Cryopreservation of 12 *Vitis* Species Using Apical Shoot Tips Derived from Plants Grown In Vitro

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Abstract. The availability of and easy access to diverse *Vitis* species are prerequisites for advances in breeding programs. Plant genebanks usually maintain collections of *Vitis* taxa as field collections that are vulnerable to biotic and abiotic stresses. Cryopreservation has been considered an ideal method of preserving these collections as safety backups in a cost-effective manner. We report a droplet vitrification method used to cryopreserve 12 *Vitis* species (*Vitis vinifera* cvs. Chardonnay and ‘Riesling, *V. actinifolia*, *V. aestivalis*, *V. jacquemontii*, *V. flexuosa*, *V. palmata*, *V. riparia*, *V. rupestris*, *V. sylvestris*, *V. ficifolia*, *V. treleasi*, and *V. ×novae angeliae*) using shoot tips excised from plants grown in vitro. Our results demonstrated wide applicability of this technique, with regrowth levels at least 43% for 13 genotypes representing 12 *Vitis* species. We demonstrated that the droplet vitrification procedure can be successfully replicated by technical staff, thus suggesting that this method is ready for implementation.

Grape (*Vitis* spp.) is one of the most economically important fruit crops cultivated and consumed worldwide (OIV, 2018). *Vitis* has a rich genetic diversity; there are more than 70 species within the genus, with most of the globally important cultivars assigned to *Vitis vinifera* (Li et al., 2017). Wild *Vitis* species as well as *Muscadinia rotundifolia* have been used for hybrid grape breeding as new cultivars and rootstocks, many of which provide resistance to pests and diseases (Carimi et al., 2011; Eibach et al., 2007; Smith et al., 2017).

The availability of and easy access to *Vitis* genetic resources are essential for breeding programs (Carimi et al., 2016). *Vitis* genebank

collections are traditionally conserved in field repositories. Field maintenance is costly and time-consuming, it requires extensive acreage, and plants are vulnerable to abiotic stresses and biotic threats (Benelli et al., 2013; Marković et al., 2013b; Pathirana et al., 2016). In vitro culture back-up is one alternative to short-term preservation of *Vitis* germplasm (Hassan et al., 2013; Postman et al., 2006), but it has some limitations because tissue culture is labor-intensive and cultures could become contaminated or undergo somaclonal variation (Kaya and Souza, 2017; Mathew et al., 2018; Souza et al., 2016).

Cryopreservation is the storage of biological materials in liquid nitrogen (LN) (−196 °C) or LN vapor (−165 to −190 °C). Cryopreservation methods have been used to securely conserve portions of plant genebank collections for long durations. Under cryopreservation conditions, viable propagules are preserved in a state in which cellular divisions and metabolic processes are minimized (Benelli et al., 2013; Wang et al., 2018). Compared with the cost of creating duplicated field collections, it is cost-effective to establish cryopreserved back-up collections (Benson, 2008).

Although there are many established cryobanks that conserve vegetative propagules such as shoot tips or dormant buds of clonally propagated genetic resources (Towill et al., 2004; Wang et al., 2014), 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). Results have been more promising for

Vitis shoot tip cryopreservation. Several works have reported successful shoot tip cryopreservation (Bi et al., 2017; Hassan and Haggag, 2013; Pathirana et al., 2016). However, genotype-specific responses have made these procedures difficult to implement (Benelli et al., 2003; Ganino et al., 2012; Pathirana et al., 2016). Most *Vitis* cryopreservation research has focused on the development of procedures using a limited number of species (Bi et al., 2017). So far, the droplet vitrification technique appears to be a promising method to overcome species-specific and genotype-specific responses to *Vitis* cryopreservation (Bi et al., 2018a; Volk et al., 2018). Droplet vitrification uses ultra-fast shoot tip cooling and warming conditions, which are an important requirement for successful cryopreservation protocols based on vitrification (Benson and Harding, 2012; Kaya et al., 2013; Souza et al., 2016).

Access to reliable cryopreservation methods that result in high levels of viability (≥40% after LN exposure) and highly skilled staff are crucial to the development of successful base collections (Volk et al., 2016). We describe the development of a widely applicable droplet vitrification procedure to cryopreserve *Vitis* shoot tips from in vitro stock plants.

