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CRYOPRESERVATION OF CITRUS SHOOT TIPS USING MICROGRAFTING FOR RECOVERY

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Abstract

The USDA-ARS National Plant Germplasm System (NPGS) and the University of California Citrus Variety Collection maintain more than 888 unique accessions representing 132 taxa of *Citrus*, *Fortunella*, and citrus wild species within field, screenhouse, and greenhouse collections. We have identified a cryopreservation method by which *Citrus* genetic resources that are not maintained *in vitro* can be successfully conserved. Shoot tips were excised from actively growing vegetative flushes of protected trees. Surface-disinfected shoot tips were precultured overnight in 0.3 M sucrose, loaded with a loading solution for 20 min and treated with PVS2 for 30 or 60 min at 0°C, prior to direct immersion in liquid nitrogen. Rewarmed shoot tips post-cultured overnight on survival medium were then micrografted on 'Carrizo' seedling rootstocks to produce whole plants. Micrografted shoot tips recovered quickly and rooted plants could be transferred to the greenhouse within months. Regrowth of whole plants after micrografting averaged 53% for cryopreserved shoot tips of cultivars representing eight *Citrus* and *Fortunella* species. This method has several advantages: it uses screenhouse or greenhouse plants as source materials, it is not dependent upon cultivar-specific recovery media, and it avoids seedling juvenility.

Keywords: citrus, cryopreservation, micrografting, shoot tip

INTRODUCTION

Citrus breeders, physiologists, and geneticists need access to diverse genetic materials to identify desirable traits for enhanced profitability. Improvements in citrus production are dependent upon availability of novel genetic resources that can provide new alleles with desired resistance, quality, or phenological traits to existing cultivars. State and Federal citrus collections preserve, evaluate and distribute pathogen-tested plant materials to the research community and provide pathogen-tested source material for the citrus industry.

The USDA-ARS National Plant Germplasm System and the University of California, Riverside (UCR), maintain a large collection of 132 taxa of *Citrus*, *Fortunella*, and citrus wild species relatives. The UCR field collection, also known as the Citrus Variety Collection (CVC), has duplicate plantings of 888 accessions within the field collection. The USDA-ARS

and CVC currently have their citrus field collections backed-up in screenhouses and greenhouses with protective screening to prevent insect entry.

The USDA-ARS protected collection has also undergone an intensive clean-up process followed by pathological screening for release of accessions from quarantine status so that pathogen-tested materials can be distributed to the user community. In many plant species, viruses can be eradicated from host plants by meristem or shoot tip culture (1, 11). However, species-specific elongation and differentiation media have not been identified for most citrus species and there is a high cultivar-to-cultivar variation in the effectiveness of regeneration medium within each species. In the 1970's, shoot tip grafting (micrografting) methods were shown to eradicate pathogens from citrus germplasm (16, 18). Shoot tips that are a fraction of a millimeter are excised and placed onto *in vitro*-grown rootstocks produced from seed (16). Pathogens such as viruses are phloem-limited from the meristematic region and *in vitro* grafted plants are potentially free of detectable disease (9).

Field collections of citrus are at risk due to the threat caused by the infestation of the Huanglongbing (HLB), which is caused by a bacterium, (*Candidatus liberibacter asiasticus*). HLB is spread by the psyllid vector *Diaphorina citri* in America and Asia (12, 28). The screenhouse and greenhouse back-up collections in California will minimize the loss of genetic diversity within these critical collections if a field HLB infestation were to occur. However, natural disasters such as hurricanes, tornadoes, and earthquakes have the potential to destroy the protective structures that house the citrus collections. Materials backed-up offsite in a secure facility are protected against biological and physical threats to critical national plant collections. Cryopreservation has been found to be a cost-effective method to conserve fruit collections in the National Plant Germplasm System (17, 23, 25). Citrus cryopreservation methods have been developed for a number of different explant types including embryonic axes (10, 21, 31, 32), embryogenic cultures (5), nucellar cells (20), somatic embryos (8) and *in vitro* shoot tips (2, 3, 7, 26, 27). However, none of these methods has been widely adopted for backing-up citrus collections either within the United States or internationally.

