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ORIGINAL ARTICLE

Citrus cryopreservation: viability of diverse taxa and histological observations

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Abstract Diverse citrus cultivars maintained clonally within gene banks serve as valuable resources for research and breeding programs worldwide. These critical collections are kept as trees within field, screenhouse, or greenhouse collections. Ex situ collections are at risk of being lost due to unforeseen environmental or biological disasters. Cryopreservation provides a secure method to back-up these important collections. Herein, we assessed the applicability of a vitrification-based cryopreservation method to conserve citrus collection cultivars. Shoot tips were excised from screenhouse-grown trees from the USDA-ARS National Clonal Germplasm Repository for Citrus and Dates. Shoot tips were then treated with cryoprotectants, plunged into liquid nitrogen (LN), warmed and then recovered by micrografting onto 'Carrizo' citrange seedling rootstocks. Of 150 cryopreserved Citrus accessions representing 32 taxa, 24 taxa had mean regrowth levels that were at least 40%. The 36 navel orange (*Citrus sinensis*) accessions had an average regrowth level of 64%. There was no decrease in viability after 3 years of LN storage for the three accessions that are part of a long-term storage experiment. Histological observations revealed high levels of cell survival after LN exposure and that cellular regrowth occurred between rootstock and shoot tips within 2 days of micrografting. We demonstrate that diverse citrus cultivars can be successfully cryopreserved within gene banks for long-term conservation.

Keywords Collection · Liquid nitrogen · Long term preservation · Micrografting

Introduction

The USDA-ARS National Plant Germplasm System maintains a diverse collection of 1372 accessions representing 96 species of Citrus and related taxa at the National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) in Riverside, California. Of these, approximately 500 accessions are pathogen-tested and apparently free of graft-transmissible pathogens. These plants are kept in duplicate within protective structures; however, they are at risk of being lost due to environmental disasters and unpredictable biological infestations.

Many clonally maintained collections of plant genetic resources have been placed into cryotanks of liquid nitrogen (LN) for secure secondary storage using various cryopreservation techniques (Wang et al. 2014). LN storage is an economical way to maintain germplasm for the long term as compared to collections maintained *in planta* in the field or within protected structures (Reed et al. 2004). For cryopreservation, shoot tips are treated with cryoprotectants and sufficiently desiccated to survive liquid nitrogen exposure (Engelmann 2004). Although most plant cryopreservation research focuses on recovery after short-term cryo-exposure, results from long-term storage experiments have also

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been published for some crops (Engelmann 1991; Volk et al. 2008).

We previously published a procedure to successfully cryopreserve Citrus shoot tips using explants excised and treated with cryoprotectants from screenhouse-grown plants as the source materials (Volk et al. 2012, 2015). After LN storage, shoot tips were warmed and micrografted onto in vitro seedling rootstocks which vastly improved the recovery procedure since optimized media were not necessary for each citrus species (Volk et al. 2015). Herein we demonstrate the applicability of the citrus cryopreservation method to 32 taxa, provide 3 year storage timepoint regrowth data, and use histological observations to document regrowth.

Materials and methods

Plant materials

Between 70 and 175 shoot tips for each of 150 citrus accessions were processed for long-term cryopreservation. Vegetative budsticks with 5–10 nodes were harvested at the triangular growth stage (3–5 mm diameter) 2–6 weeks after flushing. Leaves were removed and branches were shipped overnight in sealed plastic bags from the NCGRCD to the National Laboratory for Genetic Resources Preservation (NLGRP) in Fort Collins, CO. Upon receipt, budsticks were kept at 4 °C for up to 4 weeks prior to shoot tip excision. Budwood was cut into nodal sections, surface disinfected with 70 % isopropanol for 2 min, 0.6 % sodium hypochlorite for 10 min, followed by three rinses with sterile water. 1–1.5 mm shoot tips containing two-leaf primordia were excised from the axillary buds under aseptic conditions.

