In Vitro Cell.Dev.Biol.—Plant
DOI 10.1007/s11627-017-9822-9

INVITED REVIEW

Cryopreservation of grapevine (*Vitis* spp.)—a review

Wen-Lu Bi[1] · Chen Pan[1] · Xin-Yi Hao[1] · Zhen-Hua Cui[1] · Mafatlal M. Kher[2] · Zvjezdana Marković[3] · Qiao-Chun Wang[1] · Jaime A. Teixeira da Silva[4]

Received: 14 January 2017 / Accepted: 7 April 2017 / Editor: Ewen Mullins

Abstract Grapevine (*Vitis* genus) is one of the economically most important fruits worldwide. Some species and cultivars are rare and have only a few vines, but represent national heritages with a strong need for preservation. Field collections are labor intensive, and expensive to maintain, and are exposed to natural disasters. In addition, infection with pathogens, especially viruses, is common in grapevine because of vegetative propagation, which is conventionally used for this genus. Cryopreservation provides an alternative and ideal means for the long-term preservation of *Vitis* germplasm, which can be used as a backup to field collections for important autochthonous cultivars or only as cryo-banks for rare, native cultivars that are worthy of preservation. Cryotherapy, based on cryopreservation protocols, provides an efficient method for the eradication of grapevine viruses. This review provides comprehensive and updated information on cryopreservation for long-term preservation of genetic resources and cryotherapy for virus eradication in *Vitis*. Additional research in grapevine cryopreservation and cryotherapy is needed.

✉ Qiao-Chun Wang
qiaochunwang@nwsuaf.edu.cn

✉ Jaime A. Teixeira da Silva
jaimetex@yahoo.com

[1] State Key Laboratory of Crop Stress Biology in Arid Region, College of Horticulture, Northwest Agriculture & Forestry University, Yangling 712100, Shaanxi, People's Republic of China

[2] Post Graduate Department of Biosciences, Sardar Patel University, Sardar Patel Maidan, Vadtal Rd, P.O. Box 39, Vallabh Vidyanagar, Gujarat 388120, India

[3] Department of Viticulture and Enology, Faculty of Agriculture, University of Zagreb, Svetošimunska 15, HR-10000 Zagreb, Croatia

[4] P. O. Box 7, Miki-cho post office, Ikenobe 3011-2, Kagawa-ken 761-0799, Japan

Keywords Cryopreservation · Cryotherapy · Embryogenic cell suspensions · Genetic resource · Shoot tips · Virus-free · *Vitis*

Introduction

Grape cultivation started around 5400–5000 BC based on archaeological findings in Iran (McGovern *et al.* 1996). About 60 species of *Vitis* and 5000 cultivars of *Vitis vinifera* are available worldwide (This *et al.* 2006; Gardiman and Bavaresco 2015). To avoid the depletion of *Vitis* genetic resources, the long-term conservation of plant material can be accomplished in gene banks, and conserved material can be utilized as breeding material for future research work.

Plant cryopreservation is the storage of cells, tissues, or organs in liquid nitrogen (LN), at −196 °C, with the purpose of long-term conservation of plant germplasm (Engelmann 1997). Cryostorage of *Vitis* germplasm is attractive, but challenging. To date, several vitrification-based methods have been developed for *Vitis*, including encapsulation-dehydration, vitrification, encapsulation-vitrification, and droplet-vitrification (Yin *et al.* 2012; Benelli *et al.* 2013; Bettoni *et al.* 2016; Bi 2017). Exposing cells to such extra-low temperatures would result in freezing injury, and thus, cells need to be treated and prepared properly, prior to cryostorage in LN (Engelmann 1997; Wang *et al.* 2014a, b). Major steps in cryopreservation include (Fig. 1) (1) induction of tolerance of *in vitro* stock cultures and explants to dehydration and subsequent freezing in LN, (2) cryoprotection and loading of explants in plant vitrification solution (PVS)-mediated procedures or encapsulation and cryoprotection in encapsulation-mediated procedures, (3) exposure of explants to PVS in PVS-mediated procedures or to physical drying in dehydration-mediated procedures, (4) direct immersion of explants in LN