Buds and shoot tips are the preferred propagules for conservation for cultivars of clonally propagated crops. The published methods for citrus shoot tips rely upon having cultivars in tissue culture conditions (2, 3, 7, 26, 27, 30). *In vitro* collections are not currently available for the USDA-ARS or CVC collections and the establishment and maintenance costs for such collections make them prohibitively expensive. We have proposed using shoot tips excised directly from either screenhouse or greenhouse-grown plants as explants for the cryopreservation process. Compared to methods that require source materials to be multipled in tissue culture, our strategy decreases the labour inputs because buds are harvested directly from the materials in the germplasm collection. A modified micrografting procedure has been adopted, so species-specific media are not required for shoot tip recovery. Thus, our proposed method is applicable to diverse species maintained in protected environments and no medium optimization experiments are needed. Our results presented here show the adaptability of this methodology to a range of citrus species. Furthermore, the proposed cryopreservation method does not induce juvenility, which is a significant detriment to other tissue culture-based methods (15).

MATERIALS AND METHODS

Plant materials

Terminal shoots of *Citrus clementina* 'Clementina Fina Sodea' (PI 539186), *C. sinensis* 'Malta' blood orange (PI 654873), *C. aurantium* 'Smooth Flat Seville' sour orange hybrid (PI

654867), *C. limon* 'Allen old budline Eureka' lemon (PI 658388) were collected from growing plants maintained in screenhouses at the USDA-ARS National Clonal Germplasm Repository for Citrus and Dates (between May and November, 2010) and from field collections in the University of California Riverside Citrus Variety Collection (July 2010). Terminal shoots of *C. celebica* '*Citrus macrophylla*' papeda hybrid (PI 600628), *C. medica* 'Ethrog' citron (PI 508265), and *C. paradisi* 'Reed Marsh'grapefruit (PI 539471), and *Fortunella obovata* were sampled from greenhouse stock plants in Fort Collins, CO (between January and July, 2011).

Each of the shoots was 3 to 5 mm in diameter and contained 6 to 10 buds. The shoots were cut to a length of 15 to 20 cm, transported to the National Center for Genetic Resources Preservation in Fort Collins, CO and stored in sealed plastic bags at 4°C until use (ideally within 2 to 3 days).

Nodal sections (1 cm in length) were removed from the shoots and surface-disinfected (70% isopropanol for 2 min, 10% bleach for 10 min, followed by 3-10 min rinses with sterile water). Very tender tip material was surface sterilized with 70% isopropanol for 1 min and 5% bleach for 5 min. Shoot tips (1 mm) were excised from axillary buds for cryopreservation experiments.

Cryoexposure

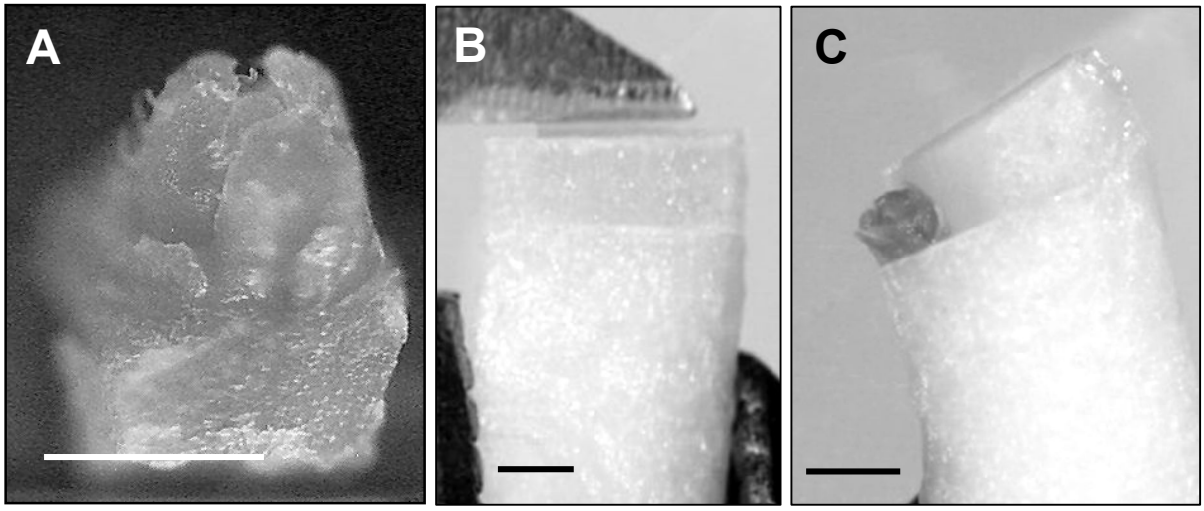
Shoot tips were cultured overnight in 0.3 M sucrose + ½ MS medium (14) in darkness at 25°C, treated with a solution of 2 M glycerol + 0.4 M sucrose, in ½ MS for 20 min at 22°C, followed by plant vitrification solution 2 [PVS2; 30% w/v glycerol, 15% (w/v) dimethylsulfoxide (DMSO), 15% (w/v) ethylene glycol and 0.4 M sucrose prepared in ½ MS (20)] for 30 min at 0°C. Shoot tips were transferred to 1.2 mL cryovials containing 1 mL PVS2 and plunged into liquid nitrogen. Cryovials were held in the liquid phase of liquid nitrogen for a minimum of 2 h prior to retrieving them.