Cryopreservation

Shoot tips were cultured overnight in 0.3 M sucrose + ½ strength MS liquid medium in darkness at 25 °C, then treated with 2 M glycerol + 0.4 M sucrose in ½ MS liquid medium for 20 min at 22 °C, followed by 30 min PVS2 (30 % w/v glycerol, 15 % w/v dimethylsulfoxide (DMSO), 15 % w/v ethylene glycol and 0.4 M sucrose prepared in ½ strength MS; Sakai et al. 1990) at 0 °C. Shoot tips were then placed into droplets of PVS2 on aluminum foil strips which were plunged directly into LN. Ten shoot tips on a foil strip were placed within each cryovial. For viability assessments, one foil strip was warmed for 122 of the accessions, and two foil strips were warmed for 28 of the accessions for micrografting. In general, a second foil strip was warmed for accessions with regrowth levels below 30 % in order to confirm the initial result. The foils containing shoot tips were maintained in LN vapor as long-term cryo back-ups.

In 2011, 240 shoot tips of three accessions (*Citrus aurantium* 'Seville sour orange' PI 654,867, *Citrus limon* 'Eureka' lemon PI 658,388, and *Citrus paradisi* 'Reed Marsh' grapefruit PI 539,471) were processed for a long-term storage experiment. After PVS2 exposure at 0 °C, shoot tips were transferred to 1.2 mL cryovials containing 1 mL PVS2 and plunged into LN. They were maintained in the vapor phase of LN for up to 3 years and three vials, each containing 10 shoot tips per accession, were warmed after 0, 3, 6, 12, 24, and 36 month intervals.

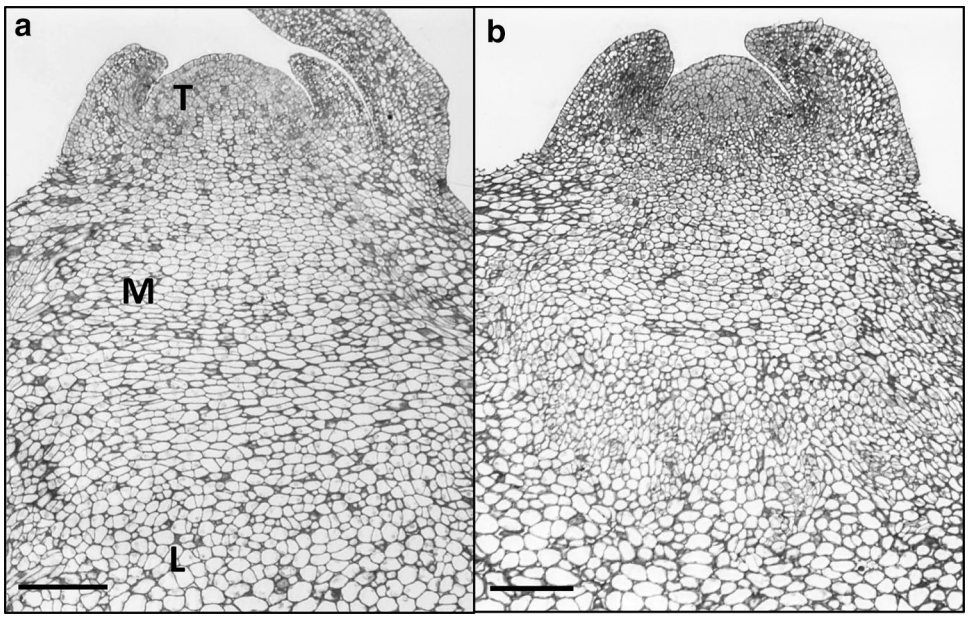

Fig. 1 Cross section of *Citrus limon* 'Eureka lemon' shoot tip excised, treated with cryoprotectants, then **a** not exposed to LN, and diluted into 1.2 M sucrose; and **b** exposed to LN on foil strips and diluted into 1.2 M sucrose. Cells in the shoot tip top (T), middle (M), and lower (L) regions were quantified for cell and protoplast size to determine the extent of plasmolysis. *Bar* 0.1 mm

Recovery by micrografting

Vials containing shoot tips in the long-term experiment were warmed in a water bath at 38 °C for 1.5 min. Foil strips with shoot tips cryopreserved in PVS2 droplets were warmed by placing foil strips directly into 1.2 M sucrose + ½ MS liquid medium at 22 °C for 15 s. After warming, all shoot tips were incubated in 1.2 M sucrose + ½ strength MS liquid medium for 20 min at 22 °C and subsequently plated onto Citrus recovery medium [Woody Plant Medium, WPM salts (McCown and Lloyd 1981; supplemented with MS vitamins, 50 g/L sucrose, 7 g/L agar at pH 5.7)] for 18 h at 25 °C in the dark.

