Cryopreservation of Citrus for Long-Term Conservation

Gayle M. Volk[1,*], Remi Bonnart[1], Ashley Shepherd[1], Robert R. Krueger[2] and Richard Lee[2]
[1]United States Department of Agriculture, Agricultural Research Service (USDA-ARS), National Center for Genetic Resources Preservation, Fort Collins, Colorado, USA
[2]United States Department of Agriculture, Agricultural Research Service (USDA-ARS), National Clonal Germplasm Repository for Citrus and Dates, Riverside, California, USA
*Corresponding author: Gayle.Volk@ars.usda.gov

Abstract

More than 850 varieties of *Citrus*, *Fortunella,* and Citrus-related species are maintained within the USDA-ARS National Plant Germplasm System and the University of California Citrus Variety Collection. These genetic resources are held within duplicated field, screenhouse, and greenhouse collections and are at risk of disease infestations, abiotic stresses, and natural disasters. We have developed a cryopreservation method by which we can back up citrus genetic resources for the long term at liquid nitrogen temperatures. We excise shoot tips directly from greenhouse or screenhouse source plants, surface sterilize, and then treat with cryoprotectants. These solutions dehydrate and allow the shoot tips to survive liquid nitrogen exposure. Plants are recovered by micrografting thawed shoot tips onto 'Carrizo' seedling rootstocks. Experiments that compared survival after freezing in cryovials vs. on aluminum foil strips reveal higher levels and more uniform survival when foil strips were utilized. High viability was maintained for at least 1 year. These data further support the use of shoot tips to conserve diverse citrus cultivars in genebank collections.

Keywords: cryopreservation, citrus, genetic resources, genebank

INTRODUCTION

World citrus collections provide researchers, breeders, and industry members access to diverse genetic resources. These collections are usually maintained in field, greenhouse, or screenhouse conditions, making them susceptible to both environmental and biotic threats. Several of the primary U.S. collections of Citrus are maintained in California. These include the Citrus Variety Collection (CVC), the Citrus Clonal Protection Program (CCPP), and the National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) (Kahn et al., 2001). More than 865 accessions of Citrus and related genera are maintained in the CVC, which is based at the University of California Riverside. The CCPP provides researchers and members of the California citrus industry with budwood of Citrus scion and rootstock varieties that are free of bud-transmitted diseases. This collection is maintained at the Lindcove Research and Education Center in the San Joaquin Valley. The NCGRCD, in Riverside, CA, facilitates and encourages the free exchange of genetic resources to qualified scientists worldwide. This collection has over 450 virus-free accessions that are maintained under screenhouse conditions (Kahn et al., 2001).

Although there are some built-in duplications among these collections, the threats of Huanglongbing, *Citrus tristeza virus*, *Citrus tatter leaf virus*, Citrus canker, Citrus black spot, and Sweet orange scab as well as extreme temperatures and stormy weather patterns have emphasized the vulnerability of these collections.

Secondary locations provide secure back-ups for key collections of genetic resources. Seed collections, such as those maintained at the USDA-ARS National Center for Genetic Resources Preservation in Fort Collins, Colorado, Kew Millennium Seed Bank housed in West Sussex, England, and the Svalbard Global Seed Vault in Longyearbyen, Norway are several examples of these key conservation sites. Crops that are clonally propagated or that have seeds that are not amenable to conventional storage

regimes require alternative strategies for security back-ups. In some cases, clonal plant collections have been physically duplicated at multiple locations, while others have been backed-up as alternative storage forms, such as in vitro cultures (Postman et al., 2006). Cryopreservation, a process by which explants (from seeds or shoots) are cooled to liquid nitrogen temperatures, has been shown to be a successful strategy for backing up some collections of plant genetic resources (Volk and Walters, 2003; Engelmann, 2011).