Cryovials retrieved from LN were immediately immersed in a water bath at 38°C for 1.5 min to warm. Rewarmed shoot tips were treated with 1.2 M sucrose + ½ MS for 20 min at 22°C and post-cultured overnight on Citrus embryo medium [Woody Plant Medium, WPM salts (13); supplemented with MS vitamins, 50 g/L sucrose, 7g/L agar at pH 5.7] for 18 h at 25°C in darkness.

Micrografting

Seeds of 'Carrizo' rootstock (PI 150916, X *Citroncirus* sp.) were peeled, surface-disinfected with 10% bleach for 10 min and cultured in 25 x 150 mm test tubes each containing 20 mL of seed germination medium (½ 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 darkness for up to 6 weeks until use as rootstocks for micrografting. Etiolated 'Carrizo' seedlings with a height of at least 3 cm were removed from culture and sliced 1 cm above the cotyledonary node. A 2 mm notch incision was made to bisect the cut surface of the epicotyl and then a perpendicular cut was made to the edge of the seedling (Fig. 1).

Figure 1. A) A typical citrus shoot tip (1 mm) used for micrografting. **B)** Etiolated 'Carrizo' seedling rootstocks were prepared by making notched incisions 1 cm above the cotyledonary node. **C)** Trimmed shoot tips were placed in the notch of the seedling rootstock. Bar = 1 mm.



After overnight post-culture on solidified citrus embryo medium, recovered shoot tips were basally trimmed (0.2 mm) to create a fresh-cut surface and then placed on the seedling rootstock ledge created by the incision. Micrografted seedlings were placed on 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 x 150 mm test tubes with 25 mL of medium per tube and cultured at 25^{o}C under 16 h light/8 h dark photoperiod provided by fluorescent light (100 μmol/m^2/s). Grafted plants were visually examined at weekly intervals. Side shoots produced from the rootstock were removed during recovery of the whole plants from cryopreserved shoot tips. For most experiments, 10 shoot tips of each cultivar were excised and processed for each treatment. Each experiment was repeated twice. After 12 weeks, micrografted, rooted plants were transplanted into potting soil (Fafard #2-V mix, Fafard Conrad, Inc., Agawam, Mass. supplemented with half-strength Osmocote Plus 15-9-12 fertilizer, Scott's Sierra Horticultural Products Company, Marysville, Ohio) and gradually equilibrated to ambient greenhouse conditions (2 weeks under shaded mist, 1 week under shade, then transferred to greenhouse conditions).

RESULTS

In general, citrus plants undergo seasonal vegetative growth phases, or flushes, under field and screenhouse conditions. Pruning also induces a flush in screenhouse/greenhouse grown citrus plants. Screenhouse budsticks harvested for experiments were taken from the terminal ends of actively growing branches. Materials introduced into tissue culture screenhouse conditions exhibited less than 5% contamination rates. *Citrus sinensis* and *C. aurantium* shoot tips excised from screenhouse-grown plants averaged 68% regrowth for untreated controls, 50% regrowth for 0.3 M sucrose overnight exposure, and 68% for 2 M glycerol + 0.4 M sucrose treatments, as measured by regrowth after micrografting. Recovered micrografted shoot tips elongated and differentiated quickly (Fig. 2). Within 12 weeks, plants could be transplanted into the greenhouse.