Table 1 Viability level (by species) of shoot tips after cryopreservation

Species	Common name	Accessions processed (no.)	Regrowth (%)
Citrus aurantifolia	Lime	2	45 ± 15
Citrus bergamia	Bergamot orange	1	80
Citrus clementina	Mandarin-clementine	8	66 ± 7
Citrus hybrid	Hybrid	3	37 ± 12
Citrus hystrix	Papeda	1	10
Citrus junos	Papdea-hybrid	1	80
Citrus latifolia	Lime	2	25 ± 15
Citrus limetta	Sweet lime	1	50
Citrus limettioides	Sweet lime	1	50
Citrus limon	Lemon	13	65 ± 5
Citrus limonia		1	50
Citrus madurensis	Calamondin	1	90
Citrus maxima	Pummelo	3	43 ± 3
Citrus medica	Citron	1	100
Citrus meyeri	Lemon hybrid	1	80
Citrus nobilis	King orange	3	63 ± 12
Citrus paradisi	Grapefruit	4	35 ± 16
Citrus pseudolimon	Gulgal	1	70
Citrus pyriformis		1	80
Citrus reticulata	Mandarin	18	60 ± 6
Citrus sinensis	Orange	36	64 ± 3
Citrus sudachi		1	90
Citrus tangerina	Mandarin	1	20
Citrus temple	Tangor	1	70
Citrus unshiu	Mandarin	18	63 ± 6
Citrus × *tangelo*	Tangelo	5	50 ± 13
Fortunella crassifolia	Kumquat	1	50
Fortunella margarita	Kumquat	2	80 ± 20
Microcitrus australasica	Microcitrus	1	10
Poncirus trifoliata	Trifoliate orange	2	40 ± 0
×*citrofortunella* spp.		2	35 ± 35
×*citroncirus* spp.	Citrumelo	5	38 ± 14
Overall		142	56 ± 4

Samples infected with bacteria were removed from final count

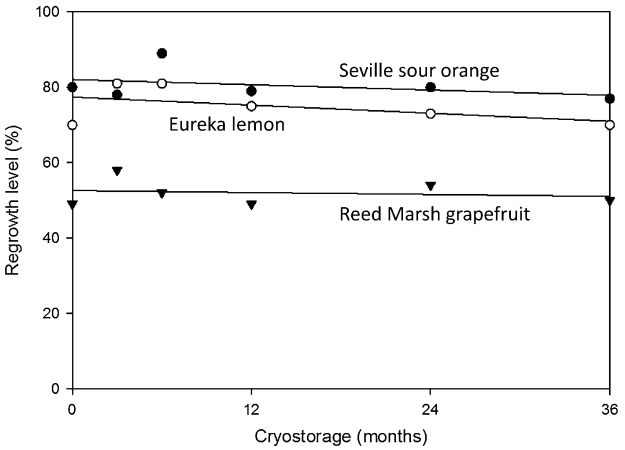

Fig. 2 Mean viability of citrus shoot tips of cultivars 'Seville sour orange', 'Eureka lemon', and 'Reed Marsh grapefruit' after 0–36 months of cryostorage. Regression lines are provided between the 0 and 36 month datapoints for each cultivar

