Vitis **58**, 71–78 (2019) DOI: 10.5073/vitis.2019.58.71-78

Cryopreservation of grapevine (*Vitis* spp.) shoot tips from growth chamber-sourced plants and histological observations

J. C. Bettoni[1), 2)], R. Bonnart[3)], A. Shepherd[3)], A. A. Kretzschmar[1)] and G. M. Volk[3)]

[1)] Santa Catarina State University, Lages, Santa Catarina, Brazil
[2)] CAPES Foundation, Ministry of Education of Brazil, Brasília, Distrito Federal, Brazil
[3)] USDA-ARS National Laboratory for Genetic Resources Preservation, Fort Collins, Colorado, USA

Summary

Many genebanks rely on cryopreservation as a method to preserve vulnerable field collections of vegetatively propagated crops. Effective cryopreservation procedures have been identified for *Vitis*; however, they usually use *in vitro* plantlets as the shoot tip source materials. It is costly to establish *Vitis* collections *in vitro* prior to cryopreservation. We sought to determine if growth chamber derived *Vitis* plants could serve as the source of shoot tips for cryopreservation. Nodal sections from growth chamber derived plants were surface-disinfected and placed in tissue culture on pre-treatment medium for 2 weeks. Uniform apical shoot tips (1 mm) were first obtained from the nodal sections and then precultured for 3 days on medium containing 0.3 M sucrose, salicylic acid, glutathione (reduced form), ascorbic acid and plant preservative mixture. Half-strength PVS2 was applied for 30 min at 22 °C, prior to full-strength PVS2 treatment at 0 °C. Cryopreserved shoot tips had the highest average regrowth of 50 and 55 % without and with cold-acclimation followed with a full-strength PVS2 exposure duration of 40 and 30 min at 0 °C, respectively. This cryopreservation protocol achieved high percentages of regrowth in *V. vinifera* 'Chardonnay' and 'Riesling' and *V. hybrid* 'Oppenheim'. Histological observations revealed that shoot tips from growth chamber plants had apical as well as multiple lateral meristems that survived LN immersion. The preservation of multiple meristems in each shoot tip may increase the capacity of shoot tip regeneration in cryopreserved *Vitis* that originates from *ex vitro* sources. The high percentage of regrowth after shoot tip cryopreservation using *Vitis* shoot tips derived from growth chamber source plants suggest that it may be possible to cryopreserve *Vitis* shoot tips without first introducing each accession into tissue culture.

K e y w o r d s : grapevine; *ex situ* conservation; droplet vitrification; germplasm.

Introduction

Grapevine is one of the most economically important temperate fruit crops. In 2017, grapevines covered 7.53 million ha and produced 73.3 million t of fruit worldwide (OIV 2018). Grapes have a rich genetic diversity; there are about 70 species within the *Vitis* genus (Li *et al.* 2017). This diversity is conserved in genebanks primarily as field plantings, and as such, these critical collections could be lost in the event of biological or environmental disasters. It is essential to have methods such as cryopreservation available to genebanks to securely conserve collections of *Vitis* genetic resources for future generations.

Cryopreservation, the storage of biological materials in liquid nitrogen (LN, -196 °C) or liquid nitrogen vapor (LNV, -165 to -190 °C), is a preferred method for the long-term storage of plant germplasm, especially to maintain the genetic integrity of genebank materials and to minimize the risk of biotic and abiotic threats. Under cryopreserved conditions, viable cells, tissues, organs and organisms are preserved in a state whereby cellular divisions and metabolic processes are stopped (Benson 2008).

Although there are many established cryobanks that preserve vegetative propagules, such as shoot tips or dormant buds of clonally propagated genetic resources (Jenderek and Reed 2017, Reed 2001, Höfer and Reed 2010, Panis *et al.* 2010, Keller 2007, Niino and Arizaga 2015), to the best of our knowledge, *Vitis* cryo-storage has not been fully implemented within genebanks. Limited results were obtained when *Vitis* dormant buds were cryopreserved (Esensee and Stushnoff 1990). Although multiple *Vitis* shoot tip cryopreservation methods have been reported, it wasn't until recently that a highly effective, widely applicable *Vitis* droplet vitrification techniques was published (Bi *et al.* 2018a, Volk *et al.* 2018). This method uses *Vitis* source plants that have been established *in vitro,* which is both time- and labor-intensive. We sought to identify a method whereby *Vitis* shoot tips could be cryopreserved directly from plants that were not in tissue culture (Bettoni *et al.* 2019a).

Correspondence to: J. C. Bettoni, Santa Catarina State University (UDESC), Lages, Santa Catarina, 88520000, Brazil. E-mail: jcbettoni@gmail.com; G. M. Volk, USDA-ARS National Laboratory for Genetic Resources Preservation, 1111 S. Mason St., Fort Collins, CO, 80521, USA. E-mail: gayle.volk@ars.usda.gov

Previously, the study of Hassan and Haggag (2013) simplified the cryo-procedure by sampling shoot tips directly from greenhouse-grown plants and regrowth percentages of 40 % for *V. vinifera* for 'Black Matrouh' and 47 % for 'Bez El-Anza' were obtained after liquid nitrogen immersion. We found that shoot tips derived directly, without nodal section preculture, exhibited a highly variable response to cryoexposure (based on regrowth percentages), which we hypothesized to be due to the use of shoot tips that were of non-uniform developmental stages. The quality and physiological state of the stock cultures and explants, as well as the preculture conditions, play key roles and are determinant to the success of *Vitis* cryopreservation techniques (Bi *et al.* 2017). Pre-treatments can differ by species and may include cold treatment exposure, the addition of osmotic agents, antioxidants and, elicitors of defense-related proteins in plants (Pathirana *et al.* 2016, Volk *et al.* 2018, Mathew *et al.* 2018). Reliable cryopreservation methods that result in high degrees of viability (≥ 40 % after LN exposure) and highly skilled staff are key to the development of successful cryopreserved base collections of clonal crops (Volk *et al.* 2016, Reed *et al.* 2004).

In addition to maintaining the viability of cryopreserved materials for long-term storage, cryopreservation protocols have been found to efficiently eradicate viruses in multiple species of economic importance (Bettoni *et al.* 2018, Bettoni *et al.* 2019b, Vieira *et al.* 2015, Yi *et al.* 2014; Helliot *et al.* 2002, Wang *et al.* 2009; Brison *et al.* 1997), including grapes (Bi *et al.* 2018b, Pathirana *et al.* 2015, Marković *et al.* 2015, Bayati *et al.* 2011, Wang *et al.* 2003). Cryotherapy is a technique whereby shoot tips of infected plants are pre-treated and then exposed to liquid nitrogen to eliminate the cells that are infected with viruses and allow the meristematic cells to regrow into healthy plants. When successful widely-applicable cryopreservation methods are available, cryotherapy procedures can be developed as tools to eradicate pathogens from grapevines (Bi *et al.* 2017, Bettoni *et al.* 2016).