Shoot tips derived from July-harvested field trees exhibited between 10 and 15% regrowth without LN exposure and between 0 and 5% regrowth with LN exposure (Table 1).

Excised shoot tips from young, hydrated screenhouse budwood were more successfully cryopreserved than from citrus trees in the field collected in July, with regrowth levels around 45% (Table 1).

Five additional *Citrus* species from the screenhouse and greenhouse were successfully cryopreserved. Across seven species, more than 50% of the shoot tips exhibited regrowth after LN exposure (Tables 1, 2). For most species, the differences between the –LN and +LN treatments were not statistically significant.

There were no significant differences among regrowth of *Citrus* or *Fortunella* shoot tips treated with PVS2 for either 30 or 60 min prior to cryoexposure (Table 3). The increased PVS2 exposure length did result in a non-significant decrease in the regrowth of non-cryoexposed controls.

Table 1. Regrowth and standard errors among replicate sets of citrus shoot tips excised from screenhouse plants and treated with PVS2 for 30 minutes, and then exposed to LN (30-PVS2+LN) or not exposed to LN (30-PVS2-LN) and recovered on 'Carrizo' seedling rootstocks using micrografting techniques. Significant differences between treatments within a species were determined using Tukey means separation tests (P<0.05).

	Screenhouse		Field	
Species	30-PVS2-LN	30-PVS2+LN	30-PVS2-LN	30-PVS2+LN
C. clementina	0.90 ± 0.10 a	0.45 ± 0.05 b	0.10 ± 0.00 bc	0.05 ± 0.05 c
C. sinensis	0.70 ± 0.10 a	0.45 ± 0.05 ab	0.15 ± 0.15 ab	0.05 ± 0.05 b
Average	0.80 ± 0.10	0.45 ± 0.00	0.13 ± 0.03	0.05 ± 0.00

Table 2. Regrowth and standard errors among replicate sets of citrus shoot tips excised from screenhouse and greenhouse plants and treated with PVS2 for 30 minutes, and then exposed (30-PVS2+LN) or not exposed to LN (30-PVS2-LN) and recovered on 'Carrizo' seedling rootstocks using micrografting techniques. Significant differences between treatments within a species were determined using Tukey means separation tests (P<0.05).

Species	30-PVS2-LN	30-PVS2+LN
C. aurantium	0.75 ± 0.15 a	0.55 ± 0.15 a
C. celebica	0.75 ± 0.05 a	0.55 ± 0.05 a
C. limon	0.75 ± 0.05 a	0.60 ± 0.10 a
C. medica	0.70 ± 0.05 a	0.65 ± 0.10 a
C. paradisi	0.60 ± 0.10 a	0.40 ± 0.00 a
Average	0.71 ± 0.05	0.55 ± 0.04

Table 3. Regrowth and standard errors among replicate citrus shoot tips excised from greenhouse plants and treated with PVS2 for 30 or 60 minutes, and then exposed (30-PVS2+LN; 60-PVS2+LN) or not exposed to LN (30-PVS2-LN; 60-PVS2-LN) and recovered on 'Carrizo' seedling rootstocks using micrografting techniques. Significant differences among treatments within a species were determined using Tukey means separation tests (P<0.05).

Species	30-PVS2-LN	30-PVS2+LN	60-PVS2-LN	60-PVS2+LN
C. aurantium	0.85 ± 0.05 a	0.45 ± 0.05 b	0.65 ± 0.05 ab	0.65 ± 0.05 ab
C. limon	0.75 ± 0.05 a	0.60 ± 0.10 a	0.57 ± 0.13 a	0.50 ± 0.10 a
F. obovata	0.85 ± 0.05 a	0.55 ± 0.15 a	0.80 ± 0.10 a	0.35 ± 0.05 a
Average	0.82 ± 0.03	0.53 ± 0.04	0.67 ± 0.07	0.50 ± 0.09