Materials and Methods

Plant materials and pretreatments. All plant materials were originally received from the USDA-ARS National Clonal Germplasm Repository for Tree Fruit, Nut Crops, and Grapes in Davis, CA, and introduced from the field to in vitro conditions. Young shoot tissue was washed with soapy water, rinsed, and treated with 70% isopropanol for 1 min, followed by two rinses with distilled water and treatment with 10% bleach (8.25% sodium hypochlorite) and 0.1% Tween 20 (v/v) for 10 min. Then, the tissue was rinsed three times in sterile distilled water. *Vitis* accessions included *Vitis vinifera* cvs. Chardonnay and Riesling, *V. actinifolia* (DVIT 2594.1), *V. aestivalis* (DVIT 1408), *V. jacquemontii* (PI 135726), *V. flexuosa* (DVIT 1385), *V. palmata* (DVIT 2228.1), *V. riparia* (PI 588214), *V. rupestris* (DVIT 8166), *V. sylvestris* (DVIT 2426.1), *V. ficifolia* (DVIT 2008.1), *V. treleasi* (DVIT 1410) and *V. ×novae angeliae* (DVIT 1457).

Stock cultures were maintained and subcultured every 8 to 12 weeks in glass culture vessels (89 × 170 mm) on MS medium (Murashige and Skoog, 1962) containing 30 g·L^{−1} sucrose, 0.175 mg·L^{−1} indole-3-acetic acid (IAA), and 2.5 g·L^{−1} gellan gum at pH 5.7 (pH 6.4 before autoclaving). Cultures were grown at 25 °C during a photoperiod of 16 h of daylight with light intensity of 40 μM·m^{−2}·s^{−1}.

Nodal sections were obtained from 2- to 3-month-old in vitro stock plants and placed in 100- × 25-mm plastic petri dishes with 50 mL pretreatment medium [MS salts/vitamins supplemented with 30 g·L^{−1} sucrose, 0.2 mg·L^{−1} 6-benzyl aminopurine (BA), 0.1 mM salicylic

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acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form), and 3 g·L⁻¹ gellan gum at pH 5.7 (pH 6.4 before autoclaving)] with a density of 40 nodal sections (1–2 cm) per plate and cultured for 2 weeks under the same conditions as the in vitro stock cultures. This step provided uniform apical shoot tips for cryopreservation experiments that were the same as those described for *V. vinifera* cv. Portland by Marković et al. (2014). Next, nodal sections were cold-acclimated at 5 °C during a 16-h photoperiod for an additional 2 weeks (when applied).

Preculture and cryopreservation. Uniform apical shoot tips (1-mm length, 1-mm width) were excised from nodal sections that either had or had not been cold-acclimated. Shoot tips were cultured on preculture medium [half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid, 1 mM glutathione (reduced form), and 3 g·L⁻¹ gellan gum at pH 5.7 (pH 6.94 before autoclaving)] for 3 d at 25 °C in darkness. Then, they were placed in loading solution [half-strength MS, 2 M glycerol, 0.4 M sucrose, pH 5.7 (pH 6.9 before autoclaving)] for 20 min at 22 °C, followed by half-strength PVS2 [filter-sterilized half-strength MS, 0.4 M sucrose, 15% (w/v) glycerol, 7.5% (w/v) ethylene glycol (EG), 7.5% (w/v) dimethyl sulfoxide (DMSO), pH 5.8] (Matsumoto and Sakai, 2003) for 30 min at 22 °C and full-strength PVS2 [filter-sterilized half-strength MS, 0.4 M sucrose, 30% (w/v) glycerol, 15% (w/v) EG, 15% (w/v) DMSO, pH 5.8] (Sakai et al., 1990) at 0 °C for 60, 75, 90, or 105 min. Two minutes before the end of each treatment, PVS2-treated shoot tips were placed on a thin layer of PVS2 on sterile aluminum foil strips (≈6 × 25 mm) and then plunged in LN.

Regrowth after cryo-exposure. After 1 hour of LN exposure (Mandal and Dixit-Sharma, 2007; Yi et al., 2018), the aluminum foil strips with shoot tips were warmed quickly by inverting the strips in unloading solution [half-strength MS, 1.2 M sucrose, pH 5.7 (pH 7.5 before autoclaving)] at 22 °C and diluted for 20 min.