We previously published a droplet vitrification protocol that successfully cryopreserved a wide range of *Vitis* species using *in vitro* plants (Volk *et al.* 2018). Herein, we modify this method to make use of *Vitis* growth chamber plants as source materials. In addition, cellular observations revealed anatomical features of cryopreserved *Vitis* shoot tips derived from growth chamber plants.

Materials and Methods

Plant materials and pre-treatments: Growth chamber plants of *V. vinifera* 'Chardonnay' and 'Riesling' and rootstock selection 'Oppenheim #4' (DVIT 8121 (SO4; *V. berlandieri* × *V. riparia*)) were originally received from the USDA-ARS National Clonal Germplasm Repository for Tree Fruit, Nut Crops and Grapes in Davis, CA, USA.

Plants were grown in pots in commercial substrate Sun Gro Professional Growing mix (Sunshine® VP Metro-Mix 250; Sun Gro Horticulture Ltd., Seba Beach, AB, Canada) at 25 °C, in a growth chamber under a photoperiod of 16 h light·day^{-1} provided by metal halide and high pressure sodium lamps. Nodal sections, approximately 2 cm long containing a single bud were harvested from the growth chamber plants (Fig. 1A), treated with 70 % isopropanol for 1 minute and rinsed twice for 1 minute with distilled water. They were then treated with 5 % sodium hypochlorite and 0.1 % Tween 20® (v/v) for 5 min and rinsed three times in sterile distilled water.

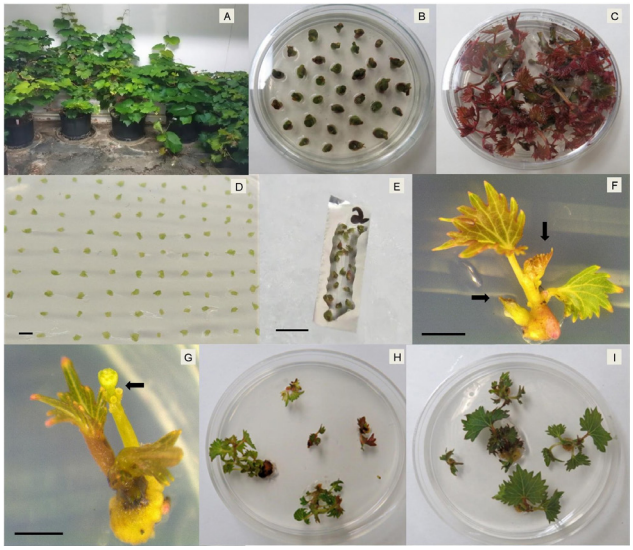

Fig. 1: Some steps of droplet vitrification procedure of *Vitis* shoot tips from growth chamber sourced plants. Nodal sections were excised from growth chamber plants (**A**), surface-disinfected and placed on pretreatment medium (**B**), and grown for 2 weeks (**C**). Shoot tips were incubated on the preculture medium for 3 d (**D**). Shoot tips were placed on foil strips in a thin layer of PVS2 prior to LN immersion (**E**). *Vitis vinifera* 'Riesling' shoot tips 40 d after cryoexposure exhibiting multiple shoots (**F**) and flower buds (**G**). Petri plates of *Vitis vinifera* 'Riesling' shoot tips grown for two months after cryoexposure (**H**) and without cryoexposure (**I**). Arrows indicate multiple shoots in **F** and inflorescence in **G**. Scale Bars: D, F and G = 2 mm; E = 5 mm.

Nodal sections were placed into 100 × 25 mm plastic Petri dishes with 50 mL pre-treatment medium Murashige and Skoog (1962) (MS) containing 30 g·L^{-1} sucrose, 0.2 mg·L^{-1} 6-benzyl aminopurine (BA[3]), 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form), 1.5 % (v/v) Plant Preservative Mixture (PPM; Plant Cell Technology, WA), and 3 g·L^{-1} gellan gum (CultureGel™, PhytoTechnology Laboratories, KS) at pH 5.7 (pH 6.4 prior to autoclaving)) with 30 nodal sections per plate (Fig. 1B). PPM™ is a broad-spectrum biocide/fungicide for plant tissue culture and was included in the medium for the growth chamber nodal sections to reduce microbial contamination. Nodal sections were cultured on pre-treatment medium for 2 weeks in a growth room at 25 °C with a 16 h photoperiod provided by fluorescent lights (40 µM m^{-2}·s^{-1}) (Fig. 1C). After initial culture at 25 °C, the influence in the regrowth percentages to cold acclimation pretreatment and without cold acclimation pretreatment prior to shoot tip desiccation was also assessed. Cold acclimation treatments were applied

by placing the plates with nodal sections in a growth chamber maintained at a constant temperature of 5 °C with a 16 h photoperiod with a light intensity of 35 $\mu M \cdot m^{-2} \cdot s^{-1}$ for an additional two weeks (when applied).

Preculture and droplet vitrification: Uniform apical shoot tips (1 mm) (Fig. 1D) were excised from nodal sections that either had or had not been cold acclimated. Shoot tips were cultured on preculture medium consisting of half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form), 1.5 % (v/v) PPM and 3 $g \cdot L^{-1}$ gellan gum at pH 5.7 (pH 6.4 prior to autoclaving) for 3 d at 25 °C in darkness. They were then placed in loading solution consisting of half-strength MS + 2M glycerol + 0.4 M sucrose at pH 5.7 (pH 6.9 prior to autoclaving) for 20 min at 22 °C followed by half-strength PVS2 (filter sterilized solution consisting half-strength MS + 15 % (w/v) glycerol + 7.5 % (w/v) ethylene glycol (EG) +7.5 % (w/v) dimethyl sulfoxide (DMSO) + 0.4 M sucrose at pH 5.8, (Matsumoto and Sakai 2003)) for 30 min at 22 °C and full-strength PVS2 (filter sterilized solution, half-strength MS + 30 % (w/v) glycerol + 15 % (w/v) EG +15 % (w/v) DMSO + 0.4 M sucrose at pH 5.8, (Sakai *et al.* 1990)) at 0 °C for 30, 40, or 50 min. Two minutes before the end of each treatment, PVS2-treated shoot tips were placed onto a thin layer of PVS2 on sterile aluminum foil strips (~6 x 25 mm) (Fig. 1E) and then plunged into LN.

