Implementation of *Citrus* shoot tip cryopreservation in the USDA-ARS National Plant Germplasm System

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Abstract

The USDA-ARS National Plant Germplasm System (NPGS) maintains a citrus genetic resource collection of 1522 accessions of *Citrus* cultivars and crop wild relatives. Of these, 540 are maintained as pathogen-free duplicate clones in a screenhouse at the National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) in Riverside, California. An additional 815 accessions of citrus and citrus relatives are maintained in greenhouses or orchards in Riverside until sanitation for pathogens can be completed. This paper describes progress in backing up the 540 pathogen-tested accessions using cryopreservation techniques and methods to accelerate processing and viability testing. Vegetative budwood was harvested from trees in the screenhouse and sent to NLGRP. At least 170 shoot tips from each accession were excised from surface-sterilized budwood, and then cryopreserved using a PVS2 droplet-vitrification technique. Viability was assessed by micrografting 10 thawed shoot tips onto in vitro grown 'Carrizo' seedling rootstock. The remaining shoot tips are held in long-term liquid nitrogen storage. A total of 451 of the pathogen-tested citrus accessions were cryoprocessed: 354 accessions had regrowth levels of 40% or greater, 17 had 0% regrowth, 47 had viability levels between 10 and 30%, and 33 had endogenous contaminants. Technical staff familiar with shoot-tip cryoprocessing could be fully trained for specific applications of citrus in about 2 months and thereafter processed a single accession of at least 170 shoot tips in about 16 h. This large-scale effort has revealed that shoot tip cryopreservation can be successfully scaled-up to secure the NPGS *Citrus* collection.

Keywords: *Citrus*, cryopreservation, PVS2, shoot tip, vitrification

INTRODUCTION

The USDA-ARS National Plant Germplasm System (NPGS) maintains a collection of 1522 accessions of citrus cultivars and crop wild relatives, representing 141 taxa, at the National Clonal Germplasm Repository for Citrus and Dates in Riverside, California. The collection is maintained by grafted cuttings grown within pots in screenhouses and/or greenhouses, with some accessions also planted as trees in the California Citrus Variety Collection and maintained in collaboration with the University of California, Riverside. The NPGS citrus collection has 540 accessions that are pathogen-tested and presumed free of all known graft-transmissible pathogens. In addition, 815 citrus and citrus relative accessions are maintained in greenhouses until sanitation for pathogens is complete.

A droplet-vitrification method, using plant vitrification solution 2 (PVS2) as cryoprotectant, was developed to cryopreserve citrus shoot tips (Volk et al., 2012, 2015, 2017a). Vegetative budwood (current season's growth) was shipped from the pathogen-tested NPGS collection from Riverside, California to the National Laboratory for Genetic Resources Preservation (NLGRP) in Fort Collins, Colorado. Bud sections were surface sterilized and shoot tips were excised directly from the sterilized budwood, thus eliminating the process of introducing each cultivar into tissue culture and undergoing a multiplication

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step prior to shoot tip excision. At least 170 shoot tips for each citrus accession were processed (Volk et al., 2017b). Viability was tested on one or two vials of 10 shoot tips each The remaining 150-160 shoot tips from each accession are currently maintained in the vapor phase of liquid nitrogen (LN) (≥-180°C) at NLGRP.

Viability tests consisted of micrografting warmed, diluted shoot tips onto in vitro 'Carrizo' seedling rootstocks, and regrowth was assessed after eight weeks of culture. This micrografting step eliminated the need to optimize regrowth conditions for each unique accession in the genebank. We report herein on our multi-year effort to implement backup of the citrus collection using the cryopreservation protocol for shoot tips of citrus accessions within the NPGS.

MATERIALS AND METHODS

Plant materials

Cryopreservation of citrus shoot tips followed Volk et al. (2017a). Briefly, stock plants (pathogen-tested, protected), maintained in a screenhouse in Riverside, CA, were pruned in March to encourage flushes of growth that occurred four to five months after pruning. Shoot tips were excised from budwood that was 3 to 5 mm in diameter in the triangular growth stage. For most cultivars, the flushes were amenable for shoot tip excision until the following January, after which flower buds began to form and axillary vegetative buds began to break. Budwood that was shipped to NLGRP was processed immediately or stored for less than three weeks in plastic bags at 4°C.