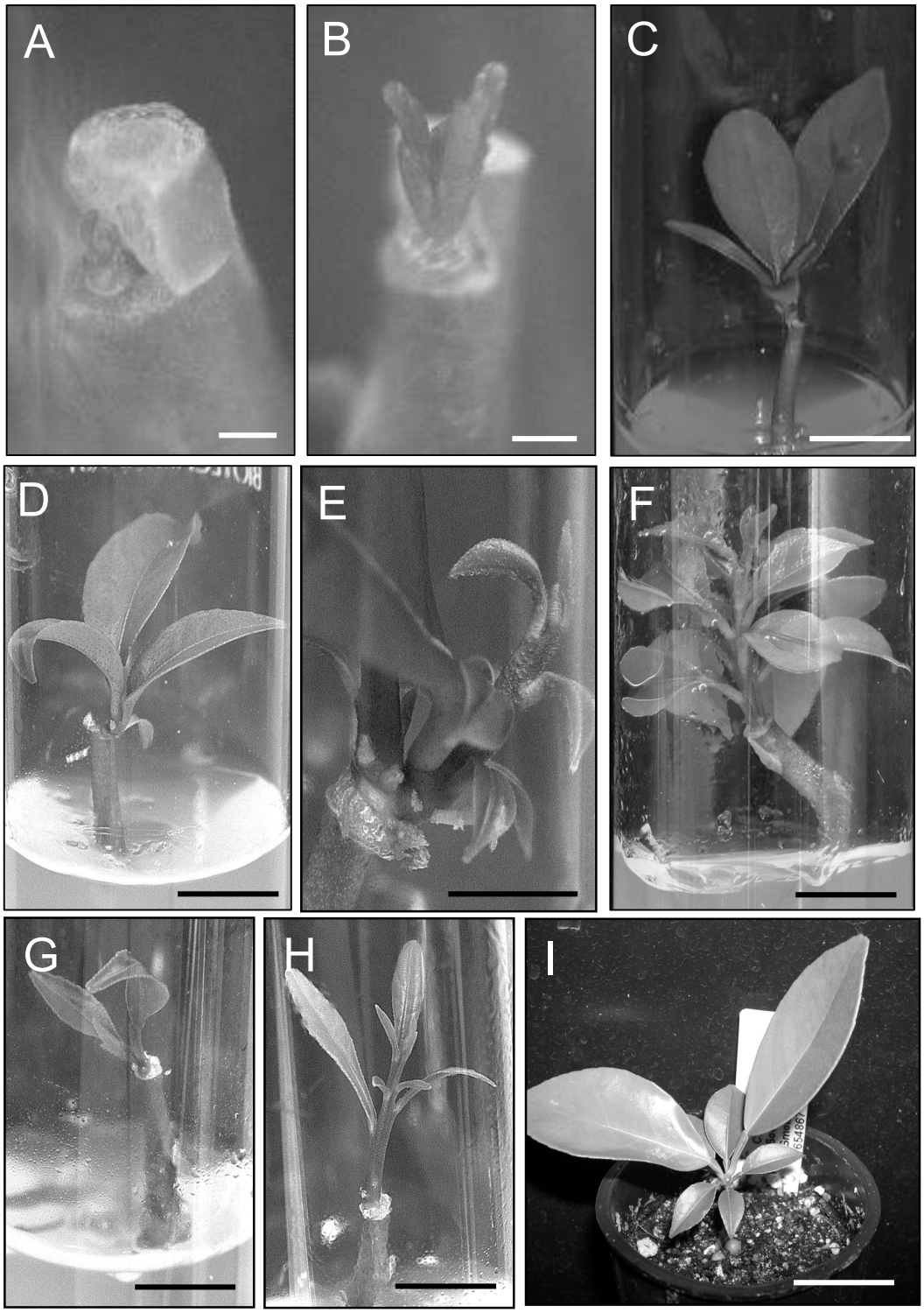
DISCUSSION

The USDA-ARS maintains a highly diverse collection of *Citrus, Fortunella*, and citrus wild species in its Riverside, CA collection. The current research focuses on selecting diverse materials within this collection to develop a successful cryopreservation strategy for conserving these genetic resources. Our proposed citrus shoot tip cryopreservation approach can be used to back-up citrus collections within the USDA genebank. By using budwood sampled directly from screenhouse or greenhouse plants, cultivar- or species-specific media are not required for proliferation in tissue culture prior to shoot tip excision. Furthermore, we applied micrografting techniques that made use of seedling rootstocks, thus eliminating the need for cultivar-specific recovery media. When accessions are routinely backed-up using cryopreservation techniques, many shoot tips are excised, treated with cryoprotectants, and placed into LN. Only a few replicate vials of shoot tips are immediately rewarmed and micrografted to confirm viability. Remaining vials of shoot tips remain in long-term storage until they are needed. Although the micrografting method is labour intensive, it is only used when accessions are retrieved from long-term storage.

Seven diverse *Citrus* species and one *Fortunella* species were selected for inclusion in cryopreservation experiments. All of these species are graft-compatible with 'Carrizo' seedling rootstocks and exhibited rapid regrowth in preliminary shoot tip grafting experiments. 'Carrizo' was selected as the rootstock in part because it was previously shown to be a superior rootstock for micrografting (4).

Plant vitrification solution (PVS) 2 was first described in 1990 for use in the cryopreservation of citrus nucellar cells (20). Since then, PVS2 has been successfully used to cryopreserve dozens of plant genera (19), including citrus shoot tips (2, 3, 26, 27). The earlier citrus vitrification methods using PVS2 as the cryoprotectant focused on rootstocks of sour orange (*C. aurantium*) and 'Troyer' citrange (*Poncirus trifoliata* x *C. sinensis*). More recently Ding *et al*. (3) used the method for cultivars of *C. sinensis, C. limon, C. reticulata*, and *C. grandis*. We found that PVS2 was an effective cryoprotectant for screenhouse- and greenhouse-derived citrus shoot tips and that reasonable levels of regrowth were obtained after either 30 or 60 min PVS2 exposure.