After one hour of LN immersion, the aluminum foil strips with shoot tips were warmed quickly by inverting the strips into unloading solution (half-strength MS + 1.2 M sucrose at pH 5.7 (pH 7.5 prior to autoclaving)) and incubating at 22 °C for 20 min (25 mL unloading solution to thaw four foil strips). For regrowth, shoot tips were placed onto recovery medium 1 (half-strength MS macroelements without ammonium, full strength MS microelements, and *Vitis* vitamins (100 $mg \cdot L^{-1}$ myo inositol, 10 $mg \cdot L^{-1}$ thiamine HCl, 1 $mg \cdot L^{-1}$ nicotinic acid, 1 $mg \cdot L^{-1}$ pyridoxine HCl, 1 $mg \cdot L^{-1}$ Ca pantothenate, 0.01 mg L^{-1} biotin, 2 mg L^{-1} glycine), supplemented with 0.6 M sucrose and 8 g L^{-1} agar at pH 5.7 (pH 7.0 prior to autoclaving)) overnight at 25 °C in the dark. They were then transferred to recovery medium 2 (half-strength MS macroelements without ammonium, full strength MS with *Vitis* vitamins), supplemented with 30 $g \cdot L^{-1}$ sucrose, 0.2 $mg \cdot L^{-1}$ BA^3 and 8 $g \cdot L^{-1}$ agar at pH 5.7 (pH 6.5 prior to autoclaving)) and cultured for 2 weeks at 25 °C in darkness. They were then transferred onto recovery medium 3 (half-strength MS macroelements and full-strength MS microelements with *Vitis* vitamins supplemented with 30 $g \cdot^{-1}$ sucrose, 0.2 $mg \cdot L^{-1}$ BA^3 and 8 $\cdot g\ L^{-1}$ agar at pH 5.7 (pH 6.5 prior to autoclaving)) and grown in the light at 25 °C (40 $\mu M \cdot m^{-2} \cdot s^{-1}$, 16 h photoperiod).

Regrowth and data analysis: Shoot tip regrowth (organized shoots with at least one leaf) was assessed 8 weeks after plating on regrowth medium 3. Each experiment was performed with at least two replicates of 20 shoot tips for each treatment. Means and standard errors were calculated across experimental replicates and analyzed using one-directional ANOVA and Tukey. $P \leq 0.05$ was considered significantly different. ($P \leq 0.05$).

Histological studies of cryopreserved and intact shoot tips: Histological studies were performed to observe cellular structural modifications pre- and post-cryopreservation for *V. vinifera* 'Riesling' shoot tips that originated from growth chamber sourced plants. The shoot tips from following treatments were sampled for control shoot tips (-LN): (1) control, shoot tips excised from pre-treatment nodal sections, (2) shoot tips treated on preculture medium for 3 d, (3) shoot tips treated with cryoprotectants (30 min at 22 °C in half-strength PVS2 followed by full-strength PVS2 at 0 °C for 30 min), and warmed with unloading solution at 22 °C for 20 min, (4) shoot tips treated with cryoprotectants (30 min at 22 °C in half-strength PVS2 followed by full-strength PVS2 at 0 °C for 30 min), and warmed with unloading solution at 22 °C for 20 min and incubated in recovery medium for 6 d. The following treatments were sampled for shoot tips exposed to LN for 1 h: (5) shoot tips treated with cryoprotectants (30 min at 22 °C in half-strength PVS2 followed by full-strength PVS2 at 0 °C for 30 min) immersed to LN, and warmed with unloading solution at 22 °C for 20 min, (6) shoot tips treated with cryoprotectants (30 min at 22 °C in half-strength PVS2 followed by full-strength PVS2 at 0 °C for 30 min), immersed to LN, and warmed with unloading solution at 22 °C for 20 min and incubated in recovery medium for 6 d.

Fifteen shoot tips per treatment were placed into fixative (1.25 % glutaraldehyde, 2 % paraformaldehyde, and 50 mM Pipes buffer [piperazine N, *N*-bis (2-ethanesulfonic acid)] overnight at 22 °C. Shoot tips were rinsed three times (10 min each) in 50 mM Pipes buffer and post-fixed with 2 % osmium tetroxide in 50 mM Pipes buffer overnight at 22 °C. Shoot tips were then rinsed three times (10 min each) in 50 mM Pipes buffer and dehydrated with 30, 50, 70, 90, 100, and 100 % ethanol for 15 min each. Samples were infiltrated with Spurr resin (Electron Microscopy Sciences, Hatfield, PA), in an ethanol: resin ratio of 3:1 for 4 h, followed 2:1 for 3 h, 1:1 for 4h, 1:2 for 4 h, 1:3 for 4h and kept in 100 % resin for 72 h (fresh resin was added every 24 h). Samples were then transferred to fresh resin, placed into embedding containers, and the resin was polymerised at 65 °C for 24 h. Thin sections (1 µm) were made with glass knives on an RMC MT-X microtome (Ventana Medical Systems Inc., Tucson, AZ) and mounted on glass slides. Sections were stained with methylene blue, visualized with an Olympus BH-2 microscope (Olympus Optical Co., Tokyo, Japan) and images captured using a Leica MC170 HD digital camera (Wetzlar, Germany).

Results

The functionality of the *Vitis* cryopreservation method described herein is dependent on the quality, uniformity, and cleanliness of the shoot tip materials. Nodal sections sampled from actively growing growth chamber plants were surface sterilized and plated on a medium containing PPM prior shoot tip excision. These strategies minimized any possible contamination.

Cryopreservation of Vitis shoot tips from growth chamber-grown plants: Nodal sections derived from growth chamber plants provided uniform shoot tips for cryopreservation. Shoot tips without LN immersion had higher regrowth percentages than those immersed in LN. There were no statistically significant differences in regrowth percentages between shoot tips that were cold acclimated at 5 °C for 2 weeks and those that were not cold-acclimated for the corresponding PVS2 exposure durations (Tabs 1 and 2). For the non-cold acclimated shoot tips without LN there were no statistically significant differences among three *Vitis* cultivars and the average regrowth percentages were 73 % for 30 min, 72 % for 40 min PVS2, and 63 % for 50 min PVS2 exposures. Similarly, with cold acclimation and without LN there were no statistically significant differences among three *Vitis* cultivars and, the highest regrowth percentages (-LN) were obtained for *V. vinifera* 'Riesling' after with 84 % after 30 min PVS2, *V. vinifera* 'Chardonnay' with 73 % after 40 min PVS2, *V. vinifera* 'Riesling' after with 64 % after 50 min PVS2 (Tab. 2). The regrowth percentages of cold-acclimated and non-cold-acclimated shoot tips after LN immersion were similar; however, the cold-acclimated treatments were less variable in regrowth percentages (Tab. 2).

