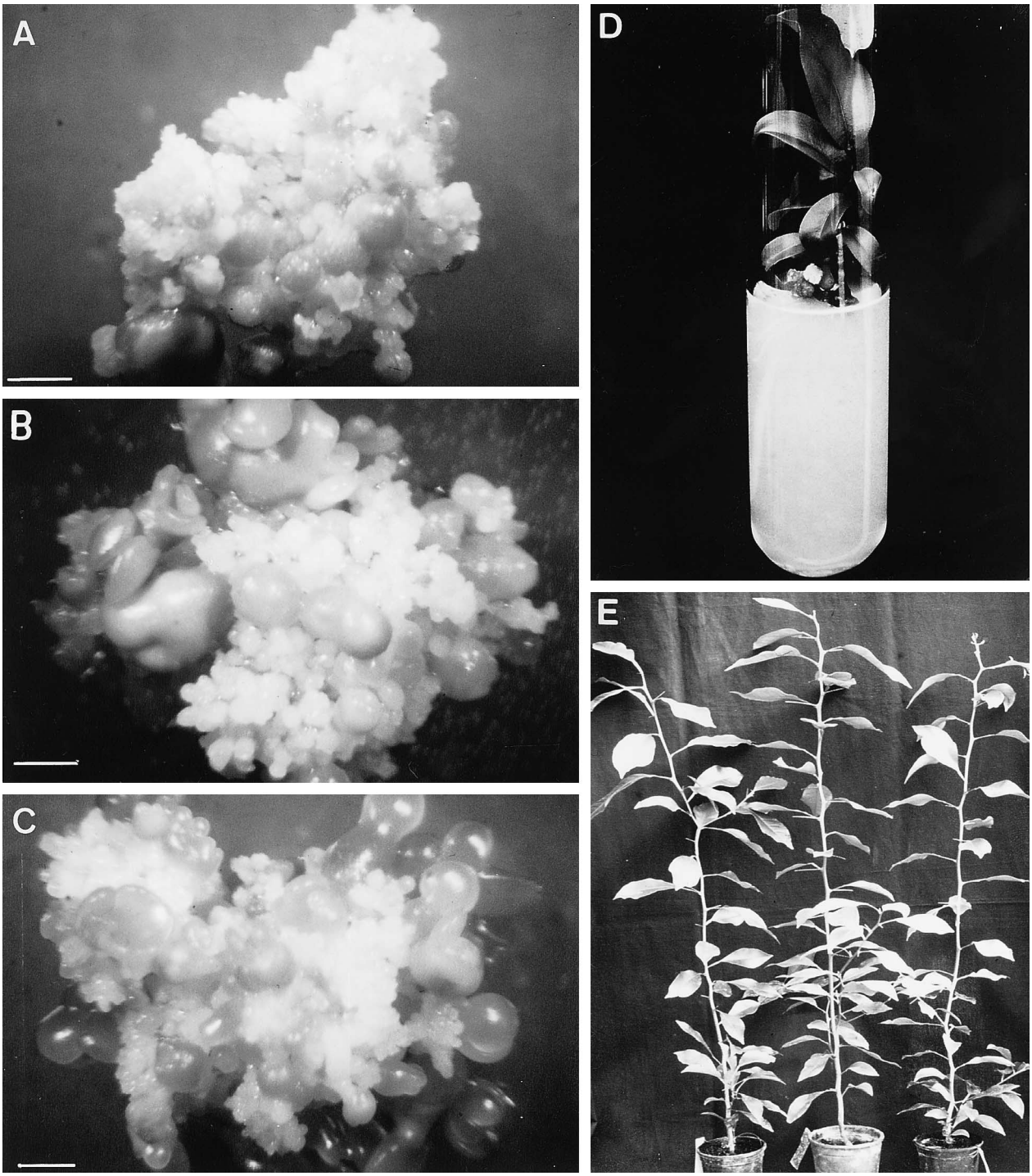

Fig. 1 Cultures of Salustiana sweet orange subjected to freezing, storage and thawing: (*bar* 5 mm) **A** unfrozen controls, **B** cultures frozen by slow cooling (R_1=0.5°C min^{-1}; R_2=0.5°C min^{-1}), stored in LN overnight and thawed by fast warming. **C** cultures frozen by slow cooling (R_1=0.5°C min^{-1}; R_2=0.5°C min^{-1}) stored in LN for 1 year and thawed by fast warming; **D** Plants from embryos recovered from cryopreserved cultures. **E** Plants 1 year after transplanting to soil from cryopreserved cultures (*left*), unfrozen control (*center*), and subjected to cryoprotection (*right*)

frozen controls. Seven of the ten cultivars assayed produced embryos that germinated into whole plants, which had the same normal morphology as those produced from the unfrozen controls. Cryopreserved cultures of Succari sweet orange, Red Marsh grapefruit, and Mexican lime did not produce embryos, nor did their unfrozen controls. Attempts to recover embryos from the source cultures which had neither been subjected to cryoprotection treatments nor frozen were also unsuccessful.