For micrografting, seeds of 'Carrizo' citrange rootstock (PI 150,916 × *Citroncirus* sp.) were peeled, surface disinfected with 0.6% sodium hypochlorite for 10 min and cultured in 25 × 150 mm test tubes with 20 mL of seed germination medium (½ strength MS inorganic salts supplemented with 13.9 mg/L ferrous sulfate heptahydrate, 18.7 mg/L disodium EDTA, 50 mg/L myo-inositol, 25 g/L sucrose and 7 g/L agar at pH 5.7). Seedlings were kept in the dark for up to 6 weeks before use as rootstocks. Seedlings with a height of at least 3 cm were cut off 1 cm above the cotyledonary node and a 2 mm deep incision was made to bisect the cut surface, followed by a perpendicular cut from the edge of the seedling. The warmed shoot tips were trimmed by removing 0.2 mm of tissue from the basal portion and placed on the rootstock ledge. Grafted seedlings were transferred to micrografting recovery medium (MS inorganic salts supplemented with 100 mg/L myo-inositol, 0.2 mg/L thiamine-HCl, 1 mg/L pyridoxine HCl, 1 mg/L nicotinic acid, 75 g/L sucrose, 7 g/L agar at pH 5.7) in 25 × 150 mm test tubes with 25 mL of medium per tube. The plants were then cultured under fluorescent lights (100 µM m^{-2} s^{-1}) with a 16 h photoperiod at 25 °C. Side shoots produced by the rootstocks were removed weekly. Regrowth data were collected 6–8 weeks after micrografting.

Microscopy

The following treatments of *C. limon* 'Eureka' lemon PI 658,388 were sampled from greenhouse-grown plants for