For recovery, shoot tips were placed on recovery medium 1 [half-strength MS macroelements without ammonium, full-strength MS microelements, and *Vitis* vitamins (100 mg·L⁻¹ myo inositol, 10 mg·L⁻¹ thiamine HCl, 1 mg·L⁻¹ nicotinic acid, 1 mg·L⁻¹ pyridoxine HCl, 1 mg·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 before autoclaving) overnight in the dark. They were transferred to recovery medium 2 (half-strength MS macroelements without ammonium and full-strength MS microelements with *Vitis* vitamins) supplemented with 30 g·L⁻¹ sucrose, 0.2 mg·L⁻¹ BA³, and 8 g·L⁻¹ agar at pH 5.7 (pH 6.5 before autoclaving) and cultured for 2 weeks at 25 °C in darkness. They were then transferred to recovery medium 3 (half-strength MS macroelements and full-strength MS microelements with *Vitis* vitamins) supplemented with 30 g·L⁻¹ sucrose, 0.2 mg·L⁻¹ BA³, and 8 g·L⁻¹ agar at pH 5.7 (pH 6.5 before autoclaving) and grown in the light at 25 °C (40 μM·m⁻²·s⁻¹, 16-h photoperiod).

Application of cryopreservation to additional *Vitis* species. The applicability of the droplet vitrification protocol was evaluated for in vitro source plants of five additional *Vitis* species: *V. vinifera* cv. Riesling, *V. actinifolia* (DVIT 2594.1), *V. ficifolia* (DVIT 2008.1), *V. treleasi* (DVIT 1410), and *V. xnovae angeliae* (DVIT 1457). This experiment was performed as described, except

that shoot tips were exposed to PVS2 for 90 min at 0 °C.

Cryopreservation by a second technical expert. The first series of experiments with eight *Vitis* species was performed by a single technical expert to identify optimum PVS2 exposure durations and the effect of cold acclimation on shoot tip regrowth after cryo-exposure. The optimized method with 90 min of full-strength PVS2 exposure was tested with five additional *Vitis* accessions. Cryopreservation experiments were then performed by a second technical expert using four of the same five additional accessions to determine the extent to which the method could be successfully performed by others.

Regrowth and data analyses. Shoot tip regrowth (organized shoots with at least one leaf) was measured 8 weeks after plating on regrowth medium. Each experiment was performed with two replicates of 20 shoot tips for each treatment. Means and SE were calculated across experimental replicates and analyzed using analysis of variance, and means separation tests were performed using Tukey's mean separation test. $P \leq 0.05$ was considered significantly different.

Results

Regrowth of cryopreserved *Vitis* shoot tips. Across the species evaluated by the first technical expert (*V. vinifera* 'Chardonnay', *V. aestivalis*, *V. Jacquemontii*, *V. flexuosa*, *V. palmata*, *V. riparia*, *V. rupestris*, and *V. sylvestris*), shoot tips that were not exposed to LN (–LN) and not cold-acclimated had higher average regrowth levels with 60, 75, 90, and 105 min of PVS2 exposure (63% to 71%) compared with the corresponding LN treatment (+LN) (37% to 53%) (Table 1). Similarly, shoot tips that were not exposed to

Table 1. Regrowth level (%) of shoot tips excised from nodal sections sourced from eight *Vitis* species grown in vitro with 60, 75, 90 and 105 min of plant vitrification solution 2 (PVS2) exposure with and without liquid nitrogen (LN) exposure and without cold acclimation.²