The non-cold acclimated shoot tips, with LN, regrowth percentages were 48 % for 30 min, 50 % for 40 min, and 41 % for 50 min PVS2 exposures (Tab. 1), with the highest regrowth percentage obtained for *V. vinifera* 'Riesling' after 30 min PVS2 (62 %) and 40 min PVS2 (64 %) (Tab. 1). With cold acclimation and LN, all three cultivars had regrowth percentages of at least 40 % with a 30 min PVS2 exposure (Tab. 2).

The optimal PVS2 treatment period was determined to be a 30 min exposure to half-strength PVS2 at 22 °C, followed by a 40 min (non-cold acclimated shoot tips) or 30 min (cold acclimated shoot tips) exposure to full-strength PVS2 at 0 °C, which resulted in an average regrowth of 50 and 55 % for shoot tips exposed to LN without and with cold-acclimation, respectively.

Histological studies of cryopreserved shoot tips: Excised shoot tips of *Vitis vinifera* 'Riesling' that originated from growth chamber plants were embedded in resin for histological observations. All of the observed shoot tips had apical meristems in addition to multiple lateral meristems (Fig. 2C). Apical meristems exhibited a characteristic dome shape and were often wider than the lateral meristems. The apical portion of the dome appeared to have cells that were smaller and more compact than those

Table 1

Regrowth percentages (%) of shoot tips excised from nodal sections sourced from *V. vinifera* cvs. 'Chardonnay' and 'Riesling' and rootstock selection 'Oppenheim' #4 grown in the growth chamber. Shoot tips were treated with half-strength PVS2 for 30 min, followed by 30, 40 or 50 min of full-strength PVS2 without cold acclimation. Data represent mean ± SE. Values followed by different letters within the set of -LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at $P < 0.05$ using the Tukey's mean separation test

Species	Plant identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		30	40	50	30	40	50
V. vinifera	Chardonnay	72 ± 4 a	68 ± 4 a	63 ± 6 a	43 ± 4 bc	37 ± 3 bc	30 ± 3 c
V. vinifera	Riesling	76 ± 4 a	72 ± 4 a	64 ± 0 a	62 ± 2 a	64 ± 4 a	50 ± 2 ab
V. hybrid	Oppenheim #4 DVIT 8121	70 ± 0 a	75 ± 0 a	63 ± 4 a	38 ± 3 bc	48 ± 3 abc	43 ± 3 bc
Average		73 ± 2	72 ± 2	63 ± 0	48 ± 7	50 ± 8	41 ± 6

Table 2

Regrowth percentages (%) of shoot tips excised from nodal sections sourced from *V. vinifera* cvs. 'Chardonnay' and 'Riesling' and rootstock selection 'Oppenheim' #4 grown in the growth chamber. Shoot tips were treated with half-strength PVS2 for 30 min, followed by 30, 40 or 50 min of full-strength PVS2 with cold acclimation. Data represent mean ± SE. Values followed by different letters within the set of –LN species/PVS2 exposure combinations and within the set of +LN species/PVS2 exposure combinations are significantly different at $P < 0.05$ using the Tukey's mean separation test

Species	Plant identifier	-LN			+LN		
		PVS2 exposure duration (min)			PVS2 exposure duration (min)		
		30	40	50	30	40	50
V. vinifera	Chardonnay	70 ± 0 ab	73 ± 3 ab	55 ± 5 b	48 ± 3 ab	43 ± 8 ab	55 ± 10 ab
V. vinifera	Riesling	84 ± 4 a	70 ± 2 ab	64 ± 4 b	68 ± 0 a	60 ± 4 ab	46 ± 6 ab
V. hybrid	Oppenheim #4 DVIT 8121	68 ± 3 ab	63 ± 3 b	60 ± 5 b	48 ± 3 ab	45 ± 5 ab	38 ± 3 b
Average		74 ± 5	69 ± 3	60 ± 3	55 ± 7	49 ± 5	46 ± 5

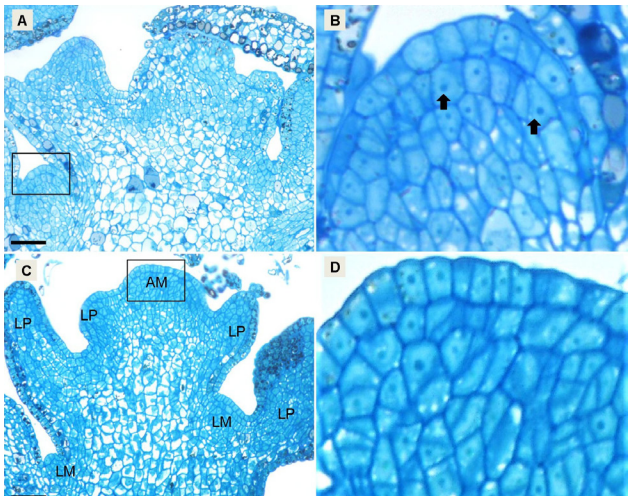

Fig. 2: Vertical section of *Vitis vinifera* 'Riesling' shoot tips sampled at (**A**) control, shoot tips excised from pre-treatment nodal sections; (**B**), a closer view of a lateral meristem in A; (**C**) shoot tips precultured on medium for 3 d; (**D**) a closer view of an apical meristem in C. Black arrows represent some cells showing nucleoli. AM, apical meristem; LM, lateral meristems; LP, leaf primordium. Scale Bars: A and C = 50 µm.

in the basal region (Fig. 2C). Many of the observed apical and lateral meristematic cells showed signs of recent cellular divisions and were highly cytoplasmic, with large nuclei, some showing nucleoli (Fig. 2B, 2D).

Both apical and lateral meristems retained their cellular integrity after PVS2 exposure, either with or without LN (Fig. 3A, 3B, 3C, 3D). Differentiated, vacuolated cortex cells in the PVS2-exposed meristems were plasmolyzed (Fig. 3E), while the cortex cells in the PVS2+LN-exposed meristems were lethally damaged, showing evidence of cell membrane disruptions (Fig. 3F). After 6 days of recovery, we observed signs of regrowth in the shoot tips that were either exposed or not exposed to liquid nitrogen (Fig. 3G, 3H).