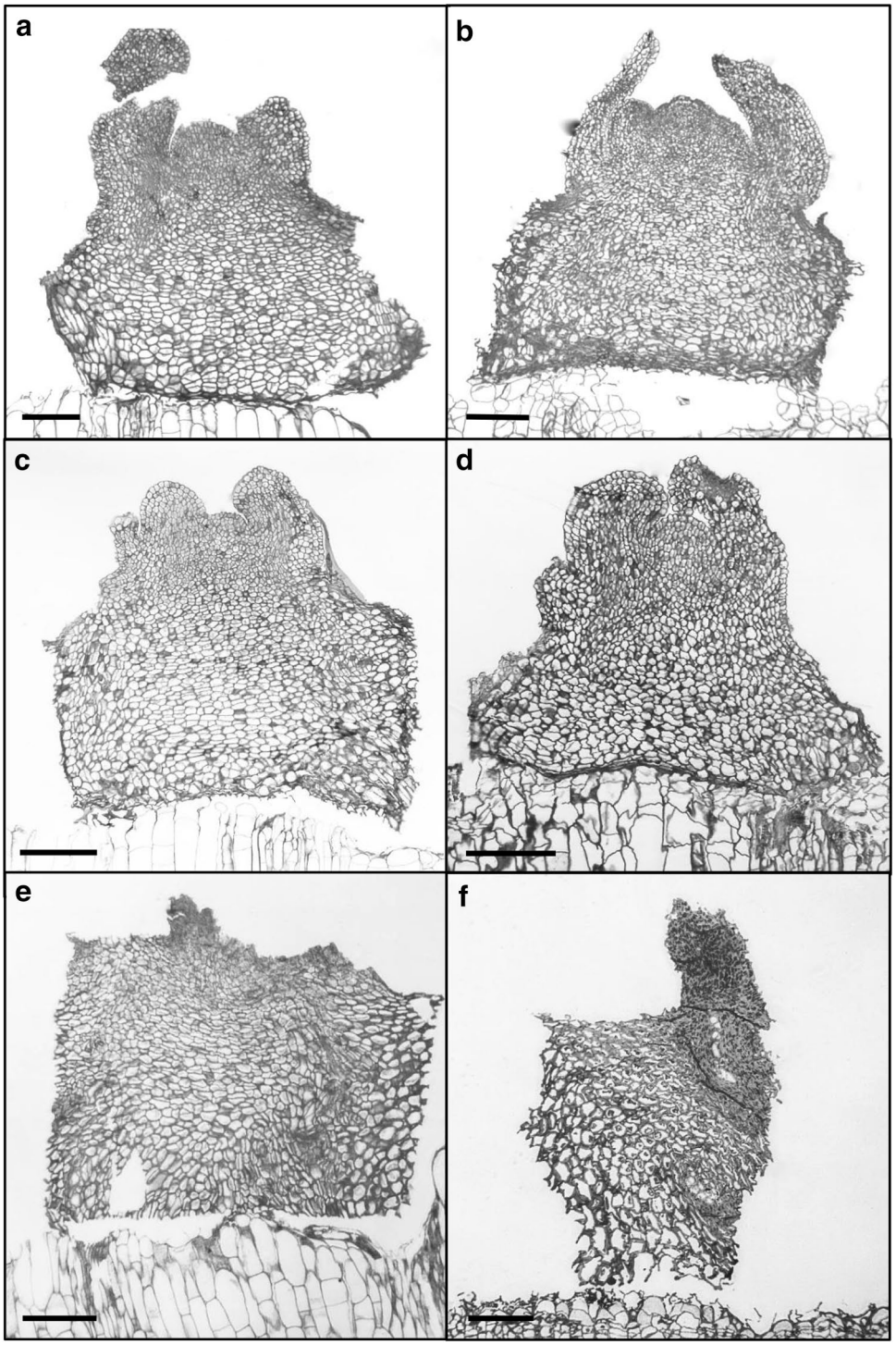

Fig. 3 Cross section of *Citrus limon* 'Eureka lemon' shoot tips sampled at either 2 or 5 days after micrografting. Shoot tips were treated with cryoprotectants, exposed to LN, warmed, micrografted and regrown for 2 (**a**) or 5 (**b**) days. Control shoot tips were treated with cryoprotectants, not exposed to LN, micrografted and regrown for 2 (**c**) or 5 (**d**) days. Dead controls were excised shoot tips plunged into LN without prior cryoprotectant treatment, warmed, micrografted, and regrown for 2 (**e**) or 5 (**f**) days. *Bar* 0.1 mm

histological observations: (1) control, fresh cut shoot tips micrografted and regrown for 5 days; (2) shoot tips treated with cryoprotectants, not exposed to LN, and diluted with 1.2 M sucrose; (3) treatment 2 shoot tips incubated overnight, micrografted and regrown for 1, 2, or 5 days. Cryo-exposed treatments were as follows (1) shoot tips treated with cryoprotectants, plunged on foils into LN, warmed and diluted with 1.2 M sucrose; (2) treatment 1 shoot tips incubated overnight, micrografted and regrown for 1, 2, or 5 days. Dead controls were excised shoot tips plunged into LN without prior cryoprotectant treatment, warmed, incubated overnight, micrografted, and cultured on rootstocks for 2 or 5 days. Regrowth levels were 100 % for fresh cut and non-LN treated shoot tips, 75 % for shoot tips treated with cryoprotectants and LN, and 0 % for dead controls.

Table 2 Cell size within three regions (top, middle, and bottom) of Citrus shoot tips

Recovery (days)	Shoot tip region	Cell area ± SE (μm^2)			
		−LN		+LN	
0	Top	42 ± 4	a	48 ± 2	a
1	Top	41 ± 4	a	44 ± 7	a
2	Top	38 ± 5	a	49 ± 1	a
5	Top	40 ± 1	a	36 ± 4	a
5-Fresh control	Top	41 ± 4	a		
0	Middle	108 ± 21	b	89 ± 21	b
1	Middle	109 ± 10	b	88 ± 12	b
2	Middle	86 ± 9	b	93 ± 8	b
5	Middle	78 ± 10	b	87 ± 3	b
5-Fresh control	Middle	78 ± 5	b		
0	Lower	763 ± 348	c	269 ± 105	c
1	Lower	532 ± 205	c	170 ± 27	c
2	Lower	126 ± 4	c	158 ± 4	c
5	Lower	137 ± 26	c	145 ± 7	c
5-Fresh control	Lower	130 ± 10	c		

Shoot tips were treated with cryoprotectants and exposed to LN (+LN) or not exposed to LN (−LN) prior to warming, micrografting and recovery for 0–5 days. Fresh control shoot tips were not treated with cryoprotectants or LN. Ten cells within each region were measured for three shoot tips