Methods for cryopreserving citrus somatic embryos, embryogenic calli, cell suspensions, and embryonic axes have been published, but their implementation in genebanks has been limited (Engelmann et al., 1994; Santos and Stushnoff, 2003; Gonzalez-Arnao et al., 2008). Citrus shoot tip cryopreservation methods have also been developed, but these methods have not been shown to have broad applicability across diverse citrus species (Gonzalez-Arnao et al., 1998; Al-Ababneh et al., 2002; Wang et al., 2002, 2003). Recently, we published a method for cryopreserving shoot tips excised from citrus vegetative buds derived from greenhouse or screenhouse-grown stock plants (Volk et al., 2012). This method has the advantage of not requiring plants to be propagated in vitro prior to cryopreservation treatments. In addition, warmed shoot tips are recovered by grafting onto in vitro seedling rootstocks (Navarro et al., 1975; Edriss and Burger, 1984; Sertkaya, 2004). This eliminates the need for optimized tissue culture media for diverse citrus cultivars. The availability of this method has increased the likelihood of adoption of citrus cryopreservation techniques in citrus collections worldwide.

We present the results of several experiments that demonstrate the effectiveness and improvement upon the published citrus cryopreservation method that utilizes screenhouse or greenhouse stock materials as budwood sources and makes use of micrografting as a recovery technique after cryoexposure.

MATERIALS AND METHODS

Citrus accessions used for cryopreservation experiments were provided by the NCGRCD from stock plants grown in screenhouses in Riverside, CA. Some species were subsequently propagated in the Colorado State University greenhouses and used for long-term liquid nitrogen exposure experiments. They included 'Seville' sour orange (*Citrus aurantium* L.), 'Eureka' lemon (*C. limon* (L.) Burm f.) and 'Reed Marsh' grapefruit (*C. paradisi* Macf.).

Vegetative budsticks at the triangular growth stage (3 to 5 mm diameter) were collected 2-6 weeks after growth flushes occurred. After harvest, budwood was kept in sealed plastic bags and delivered to NCGRP. Budsticks were kept at 4°C for up to 4 weeks prior to shoot tip excision. The budwood was cut into 1 cm nodal sections and surface-disinfested with 70% isopropanol for 2 min, 10% bleach for 10 min, followed by 3-10 min rinses with sterile water. Shoot tips were then excised from axillary buds for cryopreservation experiments.

As was previously reported, shoot tips were cultured overnight in 0.3 M sucrose + ½ MS medium in darkness at 25°C, immersed in 2 M glycerol+0.4 M sucrose in ½ MS for 20 min at 22°C, then treated with plant vitrification solution 2 (PVS2; 30% w/v glycerol, 15% w/v dimethylsulfoxide (DMSO), 15% w/v ethylene glycol and 0.4 M sucrose prepared in ½ MS; Sakai et al., 1990) for 30 min at 0°C (Volk et al., 2012). Shoot tips were either transferred to 1.2 ml cryovials containing 1 ml PVS2 and plunged into liquid nitrogen (long term exposure experiment) or placed into droplets upon aluminum foil strips which were plunged directly into LN. Vials were kept in the vapor phase of liquid nitrogen for up to 1 year. Vials and foils were kept in liquid nitrogen for a minimum of 2 h for the experiments that compared freezing vessels. For warming, vials were placed in a water bath at 38°C for 1.5 min. Shoot tips were then placed in 1.2 M sucrose + ½ MS liquid medium for 20 min at 22°C and post-cultured overnight on citrus embryo 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.

Seeds of 'Carrizo' citrange rootstock (PI 150916, × *Citroncirus* sp.) were peeled,

surface disinfected with 10% bleach for 10 min and cultured in 25×150 mm test tubes with 20 ml of seed germination medium (1/2 strength MS inorganic salts supplemented with 27.8 mg/L ferrous sulfate heptahydrate, 37.3 mg/L disodium EDTA, 50 mg/L myo-inositol, 25 g/L sucrose and 7 g/L agar at pH 5.7). Seeds were germinated in the dark for up to 6 weeks and then used as micrografting rootstocks. Seedlings with a height of at least 3 cm were sliced 1 cm above the cotyledonary node and a 2 mm deep incision was made to bisect the cut surface, followed by a perpendicular cut to the edge of the seedling. Recovered shoot tips were then trimmed (0.2 mm from the basal portion) and placed on the rootstock ledge created by the incision.

Micrografted seedlings were placed in 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. Micrografted plants were cultured at 25°C under 16 h photoperiod provided by fluorescent lights (100 μmol/m^2/s) and examined weekly. Side shoots produced by the rootstocks were removed. Two or three vials with 10 shoot tips each were thawed for each timepoint in the storage experiment. A single vial with 10 shoot tips was thawed for the foils vs vials experiment. Remaining shoot tips were kept back-ups for accessions in the NCGRCD genebank. Regrowth data were collected after at least 4 weeks.