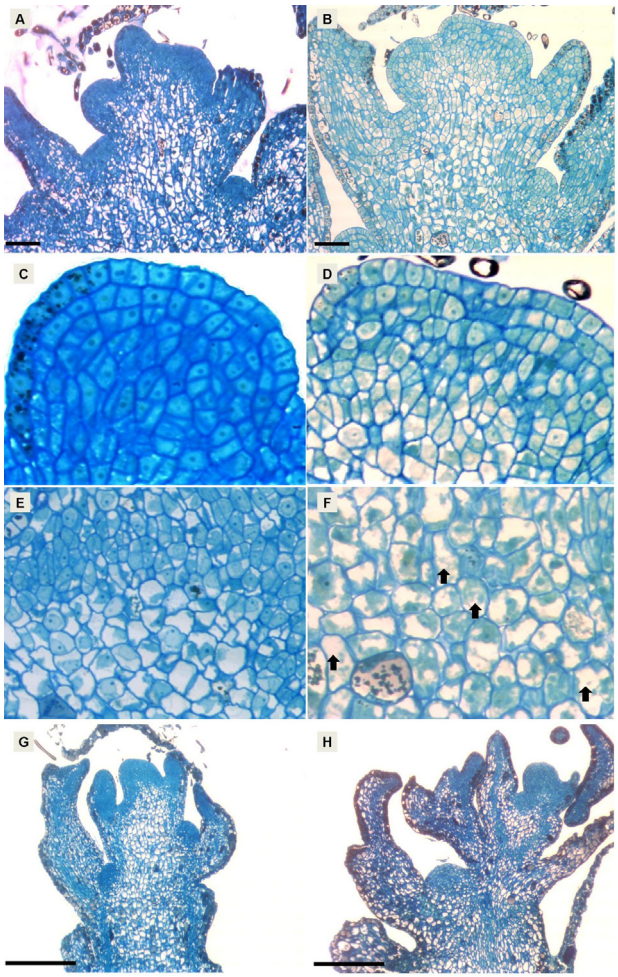

Fig. 3: Histological observations in *Vitis vinifera* 'Riesling' shoot tips after successive steps of cryopreservation procedure. Vertical section of shoot tips treated with cryoprotectants (30 min at 22 °C in half-strength PVS2 followed by full-strength PVS2 at 0 °C for 30 min), not exposed or exposed to LN, and then diluted with 1.2 M sucrose (**A**: without LN; **B**: with LN). A closer view of apical meristem (**C**) and cortex cells (**E**) in the non-LN exposed shoot tip from **A**. A closer view of apical meristem (**D**) and cortex cells (**F**) in the LN exposed shoot tip from **B**. Shoot tips treated with cryoprotectants and were either not exposed or exposed to LN, diluted with 1.2 M sucrose and were incubated in recovery medium for 6 d (**G**: without LN; **H**: with LN). Asterisks, regions where plasmolysis has occurred. Black arrows represent damaged cells. Scale Bars: A and B = 50 µm, G and H = 200 µm.

Discussion

We identified a cryopreservation protocol that did not require *Vitis* source plants to be introduced and multiplied in tissue culture prior to shoot tip excision. Nodal sections from growth chamber plants were surface sterilized and then plated on medium to produce uniform shoot tips. These shoot tips were then excised and cryopreserved using a droplet-vitrification procedure that was successfully applied to *V. vinifera* 'Chardonnay' and 'Riesling' and *V. hybrid* 'Oppenheim'. Average regrowth percentages were 48 % after shoot tips were exposed to half-strength PVS2 at 22 °C for 30 min, then full-strength PVS2 at 0 °C for 30 min, followed by liquid nitrogen immersion. Our results showed that the use of a cold acclimation treatment did not significantly improve the regrowth of *Vitis* shoot tips after cryopreservation.

The use of explants directly from the greenhouse, or possibly even from field-grown plants, for cryopreservation would alleviate the laborious steps of *in vitro* culture establishment and multiplication, thus reducing the time and increasing the efficiency of cryopreserving cultivars in genebank collections (Bettoni *et al.* 2019a, Volk *et al.* 2018, Bi *et al.* 2017, Towill *et al.* 2004). Previously, Hassan and Haggag (2013) reported the cryopreservation of shoot tips from greenhouse grapevine plants of Egyptian cultivars. These authors used shoot tips derived directly from greenhouse-grown plants, without the incubation of nodal sections on media to produce uniform shoot tips. We found that shoot tips derived directly from growth chamber plants did not exhibit uniform or repeatable responses to cryopreservation treatments (data not shown). Our proposed method of excising shoot tips from pre-treated nodal sections improved the shoot tip quality and reduced the effects of oxidation and microbial contamination.

The quality of the explant source material is a key factor for successful *Vitis* cryopreservation (Volk *et al.* 2018, Bi *et al.* 2017, Bettoni *et al.* 2016, Marković *et al.* 2014, Engelmann 2011). In our protocol, nodal sections were harvested from growth chamber plants, then surface sterilized and placed on pre-treatment medium supplemented with PPM. PPM was added during the pre-treatment and preculture steps because contamination posed the greatest challenge to using nodal sections from growth chamber plants. Preliminary trials revealed that contamination could also be reduced when nodal sections were shaken for 2 h in 5 % (v/v) PPM solution supplemented with full-strength MS salts (no pH adjustment), after nodal section sterilization and prior to plating on to pre-treatment medium (data not shown).

Successful cryopreservation procedures are dependent upon optimal pre-treatment conditions (Volk *et al.* 2018, Mathew *et al.* 2018). We included salicylic acid, glutathione (reduced form) and ascorbic acid in the pre-treatment medium to reduce the formation of reactive oxygen species (ROS) during cryoprotectant and LN immersion because oxidative damage may affect shoot tip regrowth after LN immersion (Uchendu *et al.* 2010a, Johnston *et al.* 2007). The positive effects of adding antioxidants and salicylic acid to pre-treatment and preculture media during the cryopreservation process have been reported with *Vitis* (Volk *et al.* 2018, Bi *et al.* 2018a, Bettoni *et al.* 2019a, Pathirana *et al.* 2016, Shepherd *et al.* 2013) and other species (Mathew *et al.* 2018, Reed 2014, Uchendu *et al.* 2010b).

We found that experiments that used a cold acclimation treatment (5 °C for 2 weeks) did not have higher overall regrowth levels after cryoexposure than experiments that did not include the cold acclimation treatment. For some species, cold acclimation treatments improved the regrowth and quality of regenerated plants after cryopreservation (Mathew *et al.* 2018, Panta *et al.* 2015, Kushnarenko *et al.* 2009, Kaczmarczyk *et al.* 2008, Chang and Reed 2000, 2001). This treatment step could be considered when cryopreserving other *Vitis* cultivars and/or species.