Species	Plant identifier	–LN			
		PVS2 exposure duration (min)			
		60	75	90	105
<i>V. vinifera</i>	'Chardonnay'	90 ± 0 a	85 ± 5 ab	73 ± 3 abc	73 ± 3 abcd
<i>V. aestivalis</i>	DVIT 1408	73 ± 3 abcd	70 ± 5 abcd	75 ± 5 abc	50 ± 5 bcde
<i>V. Jacquemontii</i>	PI 135726	80 ± 5 abc	85 ± 0 ab	92 ± 2 a	80 ± 5 abc
<i>V. flexuosa</i>	DVIT 1385	63 ± 8 abcde	65 ± 5 abcde	65 ± 5 abcde	68 ± 3 abcde
<i>V. palmata</i>	DVIT 2228.1	80 ± 5 abc	83 ± 13 abc	75 ± 5 abc	68 ± 18 abcde
<i>V. riparia</i>	PI 588214	48 ± 3 cde	38 ± 3 de	38 ± 3 de	33 ± 8 e
<i>V. rupestris</i>	DVIT 8166	60 ± 5 abcde	53 ± 3 bcde	65 ± 5 abcde	58 ± 3 abcde
<i>V. sylvestris</i>	DVIT 2426.1	78 ± 8 abc	68 ± 13 abcde	70 ± 0 abcde	80 ± 5 abc
Average		71 ± 5	68 ± 6	69 ± 5	63 ± 6
Species	Plant identifier	+LN			
		PVS2 exposure duration (min)			
		60	75	90	105
<i>V. vinifera</i>	'Chardonnay'	53 ± 3 abcde	68 ± 3 ab	58 ± 3 abcd	50 ± 0 abcdef
<i>V. aestivalis</i>	DVIT 1408	38 ± 3 defgh	58 ± 3 abcd	58 ± 3 abcd	33 ± 8 efgh
<i>V. Jacquemontii</i>	PI 135726	48 ± 3 abcdefg	50 ± 5 abcdef	72 ± 2 a	53 ± 8 abcde
<i>V. flexuosa</i>	DVIT 1385	33 ± 3 efgh	43 ± 3 cdefgh	48 ± 3 abcdefg	43 ± 3 cdefgh
<i>V. palmata</i>	DVIT 2228.1	30 ± 5 efgh	53 ± 3 abcde	53 ± 3 abcd	45 ± 5 bcdefgh
<i>V. riparia</i>	PI 588214	23 ± 3 h	28 ± 3 fgh	35 ± 5 defgh	25 ± 5 gh
<i>V. rupestris</i>	DVIT 8166	38 ± 8 defgh	43 ± 8 cdefgh	43 ± 8 cdefgh	33 ± 3 efgh
<i>V. sylvestris</i>	DVIT 2426.1	33 ± 3 efgh	50 ± 5 abcdef	63 ± 3 abc	48 ± 3 abcdefg
Average		37 ± 3	49 ± 4	53 ± 4	41 ± 3

²Data represent mean ± SE. Values followed by different letters within the set of –LN/PVS2 exposure combinations and within the set of +LN/PVS2 exposure combinations were significantly different at $P < 0.05$ using Tukey's mean separation test.

LN and were cold-acclimated had higher levels of regrowth with 60, 75, 90, and 105 min of PVS2 exposure (58% to 72%) compared with the corresponding LN treatment (+LN) (39% to 59%) (Table 2). There were no differences in the regrowth levels of -LN 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.

The shoot tips that were not cold-acclimated and had the lowest regrowth levels (-LN) were *V. riparia* with 60 min of PVS2 (48%), *V. riparia* (38%) and *V. rupestris* (53%) with 75 min of PVS2, *V. riparia* with 90 min of PVS2 (38%), and *V. aestivalis* (50%) and *V. riparia* (33%) with 105 min of PVS2 exposure (Table 1, Fig. 1). Similarly, with cold acclimation, the lowest regrowth levels (-LN) were obtained for *V. riparia* (48% after 60 min of PVS2 and 53% after 75 min of PVS2), *V. jacquemontii* (55% after 90 min of PVS2), *V. riparia* (43% after 90 min of PVS2), *V. aestivalis* (48% after 105 min of PVS2), *V. jacquemontii* (58% after 105 min of PVS2), *V. riparia* (38% after 105 min of PVS2), *V. rupestris* (53% after 105 min of PVS2), and *V. sylvestris* (50% after 105 min of PVS2) (Table 2). Overall, the *V. riparia* accession responded less positively to the PVS2 treatment than did the other species.

The highest regrowth levels with LN were obtained after 90 min of PVS2 exposure and no cold acclimation: *V. vinifera* 'Chardonnay', 58%; *V. aestivalis*, 58%; *V. jacquemontii*, 72%; *V. flexuosa*, 48%; *V. palmata*, 53%; *V. riparia*, 35%; *V. rupestris*, 43%; and *V. sylvestris*, 63% (Table 1, Fig. 1). The highest regrowth levels with LN were obtained after 75 min of PVS2 with cold acclimation:

V. vinifera 'Chardonnay', 70%; *V. aestivalis*, 58%; *V. jacquemontii*, 58%; *V. flexuosa*, 65%; *V. palmata*, 60%; *V. riparia*, 43%; *V. rupestris*, 55%; and *V. sylvestris*, 68% (Table 2). In general, shoot tips excised from cold-acclimated plants needed less PVS2 exposure than shoot tips that were not cold-acclimated. With 90 min of +LN treatment without cold acclimation, seven of the eight species met our goal of 40% regrowth (Table 1). With 75 min of +LN treatment with cold acclimation, all eight species met our goal of 40% regrowth (Table 2).