Budwood was cut into single-node twig sections which were then surface sterilized with 70% isopropanol for 2 min, 0.6% sodium hypochlorite for 10 min, followed by 3 rinses with sterile water. Axillary shoot tips (1-2 mm, 170 per accession) were immediately excised within laminar flow hoods and then cultured overnight in 0.3 M sucrose + ½ strength MS (Murashige and Skoog, 1962) liquid medium in darkness.

Droplet-vitrification

At least 170 shoot tips were treated with 2 M glycerol + 0.4 M sucrose in ½ strength MS for 20 min at 22°C and then 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) for 30 min at 0°C. About 10 shoot tips were positioned onto sterile aluminum foil strips containing droplets of PVS2 and plunged into LN. Each foil strip was placed in a 1.2 mL cryovial, for a total of 17 cryovials per accession. Cryovials were divided among three 'cryocanes' with five vials per cryocanes. The cryocanes have been kept in the vapor phase of liquid nitrogen within LN tanks with a 915-L capacity (Cryenco 50 MX, Chart Inc., Denver, CO). Upon transfer to the long-term storage vaults, the cryocanes will be stored in a combination of liquid phase and vapor phase cryotanks with 915 L capacities. These tanks have monitoring systems and alarm systems that are activated when the temperature within the tanks become warmer than the alarm setpoints. The remaining two cryovials for each accession, designated for regrowth assessments, were set aside in a cryo-box, stored in the vapor phase of liquid nitrogen.

Regrowth assessments

A micrografting method was used to assess the viability of each accession. Shoot tips were warmed from liquid nitrogen, diluted into sucrose solution, and then plated onto medium. They were then trimmed before they were placed onto a seedling rootstock to allow for regrowth (Volk et al., 2012, 2015, 2017a).

Vials containing foil strips with cryopreserved shoot tips were removed from LN vapor. The foil strip was immediately taken out of a single cryovial for each accession and immersed in 1.2 M sucrose at 22°C for 20 min. Shoot tips were then plated onto citrus recovery medium (woody plant medium, WPM salts (McCown and Lloyd, 1981; supplemented with MS vitamins, 50 g L^{-1} sucrose, 7 g L^{-1} agar at pH 5.7)) for 18 h at 25°C in the dark. Shoot tips were then trimmed by removing approximately 0.2 mm of tissue from

the base of the shoot tip prior to micrografting.

Seedlings of 'Carrizo' (PI 150916, × *Citroncirus* sp.) were used as a rootstock for micrografting. The seed coat was removed from purchased seeds (Lyn Citrus Seed, Inc., Arvin, CA, USA), which were then surface disinfected with 70% isopropanol for 2 min, 1.2% (w/v) sodium hypochlorite for 20 min, rinsed three times with sterile water 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^{-1} ferrous sulfate heptahydrate, 18.7 mg L^{-1} disodium EDTA, 50 mg L^{-1} myo-inositol, 25 g L^{-1} sucrose and 7 g L^{-1} 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.

Each shoot tip was removed from the recovery medium, trimmed about 0.2 mm on the base to expose fresh tissue, and placed on each rootstock. Grafted seedlings were transferred to micrografting recovery medium (MS inorganic salts supplemented with 100 mg L^{-1} myo-inositol, 0.2 mg L^{-1} thiamine-HCl, 1 mg L^{-1} pyridoxine HCl, 1 mg L^{-1} nicotinic acid, 75 g L^{-1} sucrose, 7 g L^{-1} 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. Adventitious shoots that developed from the rootstock were periodically removed near the micrograft. Regrowth data were recorded after 8 weeks. The second cryovial of 10 shoot tips per accession was kept in storage in case it was deemed necessary to repeat the viability assessment.

Survival after 5 years

In 2011-2012, shoot tips of 'Seville' sour orange (PI 539169, *C. aurantium* L.), 'Eureka' lemon (PI 658388, *C. limon*), and 'Reed Marsh' grapefruit (*Citrus* × *paradisi*) were cryopreserved with the intention of assessing regrowth after 0 to 20 years of storage. Herein we report results for up to 66 months of cryostorage. These accessions were cryopreserved as described previously except that cryovials contained 1 mL of PVS2, rather than foil strips with droplets. Three cryovials of 10 shoot tips each were warmed for each cultivar × storage time combination by placing them in a water bath at 38°C for 1.5 min. Regressions were calculated based on the mean data for each viability time point for each cultivar.