We describe a droplet-vitrification cryopreservation technique for *Vitis* that makes use of shoot tips derived from growth chamber source plants. Nodal sections were cultured on pre-treatment medium for 2 weeks, uniform apical shoot tips were excised and cultured on preculture medium for 3 d, then they were placed into loading solution for 20 min followed by half-strength PVS2 for 30 min. The optimal full-strength PVS2 exposure duration was 30 min for shoot tips derived from growth chamber plants. Our results revealed that shoot tips derived from growth chamber plants could not tolerate PVS2 incubation durations that were as long as those for shoot tips derived from *in vitro* source cultures (Volk *et al.* 2018; Bettoni *et al.* 2019a). We previously published a droplet vitrification protocol that successfully cryopreserved *Vitis* species using *in vitro* plants. In this work, *V. vinifera* cv. 'Chardonnay' had the highest regrowth percentage (43 %) after 75 min PVS2 exposure at 0 °C (Volk *et al.* 2018). Herein we demonstrate that shorter incubation times used for the growth chamber plants were sufficient for adequate percentages of shoot tip regrowth after LN immersion. This may be because in vitro plants may be more hydrated and need longer PVS2 exposures to remove the freezable water. The high percentage of regrowth after cryoexposure suggests that it may be possible to cryopreserve *Vitis* shoot tips without first introducing each accession into tissue culture.

It is interesting to note that one of the cultivars included in this work was the rootstock selection 'Oppenheim', with a similar genetic background as 'Kober 5BB' (*V. berlandieri* x *V. riparia*), which was shown by Ganino *et al.* (2012) to be recalcitrant to cryopreservation. Using our method, this rootstock cultivar had regrowth percentages of 48 % after cryoexposure.

Histological observations at the light microscope level revealed that *Vitis* shoot tips isolated from growth chamber source plants appeared to have a high degree of cellular preservation in both the apical and lateral meristems. Similarly highly preserved apical meristems were observed in cryopreserved mint and citrus shoot tips (Volk *et al.* 2007, 2017); however, in *Vitis*, we observed multiple preserved lateral meristems within each excised shoot tip. We found that multiple shoots elongated from each growth chamber derived shoot tip, indicating that both the apical and lateral shoot tips survived cryoexposure. This could increase the capacity of shoot tip regeneration in cryopreserved *Vitis* from *ex vitro* sources (Fig. 1F). Previous reports of *Vitis* shoot tip cryopreservation using explants derived from *in vitro* source plants exhibited the emergence of a single shoot from the shoot tips, suggesting that only a single meristem exhibited regrowth (Bi *et al.* 2018a, Pathirana *et al.* 2016, Marković *et al.* 2013).

In addition, we found that some lateral meristems developed floral buds (Fig. 1G). Although grape plants produce fruit in the shoot that sprouts in the growth season, the formation of the flower bud occurs over two seasons; where the floral primordia are induced in the first vegetative year and are then differentiated into the inflorescence (Li-Mallet *et al.* 2015, Vasconcelos *et al*. 2009). Apparently, when nodal sections were excised from growth chamber plants, some buds had already been induced to produce floral primordia.

Cryopreserved genebank collections require less space and fewer resources than traditional field conservation methods, particularly for clonally propagated crops (Wang *et al.* 2018, Bi *et al.* 2017, Pathirana *et al.* 2016, Bettoni *et al.* 2016, Benelli *et al.* 2013, Marcović *et al.* 2013, REED *et al.* 2004). To date, *Vitis* cryopreservation has been considered challenging due to the genotype-specific responses, low percentages of regrowth after cryopreservation and the difficulty in acquiring the same results as those that have been published by other laboratories. These characteristics have limited the implementation of grape cryopreservation in genebanks (Bi *et al.* 2017, Bettoni *et al.* 2016, Ganino *et al.* 2012, Bayati *et al.* 2011). With the application of optimized pre-treatment conditions, improvement of the shoot tip quality and uniformity, and improved regrowth medium conditions, the current challenges can be overcome.

Availability of a cryopreservation method for *Vitis* may facilitate the use of cryotherapy methods to eradicate viruses (Bi *et al.* 2017, Bettoni *et al.* 2016). To date, 5 major *Vitis* viral pathogens including *Grapevine leafroll associated virus-1* (GLRaV-1) (Pathirana *et al.* 2015), GLRaV-2 (Pathirana *et al.* 2015), GLRaV-3 (Bi *et al.* 2018b, Pathirana *et al.* 2015, Marković *et al.* 2015), *Grapevine virus A*

(GVA) (Bayati *et al.* 2011, Wang *et al.* 2003) and *Grapevine fanleaf virus* (GFLV) (Marković *et al.* 2015) have been eliminated from grapevines using cryotherapy techniques. The proposed *Vitis* cryopreservation procedure which makes use of growth chamber source plants, possibly in combination with thermotherapy, may further increase the success and applicability of cryotherapy techniques.

Conclusion

We describe a droplet-vitrification cryopreservation technique with high regrowth percentages for the cryopreservation of *Vitis* that makes use of shoot tips derived from growth chamber sourced plants. The high percentage of regrowth that we obtained in cryopreserved shoot tips suggests that it may be possible to cryopreserve *Vitis* shoot tips without first introducing each accession into tissue culture. Future work will focus on determining the applicability of the method to additional *V. vinifera* cultivars and evaluating the efficacy of using shoot tips derived from growth chamber and field plants.

Acknowledgements

The authors are grateful to the Coordination for the Improvement of Higher Education Personnel (CAPES) of the Brazilian Ministry of Education (MEC) for the Ph.D. Scholarship in Brazil and Scholarship for Ph.D. Sandwich Program Abroad (PDSE) in the United States of America granted to J. C. Bettoni to perform research at the USDA-ARS National Laboratory for Genetic Resources Preservation (NLGRP). Research was funded in part by NAS and USAID, and any opinions, findings, conclusions, or recommendations expressed are those of the authors alone, and do not necessarily reflect the views of USAID or NAS. They thank B. Prins for providing an internal review of the manuscript.

References

Bayati, S.; Shams-Bakhsh, M.; Moieni, A.; 2011: Elimination of grapevine virus A (GVA) by cryotherapy and electrotherapy. J. Agric. Sci. Technol. **13**, 443-450.

Benelli, C.; Carlo, A. D.; Engelmann, F.; 2013: Recent advances in the cryopreservation of shoot-derived germplasm of economically important fruit trees of *Actinidia*, *Diospyros*, *Malus*, *Olea*, *Prunus*, *Pyrus* and *Vitis*. Biotechnol. Adv. **31**, 175-185.

Benson, E. E.; 2008: Cryopreservation of phytodiversity: A critical appraisal of theory & practice. Crit. Rev. Plant Sci. **27**,141-219.