Application of the procedure to additional *Vitis* species. The droplet vitrification procedure with no cold acclimation and 90 min of full-strength PVS2 was tested using five additional genotypes (*V. actinifolia*, *V. ficifolia*, *V. treleasi*, *V. vinifera* 'Riesling', and *V. xnovae angeliae*) to determine its applicability to additional species. During the control treatment (-LN), shoot tips exposed to full-strength PVS2 for 90 min had an average regrowth level of 82%. The corresponding cryo-exposed (+LN) shoot tip regrowth levels ranged from 43% for *V. treleasi* to 68% for *V. vinifera* 'Riesling'. Across the five *Vitis* genotypes, the average level of regrowth was 57% (Table 3).

Replication of the method by a second technical expert. A second technical expert assessed the regrowth levels of shoot tips of *V. actinifolia*, *V. ficifolia*, *V. treleasi*, *V. vinifera* 'Riesling', and *V. xnovae angeliae* that were excised from nodal sections either with or without cold acclimation, exposed to full-strength PVS2 for 60, 75, or 90 min, and either exposed to LN (+LN) or not exposed to LN (-LN) (Tables 4 and 5).

Average regrowth levels of the noncold-acclimated and non-LN-treated shoot tips

of *V. actinifolia*, *V. treleasi*, *V. vinifera* 'Riesling', and *V. xnovae angeliae* were 74% for 60 min of PVS2, 65% for 75 min of PVS2, and 64% for 90 min of PVS2 exposure (Table 4). With LN exposure and no cold acclimation, average shoot tip regrowth levels were 54% for 60 min of PVS2, 49% for 75 min of PVS2, and 48% for 90 min of PVS2 exposure (Table 4). For noncold-acclimated shoot tips, there were no statistically significant differences among accessions with respect to regrowth levels and PVS2 treatments (Table 4).

For shoot tips that were cold-acclimated and did not undergo LN treatment, the average regrowth levels were 76%, 72%, and 71% for 60, 75, and 90 min of PVS2 exposure, respectively (Table 5). There were significant differences among accessions with respect to regrowth levels of cold-acclimated shoot tips after LN exposure (Table 5). With cold acclimation and LN exposure, *V. actinifolia* (85% regrowth) and *V. vinifera* cv. Riesling (73% regrowth) had the highest regrowth levels and *V. ficifolia* had the lowest regrowth levels (38%) after 75 min of PVS2 exposure (Table 5).

Both technical experts achieved average regrowth levels greater than the threshold level of 40% (Volk et al., 2016) for the *V. actinifolia*, *V. treleasi*, *V. vinifera* 'Riesling', and *V. xnovae angeliae* shoot tips that were exposed to full-strength PVS2 for 90 min without cold acclimation and then cryopreserved (Tables 3 and 4).

Discussion

Cryopreservation is a reliable and cost-effective method for the long-term storage of vegetatively propagated plant germplasm

Table 2. Regrowth level (%) of shoot tips excised from nodal sections sourced from eight *Vitis* species grown in vitro with 60, 75, 90, and 105 min of plant vitrification solution 2 (PVS2) exposure with and without liquid nitrogen (LN) exposure and 2 weeks of cold acclimation.²