RESULTS AND DISCUSSION

In 2011, more than 240 shoot tips of the three accessions 'Seville' sour orange, 'Eureka' lemon, and 'Reed Marsh' grapefruit were placed into long-term storage within cryotanks at NCGRP. Three vials (10 shoot tips each) for each accession will be harvested after 0 months, 3 months, 6 months, 1 year, 2 years, 3 years, 5 years, and 10 years of liquid nitrogen exposure. We present the available recovery data for the 0 month, 3 month, 6 month, and 1 year timepoints for the long-term experiment (Table 1). No decline in viability was observed after 1 year of storage at liquid nitrogen temperatures. Some variation was observed among treatments, however, these results were not significant.

Table 1. Regrowth of micrografted citrus shoot tips after 0, 3, 6, or 12 months of storage in liquid nitrogen (+LN) or control vials that were treated with cryoprotectants, but not exposed to LN (-LN).

Species	Regrowth level after storage interval (months)				
	-LN	0 +LN	3 +LN	6 +LN	12 +LN
C. aurantium	0.92 ± 0.056	0.80 ± 0.059	0.78 ± 0.027	0.89 ± 0.074	0.78 ± 0.025
C. limon	0.89 ± 0.043	0.71 ± 0.109	0.81 ± 0.095	0.81 ± 0.122	0.79 ± 0.015
C. paradisi	0.62 ± 0.062	0.49 ± 0.063	0.58 ± 0.102	0.52 ± 0.050	n/a

We previously published citrus shoot tip regrowth levels between 0.40 and 0.65 when cryovials were used as the freezing vessel (Volk et al., 2012). We sought to increase these levels and decrease the variability among accessions by performing experiments to compare recovery after freezing in cryovials vs. freezing in droplets on foil strips (droplet vitrification; Sakai and Engelmann, 2007). A total of 100 shoot tips for each accession were cryopreserved. Of these, 10 shoot tips that were frozen on foil strips and a cryovial containing 10 shoot tips that were frozen directly in PVS2 in the vial were thawed to ascertain viability. Across the seven accessions, regrowth levels after LN exposure ranged from 0.70 to 1.00 for shoot tips frozen using the droplet vitrification technique (foil strips) and from 0.30 to 0.90 for shoot tips frozen within solution in cryovials; significantly higher mean recovery levels were obtained using the droplet vitrification method (Table 2). Previously, we reported freezing rates of -106°C/s when shoot tips were frozen on foil strips, compared to freezing rates of -3°C/s with vials (Volk and Walters, 2003). These

faster freezing rates appear to unify the regrowth across diverse accessions, thus making the method more easily implemented within citrus collections. Others have reported high levels of success using droplet vitrification methods for plant shoot tips of other species (Volk et al., 2004; Lee et al., 2011; Kim and Lee, 2012).

Table 2. Regrowth of micrografted citrus shoot tips after freezing on foil strips (droplet vitification) or in PVS2 solution in cryovials.

Species	Cultivar	Accession number	Regrowth level	
			Foil strips	Cryovials
C. sinensis	Fukumoto	PI 539577	0.70	0.50
C. reticulata	Gold Nugget	PI 539532	1.00	0.30
C. reticulata	Seedless Kishu	PI 539530	0.70	0.30
C. unshiu	Owari (Frost #1)	PI 539689	1.00	0.90
C. clementina	Sidi Aissa	PI 539185	0.90	0.40
C. clementina	Clemenules	PI 654845	0.80	0.60
× *citroncirus* sp.	Carrizo	PI 150916	0.70	0.70
Mean			0.83 ± 0.052 a	0.53 ± 0.083 b

The current data demonstrate that shoot tips cryopreserved within vials using the previously published method by Volk et al. (2012) remain viable for at least 1 year when kept at liquid nitrogen temperatures. In addition, regrowth improved when shoot tips were cryopreserved using the droplet vitrification technique, compared to freezing shoot tips within vials. These data further support the use of shoot tips cryopreservation methods to conserve diverse cultivars of citrus in genebank collections.

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