Bettoni, J. C.; Bonnart, R.; Shepherd, A.; Kretzschmar, A. A.; Volk, G. M.; 2019a: Successful cryopreservation of *Vitis vinifera* 'Chardonnay' from both *in vitro* and growth chamber source plants. Acta Hortic. **1234**, 211-218.

Bettoni, J. C.; Souza, J. A.; Volk, G. M.; Dalla Costa, M.; da Silva, F. N.; Kretzschmar, A. A.; 2019b. Eradication of latent viruses from apple cultivar 'Monalisa' shoot tips using droplet-vitrification cryotherapy. Sci. Hortic. **250**, 12-18.

Bettoni, J. C.; Costa, D. C.; Souza, J. A.; Volk, G. M.; Nickel, O.; da Silva, F. N.; Kretzschmar, A. A.; 2018: Cryotherapy by encapsulation-dehydration is effective for *in vitro* eradication of latent viruses from 'Marubakaido' apple rootstock. J. Biotechnol. **269**, 1-7.

Bettoni, J. C.; Costa, M. D.; Gardin, J. P. P.; Kretzschmar, A. A.; Pathirana, R.; 2016: Cryotherapy: a new technique to obtain grapevine plants free of viruses. Rev. Brasil. Frutic, **38**, e-833.

Bi, W. L.; Hao, X. Y.; Cui, Z. H.; Volk, G. M.; Wang, Q. C.; 2018a: Droplet-vitrification cryopreservation of *in vitro*-grown shoot tips of grapevine (*Vitis* spp.). In Vitro Cell Dev. Biol.-Plant **54**, 590-599.

Bi, W. L.; Hao, X. Y.; Cui, Z. H.; Pathirana, R.; Volk, G. M.; Wang, Q. C.; 2018b: Shoot tip cryotherapy for efficient eradication of grapevine leafroll-associated virus-3 from diseased grapevine *in vitro* plants. Ann. Appl. Biol. **173**, 261-270.

Bi, W. L.; Pan, C.; Hao, X. Y.; Cui, Z. H.; Kher, M. M.; Marković, Z.; Wang, Q. C.; Teixeira da Silva, J. A.; 2017: Cryopreservation of grapevine (*Vitis* spp.) - a review. In Vitro Cell Dev. Biol.-Plant **53**, 449-460.

Brison, M.; Boucaud, M. T.; Pierronnet, A.; Dosba, F.; 1997: Effect of cryopreservation on the sanitary state of a cv. *Prunus* rootstock experimentally contaminated with *Plum Pox Potyvirus*. Plant Sci. **123**, 189-196.

Chang, Y.; Reed, B. M.; 2000: Extended alternating-temperature cold acclimation and culture duration improve pear shoot cryopreservation. Cryobiology **40**, 311-322.

Chang, Y.; Reed, B. M.; 2001: Preculture conditions influence cold hardiness and regrowth of *Pyrus cordata* shoot tips after cryopreservation. HortScience **36**, 329-333.

Engelmann, F.; 2011: Cryopreservation of embryos: an overview. Methods Mol. Biol .**710**, 155-184.

Esensee, V.; Stushnoff, C.; 1990: Cryoconservation of dormant grape (*Vitis* sp.) buds. HortScience **25**, 90.

Ganino, T.; Silvanini, A.; Beghé, D.; Benelli, C.; Lambardi, M.; Fabbri, A.; 2012: Anatomy and osmotic potential of *Vitis* rootstock shoot tips recalcitrant to cryopreservation. Biol. Plant. **56**, 78-82.

Hassan, N. A.; Haggag, A. M.; 2013: Cryopreservation of two Egyptian grape (*Vitis vinifera*) cultivars using two steps vitrification protocol. World Appl. Sci. J. **28**, 254-258.

Helliot, B.; Panis, B.; Poumay, Y.; Swennen, R.; Lepoivre, P.; Frison, E.; 2002: Cryopreservation for the elimination of cucumber mosaic and banana streak viruses from banana (*Musa* spp.). Plant Cell Rep. **20**, 1117-1122.

Höfer, M.; Reed, B. M.; 2010: Cryopreservation of strawberry genetic resources in Germany. Acta Hortic. **918**, 139-146.

Jenderek, M. M.; Reed, B. M.; 2017: Cryopreserved storage of clonal germplasm in the USDA National Plant Germplasm System. In Vitro Cell Dev. Biol.-Plant **53**, 299-308.

Johnston, J. W.; Harding, K.; Benson, E. E.; 2007: Antioxidant status and genotypic tolerance of *Ribes in vitro* cultures to cryopreservation. Plant Sci. **172**, 524-534.

Kaczmarczyk, A.; Shvachko, N.; Lupysheva, Y.; Hajirezaei, M. R.; Keller, E. R. J.; 2008: Influence of alternating temperature preculture on cryopreservation results for potato shoot tips. Plant Cell Rep. **27**, 1551-1558.

Keller, E. R. J.; 2007: Cryopreservation for maintenance of plant germplasm in Germany. Adv. Hortic. Sci. **21**, 228-231.

Kushnarenko, S. V.; Romadanova, N. V.; Reed, B. M.; 2009: Cold acclimation improves regrowth of cryopreserved apple shoot tips. CryoLetters **30**, 47-54.

Li, B.; Jiang, J.; Fan, X.; Zhang, Y.; Sun, H.; Zhang, G.; Liu, C.; 2017: Molecular characterization of Chinese grape landraces (*Vitis* L.) using microsatellite DNA markers. HortScience **52**, 533-540.

Li-Mallet, A.; Rabot, A.; Geny, L.; 2015: Factors controlling inflorescence primordia formation of grapevine: their role in latent bud fruitfulness? A review. Botany **94**, 147-163.

Marković, Z.; Chatelet, P.; Peyrière, A.; Preiner, D.; Engelmann-Sylvestre, I.; Karoglan, J.; Engelmann, F.; 2013: Effect of proline pretreatment on grapevine shoot-tip response to a droplet-vitrification protocol. Am. J. Plant Sci. **2**, 2414-2417.

Marković, Z.; Chatelet, P.; Preiner, D.; Sylvestre, I.; Kontić, J. K.; Engelmann, F.; 2014: Effect of shooting medium and source of material on grapevine (*Vitis vinifera* L.) shoot tip recovery after cryopreservation. CryoLetters **35**, 40-47.

Marković, Z.; Preiner, D.; Stupić, D. S.; Andabaka, Ž.; Šimon, S.; Vončina, D.; Maletić, E.; Kontić, J. K.; Chatelet, P.; Engelmann, F.; 2015: Cryopreservation and cryotherapy of grapevine (*Vitis vinifera* L). Vitis **54**, 247-251.