Species	Plant identifier	-LN			
		PVS2 exposure duration (min)			
		60	75	90	105
<i>V. vinifera</i>	'Chardonnay'	85 ± 0 ab	85 ± 0 Ab	83 ± 3 abc	83 ± 8 abc
<i>V. aestivalis</i>	DVIT 1408	60 ± 0 abcdefgh	72 ± 3 abcdef	63 ± 3 abcdefgh	48 ± 3 fgh
<i>V. jacquemontii</i>	PI 135726	78 ± 3 abcde	68 ± 3 abcdefg	55 ± 15 cdefgh	58 ± 8 bcdefgh
<i>V. flexuosa</i>	DVIT 1385	88 ± 3 a	85 ± 5 Ab	75 ± 5 abcdef	80 ± 0 abcd
<i>V. palmata</i>	DVIT 2228.1	75 ± 5 abcdef	68 ± 8 abcdefg	68 ± 13 abcdefg	60 ± 0 abcdefgh
<i>V. riparia</i>	PI 588214	48 ± 3 fgh	53 ± 3 defgh	43 ± 3 gh	38 ± 3 h
<i>V. rupestris</i>	DVIT 8166	70 ± 5 abcdefg	60 ± 0 abcdefgh	70 ± 5 abcdefg	53 ± 3 defgh
<i>V. sylvestris</i>	DVIT 2426.1	70 ± 5 abcdefg	68 ± 3 abcdefg	73 ± 8 abcdef	50 ± 0 efgh
Average		72 ± 5	70 ± 4	66 ± 4	58 ± 6
Species	Plant identifier	+LN			
		PVS2 exposure duration (min)			
		60	75	90	105
<i>V. vinifera</i>	'Chardonnay'	58 ± 3 abcde	70 ± 0 a	58 ± 8 abcde	45 ± 0 abcdefg
<i>V. aestivalis</i>	DVIT 1408	48 ± 3 abcdefg	58 ± 3 abcde	40 ± 5 cdefg	28 ± 8 g
<i>V. jacquemontii</i>	PI 135726	55 ± 5 abcdef	58 ± 3 abcde	53 ± 3 abcdefg	38 ± 3 defg
<i>V. flexuosa</i>	DVIT 1385	55 ± 5 abcdef	65 ± 5 abc	53 ± 3 abcd	55 ± 10 abcdef
<i>V. palmata</i>	DVIT 2228.1	55 ± 5 abcdef	60 ± 0 abcd	58 ± 3 abcdefg	30 ± 5 fg
<i>V. riparia</i>	PI 588214	33 ± 3 efg	43 ± 3 bcdefg	33 ± 3 efg	28 ± 3 g
<i>V. rupestris</i>	DVIT 8166	45 ± 0 abcdefg	55 ± 5 abcdef	58 ± 8 abcde	40 ± 5 cdefg
<i>V. sylvestris</i>	DVIT 2426.1	55 ± 5 abcdef	68 ± 3 ab	58 ± 8 abcde	48 ± 3 abcdefg
Average		50 ± 3	59 ± 3	51 ± 3	39 ± 4

²Data represent mean ± SE. Values followed by different letters within the set of -LN /PVS2 exposure combinations and within the set of +LN /PVS2 exposure combinations are significantly different at *P* < 0.05 using Tukey's mean separation test.

Fig. 1. Grapevine plants recovered after 90 min of plant vitrification solution 2 (PVS2) treatment with liquid nitrogen (+LN) or without liquid nitrogen (–LN) exposure. *V. aestivalis* shoot tips exposed to LN and recovered for 30 d (A) and 2 months (B) and without LN exposure after 2 months of regrowth (C). *V. Jacquemontii* shoot tips exposed to LN and recovered for 30 d (D) and 2 months (E) and without LN exposure after 2 months of regrowth (F). ‘Chardonnay’ shoot tips exposed to LN and recovered for 30 d (G) and 2 months (H) and without LN exposure after 2 months of regrowth (I). *V. flexuosa* shoot tips exposed to LN and recovered for 30 d (J) and 2 months (K) and without LN exposure after 2 months of regrowth (L). *V. palmata* shoot tips exposed to LN and recovered for 42 d (M) and 2 months (N) and without LN exposure after 2 months of regrowth (O). Scale bars: A = 1 mm; D, G, J, M = 2 mm.

because when collections are established in LN, cryopreserved collections require minimal space, labor, and maintenance (Benelli et al., 2013; Wang et al., 2018). Our droplet vitrification technique was effective for the cryopreservation of *Vitis* species using apical shoot tips from in vitro plants. This method resulted in regrowth levels of at least 43% for 13 genotypes representing 12 *Vitis* species, including two *V. vinifera* cultivars.

Vitis shoot tip cryopreservation protocols have been previously reported using encapsulation dehydration (Marković et al., 2013b; Plessis et al., 1993; Wang et al., 2003a, 2003b; Zhai et al., 2003; Zhao et al., 2001), encapsulation vitrification (Benelli et al., 2003), vitrification (Ganino et al., 2012; Matsumoto and Sakai, 2003; Wang et al., 2003a, 2003b), droplet vitrification (Bi et al., 2018b; Hassan and Haggag, 2013; Pathirana

et al., 2015, 2016; Marković et al., 2013a, 2013b, 2014; Volk et al., 2018; Bettoni et al., 2019a), and V cryo-plate (Bettoni et al., 2019b) methods. For *Vitis*, previous reports focused on developing and applying methods primarily to *V. vinifera* cultivars, and it has been difficult to extend these procedures to the diverse range of *Vitis* species conserved within plant genebanks (Bi et al., 2017). Our goal was to identify a protocol that effectively cryopreserved diverse *Vitis* species to help eliminate the genotype-specific responses that limit the application of *Vitis* cryopreservation in genebanks.