Figure 2. Cryopreserved *C. sinensis* shoot tips were micrografted and allowed to recover for **A)** 5 days **B)** 13 days and **C)** 8 weeks. Cryopreserved, micrografted: **D)** *C. clementina* after 8 weeks recovery; **E)** *C. limon* after 7 weeks recovery; **F)** *C. aurantium* after 8 weeks recovery; **G)** *C. paradisi* after 4 weeks recovery; **H)** *C. celebica* after 4 weeks recovery; **I)** *C. aurantium* 8 weeks after greenhouse acclimation. **A, B)** Scale bar, 1 mm; **C, D, E, F, G, H)** Scale bar, 1 cm; **I)** Scale bar 2 cm.



When seeds or embryos are cryopreserved and recovered, resulting plants retain juvenility characteristics for many years (7). Shoot tips excised from adult plants that are micrografted do not exhibit undesirable juvenile characteristics (6, 15). This advantage allows collection curators to produce mature plants capable of fruit production more quickly than if plants revert to the juvenile state.

When citrus shoot tips are micrografted for pathogen-eradication purposes, excised shoot tips are less than 0.5 mm (3, 15, 22). Successful regrowth of these small shoot tips is challenging; however, essential for successful eradication (22). The larger 1 mm citrus shoot tips excised for cryopreservation are easier to handle and result in higher regrowth levels than the smaller shoot tips excised for pathogen-eradication.

Recently, cryotherapy has become a new biotechnology for production of pathogen-free plants (28, 29). Compared with the more traditional meristem culture, cryotherapy consistently produces a higher frequency of pathogen-free plants, which is independent of cryogenic methods and size of shoot tips, a key factor for eradication of plant pathogens by the meristem culture (28, 29). Wang *et al*. (28) proposed that the expanded cells within 1 mm shoot tips harbour the pathogen and are killed by cryoexposure. These larger cells do not withstand the cryopreservation process (24). Future research will determine if 1 mm cryopreserved citrus shoot tips serve as an effective method to eradicate pathogens from the USDA-ARS citrus collection. There is precedence for effective cryotherapy in citrus. Ding *et al.* (3) demonstrated that HLB infected shoot tips could be cryopreserved and regenerated free of HLB.

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