RESULTS AND DISCUSSION

Implementation of the cryopreservation procedure

A total of 451 citrus accessions were cryopreserved between 2012 and 2017 at the NLGRP (Table 1). Of these, 354 accessions had viability levels that met our standards of at least 40% viability and 60 viable shoot tips (predicted) per accession (Table 2) (Jenderek and Reed, 2017). The taxa that were successfully cryopreserved represented many commercially important citrus cultivars, including *C. clementina* (clementine), *C limon* (lemon), *C. maxima* (pomelo), *C. paradisi* (grapefruit), *C reticulata* (tangerine), *C. sinensis* (sweet orange), × *Citroncirus* (citrange), and *C. unshiu* (mandarin). Some of the more distant crop wild relatives, including *Microcitrus australasica, C. daoxianensis, Fortunella obovata*, and *C. obovoidea* had low viabilities after cryopreservation; there were only one or two accessions representing each of those cultivars (Table 2).

Table 1. The number of unique citrus accessions processed for cryopreservation in the 2012-2013 to 2016-2017 seasons.

Processing year	Accn. processed (no.)
2012-2013	10
2013-2014	45
2014-2015	34
2015-2016	42
2016-2017	320
Total	451

Table 2. The number of accessions processed for cryopreservation for each taxon between 2012 and 2017. The number of accessions that met the 40% viability/60 predicted viable shoot tips are provided.

Taxon	Total accn. processed (no.)	Accn that met criteria (no.)	Regrowth level of accn. that met criteria (%)	Accn that did not meet criteria (no.)	Regrowth level of accn. that did not meet criteria (%)	Accn. that met criteria (%)
C. asahikan	1	1	70			100
C. aurantiifolia	4	2	60	2	15	50
C. aurantium	6	4	83	2	0	67
C. bergamia	2	2	75			100
C. clementina	19	15	71	4	10	79
C. daoxianensis	1			1	30	0
C. deliciosa	3	1	60	2	10	33
C. hassaku	1	1	80			100
C. hiroshimana	1	1	80			100
C. hybrid	19	12	75	7	10	63
C. hystrix	1			1	10	0
C. iyo	2	1	90	1	0	50
C. jambhiri	4	3	97	1	30	75
C. junos	1	1	80			100
C. latifolia	3	1	40	2	0	33
C. limetta	2	2	55			100
C. limettioides	3	2	75	1	20	67
C. limon	43	39	78	4	16	91
C. limonia	2	1	50	1	0	50
C. macroptera	1	1	60			100
C. madurensis	2	1	90	1	0	50
C. maxima	24	18	64	6	3	75
C. medica	5	4	80	1	0	80
C. meyeri	1	1	80			100
C. myrtifolia	2	1	90	1	0	50
C. nobilis	6	6	73			100
C. obovoidea	1			1	0	0
C. paradisi	19	12	58	7	6	63
C. pseudolimon	1	1	80			100
C. pyriformis	1	1	80			100
C. reshni	1	1	70			100
C. reticulata	48	36	76	12	20	75
C. sinensis	117	106	75	11	15	91
C. sp.				1	10	10
C. sphaerocarpa	1	1	90			100
C. sudachi	1	1	90			100
C. sunki	1	1	90			100
C. tangerina	2			2	25	0
C. temple	1	1	70			100
C. unshiu	43	33	74	10	9	77
Fortunella crassifolia	1	1	50			100
Fortunella margarita	3	2	80	1	30	67
Fortunella obovata	1			1	0	0
Microcitrus australasica	1			1	10	0
Poncirus trifoliata	7	5	62	2	30	71
× *Citrofortunella* sp.	4	2	80	2	0	50
× *Citroncirus* sp.	24	15	71	5	12	63
× *Citroncirus webberii*	2	2	75			100
× *tangelo*	13	10	71	3	10	77
× *tangelo* hybrid	2	2	50			100
Total	451	354		97		
Mean			73		11	69

There were 47 accessions with regrowth levels between 10 and 30%. The viability of these accessions will be reassessed using the second vial of 10 shoot tips and repeating the micrografting process. Fifty accessions had 0% regrowth, of which 33 were contaminated

with bacteria. These 50 accessions will be reprocessed from screenhouse-grown trees in the coming years. Testing is underway to identify the bacterial contaminants.

A total of 89 accessions that have not been previously processed will be cryopreserved as soon as possible, as the project experienced a setback when the Riverside collection was placed under quarantine in 2017 due to the occurrence of Citrus Greening disease within Riverside County.

A total of 815 additional accessions in the NPGS collection have not yet been completely screened for pathogens. Those accessions will be cryopreserved when they are determined to be free of pathogens. A subset of those accessions represent crop wild relatives, for which seed or pollen preservation could be considered instead of shoot tips.