Table 4 Growth of embryogenic callus of several citrus cultivars subjected to cryopreservation. Growth was evaluated as the frequency of growing cultures after freezing and thawing. Cryopreserved cultures were subjected to slow cooling (R_1=0.5°C min^{-1}; R_2=0.5°C min^{-1}), stored in LN overnight and thawed by fast warming. Control cultures were subjected to cryoprotection but they were not frozen or thawed. Figures in parantheses indicate the number of cultures assayed in each treatment

Cultivar	Percent viable (number of cultures)		Embryogenesis		Plants	
	Control	Frozen	Control	Frozen	Control	Frozen
Sweet orange:						
Washington Navel	100 (10)	100 (20)	+	+	+	+
Pineaple	100 (10)	100 (20)	+	+	+	+
Succari	100 (10)	100 (20)	–	–	–	–
Cleopatra mandarin	100 (10)	100 (20)	+	+	+	+
Sour orange	100 (10)	100 (20)	+	+	+	+
Grapefruit:						
Star Ruby	100 (10)	100 (20)	+	+	+	+
White	100 (10)	100 (20)	+	+	+	+
Red Marsh	100 (10)	100 (20)	–	–	–	–
Lemon:						
Lac	100 (10)	100 (20)	+	+	+	+
Mexican lime	100 (10)	100 (20)	–	–	–	–

Cryoconservation is considered today the most promising alternative for germplasm conservation of species with recalcitrant seeds and of vegetatively propagated plants (Withers and King 1980). In citrus, survival of seeds, embryos, ovules, and embryonic axes after freezing and thawing has been demonstrated with different levels of success (Mumford and Grout 1979; Bajaj 1984; Marín and Duran-Vila 1988; Marín et al. 1993; Radhamani and Chandel 1992). In addition, embryogenic cells from Washington navel sweet orange and common mandarin have been shown to survive subjection to a wide range of cryopreservation protocols ranging from conventional cryoprotection and slow cooling (Kobayashi et al. 1990; Aguilar et al. 1993) to vitrification followed by fast cooling (Sakai et al. 1990, 1991a). The protocol developed in this study requires the application of cryoprotection treatments with DMSO and slow cooling at 0.5°C min^{-1} down to –40°C followed by further cooling at 20°C min^{-1} down to –150°C, prior to immersion in LN. The cryopreserved cultures showed very high survival rates provided the cooling rates applied were sufficiently low, thus confirming previous reports using a comparable protocol to cryopreserve navel sweet orange (Kobayashi et al.1990) and common mandarin (*C. deliciosa* Tan.) (Aguilar et al. 1993). In addition, the results of this study using ten different genotypes from six citrus species expand earlier results to a wide range of polyembryonic species and cultivars. This indicates that the same protocol could probably be applied to cryopreserve most polyembryonic citrus. The development of embryogenic cultures from monoembryonic citrus must be initiated through the culture of nucellus isolated from immature seeds (Rangan et al. 1968; Esan 1973) and many of the plants recovered by this process are not true to type (Juárez et al. 1976; Navarro et al. 1985). Therefore, these cryopreservation techniques should not be used for the conservation of monoembryonic citrus.

The use of a programmable freezing unit allows control of the cooling curve and standardization of the overall cooling process; however, as shown in this study, adequate cooling conditions can also be achieved with less sophisticated equipment. Other cooling methods that only require commercial freezers at –30°C or at –80°C have also been used successfully (Sakai et al. 1991b, Engelman et al. 1994). Alternative approaches to cryopreserve navel sweet orange were based on direct immersion in LN after vitrification (Sakai et al. 1990; 1991a) which is simple and fast but requires elaborate vitrification pretreatments. The information available suggests that embryogenic cell cultures offer excellent plant material for cryopreservation under a wide range of conditions. The method has the advantage of preserving cultures in a form that can be readily used for the application of technologies of protoplast isolation and culture and genetic transformation.

In other studies, the viability of cryopreserved cultures has been estimated as the percent survival over nontreated controls by means of FDA staining (Widholm 1972; Nadel 1989) immediately after thawing. Our results show that FDA staining performed immediately after thawing overestimates cell survival, thus confirming a previous observation by Aguilar et al. (1993) with cryopreserved mandarin cultures. The FDA test to estimate cell survival has been widely used, but requires caution when evaluating cryopreservation protocols when the ultimate purpose is the recovery of growing cultures and whole plants. Performing cell survival assessment tests 1 week after thawing would provide more realistic results; however, growth recovery and evaluation of the embryogenic potential must remain the most desirable test for the purpose of germplasm conservation. With the protocol proposed in this study, 100% of the cultures subjected to freezing and thawing grew and the embryogenic potential was comparable to that of the control cultures. However callus lines to be used for cryopreservation must be capable of producing embryos routinely before being considered good candidates for germplasm preservation.

The results presented here demonstrate that cryopreserved cultures remain viable when maintained in LN for up to 2 years, confirming that long-term conservation is

feasible. Since this type of storage relies on the periodic replenishing of LN, storage of cryopreserved cultures was also assayed with success using a commercial freezer kept at –150°C. Samples of the cryopreserved cultures from the ten citrus sources studied are now being maintained in a LN tank and in a –150°C commercial freezer for further evaluation of long-term storage. Some of them have been thawed after 6–8 months storage and successfully used for protoplast isolation (O. Olivares, unpublished results) and genetic tranformation (L. Peña, unpublished). An ongoing project has been initiated to produce embryogenic cell cultures from all the polyembrionic species and cultivars of the germplasm bank maintained at IVIA with the aim of establishing a germplasm bank of cryopreserved cultures that may replace totally or partially some genotypes presently maintained as living plants.

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