Mathew, L.; McLachlan, A.; Jibran, R.; Burritt, D. J.; Pathirana, R.; 2018: Cold, antioxidant and osmotic pre-treatments maintain the

structural integrity of meristematic cells and improve plant regeneration in cryopreserved kiwifruit shoot tips, Protoplasma **255**, 1-13.

Matsumoto, T.; Sakai, A.; 2003: Cryopreservation of axillary shoot tips of *in vitro*-grown grape (*Vitis*) by a two-step vitrification protocol. Euphytica **131**, 299-304.

Murashige, T.; Skoog, F.; 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473-497.

Niino, T.; Arizaga, M. V.; 2015: Cryopreservation for preservation of potato genetic resources. Breed. Sci. **65**, 41-52.

OIV (International Organisation of Vine and Wine); 2018: World Viticulture Situation. OIV Statistical Report on World Vitiviniculture. O I V (Off. Int. Vigne Vin), Paris, France.

Panis, B.; Garming, H.; Piette, B.; Roux, N.; Swennen, R.; Van den Houve, L.; 2010: Banana conservation activities in the Bioversity International Transit Centre (ITC) Belgium. CryoLetters **31**, 76-94.

Panta, A.; Panis, B.; Ynouye, C.; Swennen, R.; Roca, W.; Tay, D.; Ellis, D.; 2015: Improved cryopreservation method for the long-term conservation of the world potato germplasm collection. Plant Cell Tiss. Org. Cult. **120**, 117-125.

Pathirana, R.; McLachlan, A.; Hedderley, D.; Carra, A.; Carimi, F.; Panis, B.; 2015: Removal of leafroll viruses from infected grapevine plants by droplet vitrification. Acta Hortic. **1083**, 491-498.

Pathirana, R.; McLachlan, A.; Hedderley, D.; Panis, B.; Carimi, F.; 2016: Pretreatment with salicylic acid improves plant regeneration after cryopreservation of grapevine (*Vitis* spp.) by droplet vitrification. Acta Physiol. Plant **38**, 1-11.

Reed, B. M.; 2014: Antioxidants and cryopreservation, the new normal? Acta Hortic. **1039**, 41-48.

Reed, B. M.; Kovalchuk, I.; Kushnarenko, S.; Meier-Dinkel, A.; Schoenweiss, K.; Pluta, S.; Straczynska, K.; Benson, E. E.; 2004: Evaluation of critical points in technology transfer of cryopreservation protocols to international plant conservation laboratories. CryoLetters **25**, 341-352.

Reed, B. M.; 2001: Implementing cryogenic storage of clonally propagated plants. Cryoletters **22,** 97-104.

Sakai, A.; Kobayashi, S.; Oiyama, I.; 1990: Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. *brasiliensis* Tanaka) by vitrification. Plant Cell Rep. **9**, 30-33.

Shepherd, A.; Bonnart, R.; Volk, G. M.; 2013: Cryopreservation of *Vitis* shoot tips from diverse species. In: B. M. Reed (Ed.): 2nd Int. Symp. Plant Cryopreservation. Fort Collins, USA.

Towill, L. E.; Forsline, P. L.; Walters, C.; Waddell, J. W.; Laufmann, J.; 2004: Cryopreservation of *Malus* germplasm using a winter vegetative bud method: results from 1915 accessions, CryoLetters **25**, 323-334.

Uchendu, E. E.; Leonard, S. W.; Traber, M. G.; Reed, B. M.; 2010b: Vitamins C and E improve regrowth and reduce lipid peroxidation of blackberry shoot tips following cryopreservation. Plant Cell Rep. **29**, 25-35.

Uchendu, E. E.; Muminova, M.; Gupta, S.; Reed, B. M.; 2010a: Antioxidant and anti-stress compounds improve regrowth of cryopreserved *Rubus* shoot tips. In Vitro Cell Dev. Biol.-Plant **46**, 386-393.

Vasconcelos, M. C.; Greven, M.; Winefield, C. S.; Trought, M. C. T.; Raw, V.; 2009: The Flowering Process of *Vitis vinifera*: A Review. Am. J. Enol. Vitic. **60**, 411-434.

Vieira, R. L.; da Silva, A. L.; Zaffari, G. R.; Steinmacher, A.; Fraga, H. P. F.; Guerra, M. P.; 2015: Efficient elimination of virus complex from garlic (*Allium sativum* L.) by cryotherapy of shoot tips. Acta Physiol. Plant **37**, 1-11.

Volk, G. M.; Bonnart, R.; Shepherd, A.; Yin, Z.; Lee, R.; Polek, M.; Krueger, R.; 2017: Citrus cryopreservation: viability of diverse taxa and histological observations. Plant Cell Tiss. Org. Cult. **128**, 327-334.

Volk, G. M.; Caspersen, A. M.; 2007: Plasmolysis and recovery of different cell types in cryoprotected shoot tips of *Mentha* X *piperita*. Protoplasma **231**, 215-226.

Volk, G. M.; Henk, A. D.; Jenderek, M.; Richards, C. M.; 2016: Probabilistic viability calculations for cryopreserving vegetatively propagated collections in genebanks. Genet. Resour. Crop Evol. **64**, 1613-1622.

Volk, G. M.; Shepherd, A. N.; Bonnart, R.; 2018: Successful cryopreservation of *Vitis* shoot tips: Novel pretreatment combinations applied to nine species, CryoLetters **39**, 322-330.

Wang, M. R.; Chen, L.; Teixeira da Silva, J. A.; Volk, G. M.; Wang, Q. C.; 2018: Cryobiotechnology of apple (*Malus* spp.): development, progress and future prospects. Plant Cell Rep. **37**, 689-709.

Wang, Q. C.; Panis, B.; Engelmann, F.; Lambardi, M.; Valkonen, J. P. T.; 2009: Cryotherapy of shoot tips: a technique for pathogen eradication to produce healthy planting materials and prepare healthy plant genetic resources for cryopreservation. Ann. Appl. Biol. **154**, 351-363.

Wang, Q. C.; Mawassi, M.; Li, P.; Gafny, R.; Sela, I.; Tanne, E.; 2003: Elimination of grapevine virus A (GVA) by cryopreservation of in vitro-grown shoot tips of *Vitis vinifera* L. Plant Sci. **165**, 321-327.

Yi, J. Y.; Lee, G. A.; Jeong, J. W.; Lee, S. Y.; Lee, Y. G.; 2014: Eliminating *Potato virus Y* (PVY) and *Potato leaf roll virus* (PLRV) using cryotherapy of *in vitro* grown potato shoot tips. Korean J. Crop Sci. **59**, 498-504.

Received January 28, 2019
Accepted March 27, 2019