Vitis cryopreservation protocols have been established using apical and axillary shoot tips excised directly from 3-week-old to 5-month-old in vitro stock plants (Benelli et al., 2003; Matsumoto and Sakai, 2003; Wang et al., 2000) or axillary shoot tips excised directly from 1-year-old greenhouse plants (Hassan and Haggag, 2013). In a recent study, Pathirana et al. (2016) found that shoot tips located at different positions along the shoot have differing regrowth responses to PVS2 exposure. The quality and physiological state of the stock cultures were thought to be key factors in successful *Vitis* cryopreservation (Bi et al., 2017; Marković et al., 2014). Our protocol used in vitro plants as source materials. Nodal sections from in vitro plants were placed on shooting medium for 2 weeks to generate shoots from which uniform 1-mm apical shoot tips could be excised. Previously, Marković et al. (2014) observed that shoot tips sampled from micro-cuttings had higher regrowth compared with shoot tips sampled directly from in vitro stock plants.

Successful cryopreservation procedures are dependent on having optimized pretreatment conditions (Volk et al., 2018). Pretreatments can differ by species and may include cold treatment exposure (Mathew et al., 2018), adding osmotic agents such as sorbitol and sugars, antioxidants such as glutathione reductase and ascorbic acid (Mathew et al., 2018; Reed, 2014; Volk et al., 2018), and elicitors of defense-related proteins in plants such as salicylic acid (Pathirana et al., 2016; Volk et al., 2018). We previously determined that osmotic agents and salicylic acid improved the cryopreservability of *Vitis* species (Volk et al., 2018). It has been reported that the addition of BA to the shooting medium has a positive effect on regrowth after LN exposure (Marković et al., 2014). In addition to BA, we included salicylic acid, glutathione (reduced form), and ascorbic acid to the pretreatment medium in an attempt to reduce the formation of reactive oxygen species during exposure to cryoprotectant and LN. The influence of cold acclimation pretreatment (5 °C for 2 weeks) before shoot tip desiccation was also assessed. Our results showed that the use of cold pretreatment did not significantly improve the regrowth of *Vitis* shoot tips after cryopreservation. For some species, cold acclimation pretreatments improved the regrowth and quality of regenerated plants after cryopreservation (Chang

Table 3. Regrowth levels (%) of shoot tips excised from nodal sections sourced from five *Vitis* species grown in vitro with 90 min of plant vitrification solution 2 (PVS2) exposure with and without liquid nitrogen (LN) exposure and without cold acclimation.²

Species	Plant identifier	PVS2 exposure time (min)	
		-LN	+LN
		90	
<i>V. actinifolia</i>	DVIT 2594.1	73 ± 3 a	63 ± 3 ab
<i>V. ficifolia</i>	DVIT 2008.1	83 ± 3 a	53 ± 3 bc
<i>V. treleasi</i>	DVIT 1410	83 ± 8 a	43 ± 3 c
<i>V. vinifera</i>	'Riesling'	88 ± 3 a	68 ± 3 ab
<i>V. × novae angeliae</i>	DVIT 1457	83 ± 3 a	58 ± 3 ab
Average		82 ± 1	57 ± 0

²Data represent mean ±SE. Values followed by different letters within each column are significantly different at $P < 0.05$ using Tukey's mean separation test.

Table 4. Regrowth level (%) of shoot tips excised from nodal sections sourced from four *Vitis* species grown in vitro with 60, 75, and 90 min of plant vitrification solution 2 (PVS2) exposure with and without liquid nitrogen (LN) exposure and without cold acclimation.²