Table 3 Extent of plasmolysis in shoot tip cross sections

Recovery (days)	Liquid nitrogen exposure	Location	Plasmolyzed cells (%)	Fraction of cross-sectional cell area that is not plasmolyzed
0	−LN	Lower	30	0.45 ± 0.05
1	−LN	Lower	23	0.44 ± 0.06
1	+LN	Lower	7	0.35 ± 0.06

Of the 30 cells observed (from 3 shoot tips) for each of the conditions in the table, three treatments resulted in cells that exhibited plasmolysis. The percent of cells that were plasmolyzed and the fraction of the cross-sectional cytoplasm that was retained after plasmolysis are provided

A minimum of 10 shoot tips from each treatment were placed into fixative (1.25% glutaraldehyde, 2% paraformaldehyde, and 50 mM Pipes buffer) overnight. After an overnight incubation in fixative at 4 °C, samples were rinsed three times (10 min each) with 50 mM Pipes buffer, then post-fixed with 2% osmium tetroxide in 50 mM Pipes buffer overnight at room temperature. Samples were then rinsed three times with 50 mM Pipes buffer, 10 min per rinse, and dehydrated with 30, 50, 70, 90, 100% acetone (two times), for 15 min each. Samples were infiltrated with Spurr resin (Electron Microscopy Sciences, Hatfield, PA) over several days and polymerized at 70 °C overnight. Samples were sectioned with glass knives on an RMC MT-X microtome (Ventana Medical Systems Inc., Tucson, AZ) and mounted on glass slides. Sections were counterstained with Stevenel's Blue (del Cerro et al. 1980) and visualized with an Olympus BH-2 microscope (Olympus Optical Co., Tokyo, Japan). Images were digitally captured with a Leica MC170 HD digital camera (Wetzlar, Germany).

Cells were quantified using ImageJ software (Abramoff et al. 2004). Ten representative cells were measured from three regions (top-meristem, mid-portion of shoot tip, lower region of shoot tip; Fig. 1) for each of three shoot tips for each treatment. Cell cross-sectional area and protoplast cross-sectional area were measured for each cell. Means were calculated for the 10 cells within each region of each shoot tip, and the three means for each shoot tip were used for statistical comparisons among cell types and treatments. The fraction of cross-sectional cell area that was not plasmolyzed was calculated by dividing the protoplast cross sectional area by the cell cross sectional area in cells that exhibited plasmolysis.

Results

Citrus cryopreservation

Shoot tip regrowth levels averaged 56% after LN exposure across the 32 citrus species, relatives, and hybrids processed for cryopreservation. Of the 150 accessions processed, eight exhibited bacterial contamination and had low viability levels (0% regrowth for five accessions and 10, 15, and 50% regrowth for the remaining three accessions). These contaminated accessions were not included in the regrowth data summaries. The largest numbers of accessions per species were processed for *C. limon*, with 65% regrowth for 13 accessions, *C. reticulata* with 58% average regrowth for 18 accessions, *C. sinensis* with 64% average regrowth for 36 accessions, and *C. unshiu* with 61% regrowth for 18 accessions (Table 1).

A multi-year experiment was designed to revive cryopreserved shoot tips after extended storage intervals. No significant changes in regrowth levels occurred after *C. aurantium* 'Seville sour orange', *C. limon* 'Eureka' lemon, and *C. paradisi* 'Reed Marsh grapefruit' shoot tips were stored in LN vapor for 3 years (Fig. 2).