Long-term storage

Regrowth of shoot tips after five years of storage in LN vapor are shown in Figure 1. Shoot tip viability did not statistically change during the test period, as shown in Figure 1, where the regression lines have non-significant slopes.

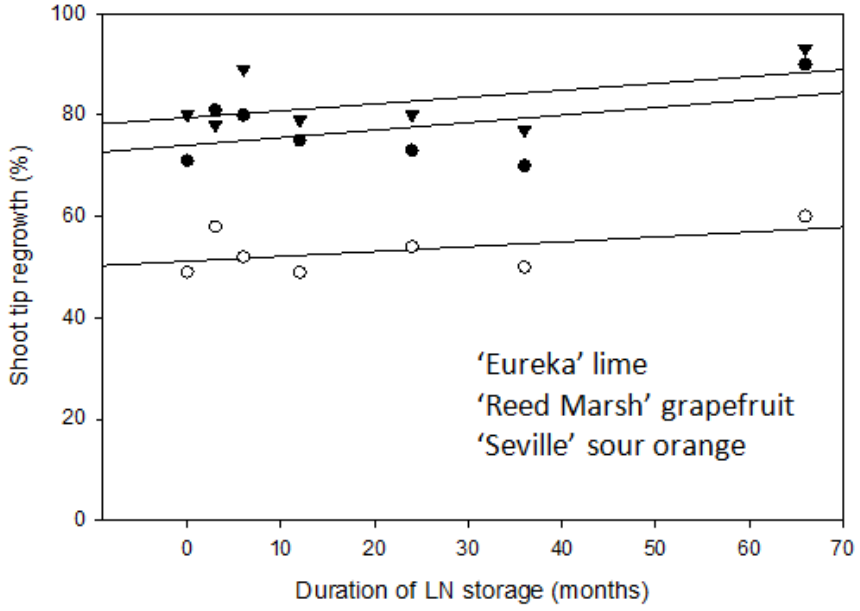

Figure 1. Viability of three citrus accessions that were cryopreserved and stored in LN vapor for 66 months. Mean viability for each timepoint and regression line provided.

Technical time commitments

We calculated the hands-on technical time required for the cryopreservation and micrografting processes. At the start of a concerted effort to process citrus in 2016, two technicians were considered experts with cryoprocessing citrus, two additional technicians were skilled in shoot tip cryopreservation of other species and required approximately three weeks of training and practice to acquire the skills needed to excise high quality shoot tips. A fifth technician was skilled in tissue culture, and required additional time for training (two one-week sessions), followed by four weeks of practice. Shoot tip micrografting skills were acquired after 4-5 weeks of practice for skilled tissue culture technicians.

Each technician cryopreserved three citrus accessions in a five day workweek. Shoot tips were excised on Mondays, Tuesdays, and Wednesdays, and cryoprocessing treatments were performed on Tuesdays, Wednesdays, and Thursdays for the previous day's excised shoot tips. This left one day (Friday) to prepare media and solutions, collect data, and perform other laboratory duties.

The micrografting portion of the project was performed when budwood was not

available for cryoprocessing (between May and July). Two expert technicians performed most of the micrografts and were each able to micrograft four accessions with 10 shoot tips per vial each in one day, so a total of 12 to 16 accessions each week per technician. Time for trimming micrografts and collecting data was considered nominal. One technician prepared and cultured the rootstock seeds, which required 4 h every two weeks. Thus the total time for citrus cryopreservation per accession is approximately 16 h, which is dependent upon the difficulty of the excision process for each *Citrus* species. This is less than half of the 35-40 h often required per accession for shoot tip cryopreservation (Volk and Walters, unpublished). The reduction in labor is primarily due to the use of budwood collected directly from the screenhouse, rather than going through a tissue culture multiplication step prior to shoot tip excision.

CONCLUSIONS

The citrus cryopreservation method employed was highly successful, particularly for the commercially important cultivars. The method was developed using diverse citrus species to ensure that it was widely applicable and a single rootstock cultivar could be used for most, if not all of the citrus accessions in the collection. Given the high labor inputs of shoot tip cryopreservation procedures, we identified modifications from cryopreservation procedures that would increase the procedural efficiency when it was implemented. Many hours of labor were saved by excising shoot tips directly from budwood and not introducing it into tissue culture prior to the cryopreservation regrowth-step. Overall, we demonstrate the implementation of a successful citrus cryopreservation procedure at NLGRP.

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