Species	Plant identifier	PVS2 exposure duration (min)		
		-LN		
		60	75	90
<i>V. actinifolia</i>	DVIT 2594.1	75 ± 10 a	70 ± 10 a	65 ± 10 a
<i>V. treleasi</i>	DVIT 1410	57 ± 4 a	47 ± 7 a	44 ± 4 a
<i>V. vinifera</i>	'Riesling'	83 ± 3 a	64 ± 9 a	69 ± 19 a
<i>V. × novae angeliae</i>	DVIT 1457	83 ± 7 a	82 ± 7 a	78 ± 2 a
Average		74 ± 2	65 ± 1	64 ± 4
Species	Plant identifier	PVS2 exposure duration (min)		
		+LN		
		60	75	90
<i>V. actinifolia</i>	DVIT 2594.1	73 ± 13 a	62 ± 18 a	58 ± 2 a
<i>V. treleasi</i>	DVIT 1410	34 ± 1 a	32 ± 2 a	40 ± 0 a
<i>V. vinifera</i>	'Riesling'	50 ± 6 a	54 ± 6 a	35 ± 5 a
<i>V. × novae angeliae</i>	DVIT 1457	58 ± 3 a	50 ± 6 a	59 ± 7 a
Average		54 ± 3	49 ± 4	48 ± 1

²Data represent mean ±SE. Values followed by different letters within the set of -LN/PVS2 exposure combinations and within the set of +LN/PVS2 exposure combinations are significantly different at $P < 0.05$ using Tukey's mean separation test. These data were obtained by a second technical expert.

Table 5. Regrowth level (%) of shoot tips excised from nodal sections sourced from four *Vitis* species grown in vitro with 60, 75, and 90 min of plant vitrification solution 2 (PVS2) exposure with and without liquid nitrogen (LN) exposure and 2 weeks of cold acclimation.²

Species	Plant identifier	PVS2 exposure duration (min)		
		-LN		
		60	75	90
<i>V. actinifolia</i>	DVIT 2594.1	84 ± 4 a	80 ± 13 a	76 ± 11 a
<i>V. ficifolia</i>	DVIT 2008.1	54 ± 19 a	58 ± 8 a	73 ± 8 a
<i>V. treleasii</i>	DVIT 1410	90 ± 0 a	73 ± 8 a	72 ± 2 a
<i>V. vinifera</i>	'Riesling'	76 ± 9 a	77 ± 4 a	65 ± 15 a
Average		76 ± 4	72 ± 2	71 ± 3
Species	Plant Identifier	PVS2 exposure duration (min)		
		+LN		
		60	75	90
<i>V. actinifolia</i>	DVIT 2594.1	54 ± 7 ab	85 ± 0 a	70 ± 5 ab
<i>V. ficifolia</i>	DVIT 2008.1	45 ± 5 b	38 ± 13 b	47 ± 3 b
<i>V. treleasii</i>	DVIT 1410	42 ± 7 b	43 ± 13 b	43 ± 3 b
<i>V. vinifera</i>	'Riesling'	63 ± 3 ab	73 ± 3 ab	68 ± 3 ab
Average		51 ± 1	60 ± 3	57 ± 1

²Data represent mean ±SE. Values followed by different letters within the set of -LN/PVS2 exposure combinations and within the set of +LN/PVS2 exposure combinations are significantly different at $P < 0.05$ using Tukey's mean separation test. These data were obtained by a second technical expert.

and Reed, 2000; Kushnarenko et al., 2009; Mathew et al., 2018; Panta et al., 2015) and may be helpful for the cryoprocessing of some untested *Vitis* species and/or cultivars.

We investigated whether the described droplet vitrification procedure could be per-

formed by another technician in the laboratory. We obtained similar regrowth levels for the cryopreserved shoot tips across all the evaluated accessions when the experiments were performed by two technicians skilled in tissue culture and cryopreservation proce-

dures. The reproducibility of results across technicians helped to demonstrate that the described procedures can be replicated, thus increasing the likelihood of success for genebanks that may adopt this method for *Vitis* cryopreservation.

In addition to the shoot tip cryopreservation method described herein, *Vitis* also could be cryopreserved as seeds or pollen collected from the wild or by performing controlled crosses among accessions in the vineyard (Bi et al., 2017; Ganeshan, 1985; Volk et al., 2005, 2017). *Vitis* is genetically heterozygous; therefore, there will be some instances when clonal preservation will be needed, specifically for hybrids, single accessions of a species, and specific genotypes of interest. In these cases, shoot tip preservation is preferred over seeds or pollen (Bi et al., 2017).

Conclusions

Our droplet vitrification cryopreservation technique resulted in high regrowth levels of cryopreserved *Vitis* species using shoot tips derived from in vitro stock plants. The high regrowth levels that we obtained for cryopreserved shoot tips could be independently replicated by two technicians skilled in tissue culture and cryopreservation procedures, thus suggesting that this method may be ready for implementation in *Vitis* genebank collections.

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