Histology

Histological observations revealed a high level of cellular preservation after LN exposure and subsequent micrografting in citrus. Micrografted shoot tips were observed after 2 and 5 days of regrowth (Fig. 3). There were no differences in

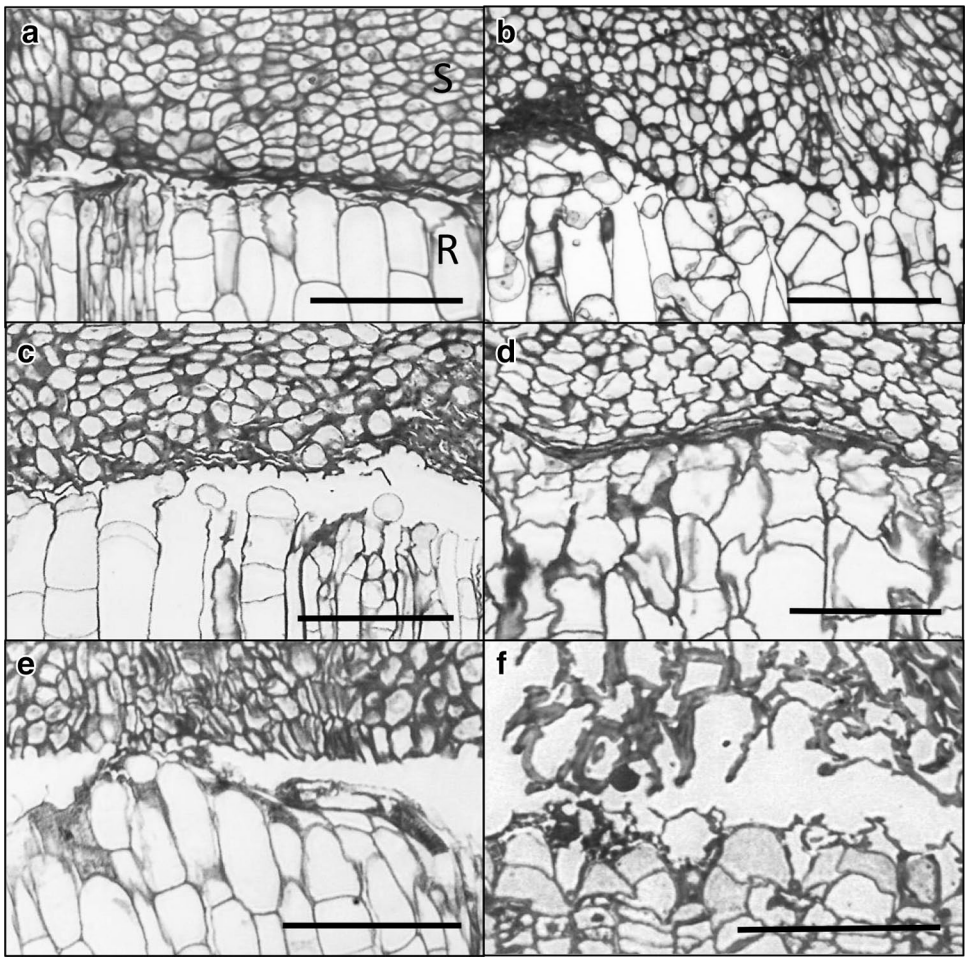

Fig. 4 Graft union interface between the shoot tip (S) and the rootstock (R) of *Citrus limon* 'Eureka lemon' shoot tips treated with cryoprotectants, exposed to LN, warmed, micrografted and regrown for 2 (**a**) or 5 (**b**) days. Control shoot tips were treated with cryoprotectants, not exposed to LN, micrografted and regrown for 2 (**c**) or 5 (**d**) days. Dead controls were excised shoot tips plunged into LN without prior cryoprotectant treatment, warmed, micrografted, and regrown for 2 (**e**) or 5 (**f**) days. *Bar* 0.1 mm

meristematic cell cross sectional area (shoot tip region = top; mean = 42 μm^2) in all the treatments (Table 2). Similarly, there were no differences in the cell cross sectional area in the cells sampled from the middle region of the shoot tips (shoot tip region = middle; mean = 90 μm^2); however, these cells were significantly larger than those in the meristematic region (Table 2). None of the cells in the meristematic or middle regions exhibited plasmolysis. Cells in the lowermost region (shoot tip region = lower; mean = 270 μm^2) were not significantly different from one another, but were larger than those in the upper and middle regions (Table 2). Some of the −LN 0 day regrowth, −LN 1 day regrowth, and +LN 1 day regrowth cells were plasmolyzed. These cells retained protoplasts (in cross sectional area) that were 0.35–0.45 of the cell wall surface area (Table 3).

Investigation of the interface between the seedling rootstock and shoot tip cells revealed early signs of cell growth from the rootstock at the shoot tip interface as early as 2 days after micrografting (Fig. 4). Five days after micrografting cells appear to have grown together between the rootstock and the shoot tip in some regions of the interface. Cells of the dead control shoot tips did not exhibit regrowth from either the rootstock or shoot tip (Fig. 4).

Discussion

We demonstrate that a single cryopreservation method was effective for cryopreserving diverse citrus genetic resources. Viability data show a wide applicability of this method with mean regrowth levels of more than 40% in 24 of the 32 taxa. Accessions of key species such as *C. limon, C. reticulata, C. sinensis*, and *C. unshiu* had high viabilities.

Two primary reasons for the efficiency and success of this citrus cryopreservation effort were the use of budwood collected from pathogen-tested trees maintained in a protected environment (screenhouse) and the use of micrografting for shoot tip recovery. Successful citrus micrografting was described by Navarro et al. (1975). It has since been used in many applications including recovery of shoot tips after pathogen elimination, regeneration after *Agrobacterium tumefaciens*-mediated transformation, multiplication of difficult-to-root plants, and recovery of haploid or tetraploid plants as well as abnormally and normally developed cybrid embryos (Aleza et al. 2016; Chae et al. 2013; Helliot et al. 2002; Hussain et al. 2014; Navarro 1988; Peña et al. 1995, 1997; Vidalakis et al. 2010). Our work in citrus

cryopreservation demonstrates the effectiveness of micrografting for recovery after cryopreservation (Volk et al. 2012, 2015).

Histological observations at the light microscope level revealed that citrus shoot tips appeared to be mostly undamaged by LN exposure. Cells within the shoot tip, including in the meristem, middle, and lower shoot tip regions were primarily unplasmolyzed (with the exception of some of the larger cells in some of the lower regions in some treatments). The apparently undamaged cells (as observed in the light microscope) in citrus differ from cells observed in other cryopreserved shoot tip systems. For example, lily shoot tips had some damaged cells with weakly stained cytoplasm in the meristematic region and many damaged cells in the lower part of the apical dome and in expanded cells of the leaf primordia after LN exposure (Yin et al. 2014). Similarly, ultrastructural studies of cryopreserved banana shoot tips revealed that small areas of cells in the meristematic dome and at the base of the leaf primordia survived LN exposure (Helliot et al. 2003). In potato, regrowth after LN exposure appeared to be primarily in the regions of the leaf primordia (Kaczmarczyk et al. 2008). In apple, many of the apical dome and leaf primordia cells survived LN, whereas other cells in the shoot tips were severely damaged (Li et al. 2015). In cryopreserved mint shoot tips, plasmolysis occurred in most cell types, including regions of the meristem. Meristem cells appeared to recover; and more elongated cell types were permanently damaged (Volk and Caspersen 2007). In citrus, the many undamaged shoot tip cells may have helped provide nutrients from the micrografted rootstock to the growing meristem.

Observations of the graft union revealed that cells appear to proliferate from the rootstock to the shoot tip within 2 days of micrografting, allowing the shoot tip to adhere to the rootstock throughout the embedding process. One-day micrografted shoot tips did not remain attached to the rootstock during the embedding process (data not shown). These observations were similar to those observed 3 days after micrografting in passionfruit (Ribeiro et al. 2015).

This work demonstrates that our described citrus cryopreservation procedure can be applied to diverse citrus genetic resources. The availability of this method for long-term preservation of citrus is critical, particularly because key cultivars and breeding lines may be lost as a result of their susceptibility to pathogens.

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Author contributions G. V. designed project, analyzed data and wrote the manuscript. R. B., A. S., and Z. Y. designed and performed experiments and edited manuscript. R. L. and M. P. provided critical project suggestions and edited manuscript. R. K. provided prioritized plant materials and edited manuscript.

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