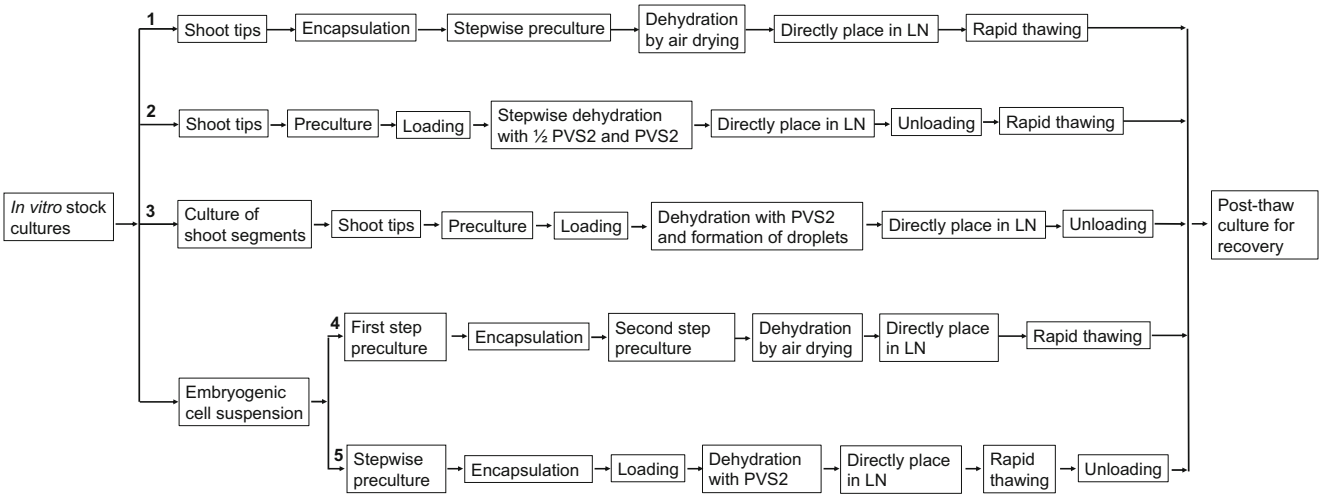

Fig. 1. Major steps involved in cryogenic procedures most frequently used for cryopreservation of shoot tips and somatic embryogenic cell suspensions in *Vitis*: (*1*) encapsulation-dehydration (Wang *et al.* 2000); (*2*) vitrification (Matsumoto and Sakai 2003); (*3*) droplet-vitrification (Marković *et al.* 2013a, b; Bi 2017; (*4*) encapsulation-dehydration (Wang *et al.* 2002); (*5*) encapsulation-vitrification (Wang *et al.* 2004). *LN* liquid nitrogen, *PVS2* plant vitrification solution 2 (Sakai *et al.* 1990).

for cryostorage, (5) rewarming in dehydration-mediated procedures or rewarming and unloading to remove cryoprotectants in PVS-mediated procedures, and (6) post-thaw culture for recovery. Greater details of these techniques can be found in several documents (Reed 2008; Yin *et al.* 2012; Wang *et al.* 2014b; Bettoni *et al.* 2016).

For long-term preservation of genetic resources, shoot tips that are capable of maintaining unique strains of propagated materials are preferred over seeds, embryos, cells, and callus, because they are identical to the mother plants (Engelmann 1997; Wang *et al.* 2014b). Somatic embryogenic tissues have great potential for micropropagation, genetic transformation, and production of artificial seeds in grapevine (Wang *et al.* 2005, 2014b; Martinelli and Gribaudo 2009).

Viral diseases have long threatened the sustainable development of agricultural production including grapevine (Wang *et al.* 2011; Naidu *et al.* 2014). Like most fruit crops, grapevine is vegetatively propagated to maintain the unique nature of cultivars, but this makes it vulnerable to virus infection. Viruses can be transmitted and accumulate in propagated plant materials over generations. In practice, the use of pathogen-free plants is an effective means to control virus-induced diseases, and the development of simple, efficient techniques is a prerequisite for the production of virus-free plants. Cryotherapy, a biotechnology based on cryopreservation, refers to treatment of infected material for a short period in LN to cure infected plants and has proven to be an efficient means to eradicate plant pathogens including grapevine viruses (Wang *et al.* 2003a, 2009; Wang and Valkonen 2009; Marković *et al.* 2015; Pathirana *et al.* 2015; Bettoni *et al.* 2016). The precise mechanism of how cryotherapy efficiently eradicates plant pathogens, including viruses, can be found elsewhere (Wang and Valkonen 2009; Wang *et al.* 2009, 2014b).

Cryopreservation of Grapevine

The pioneer studies were conducted by Parfitt and Almehdi (1983), Ganeshan (1985), and Ganeshan and Alexander (1990), who reported successful cryopreservation of grapevine pollen. Cryopreservation of shoot tips was first published by Ezawa *et al.* (1989), followed by Esensee and Stushnoff (1990) and Plessis *et al.* (1991, 1993). A few years later, Dussert *et al.* (1991, 1992) successfully described the cryopreservation of somatic embryogenic cell suspensions. Vitrification-based cryotechnologies developed in the early 1990s that allowed samples to be directly immersed in LN and avoided the use of a programmable freezer (Engelmann 1997), greatly accelerated studies on cryopreservation of plants including grapevine. Since the 1990s, novel cryogenic procedures have been described for grapevine, including encapsulation-dehydration, vitrification, encapsulation-vitrification, and droplet-vitrification, which are among the most frequently used vitrification-based cryoprocedures for plants (Engelmann 1997; Reed 2008). Recently, cryotherapy has also been described for the efficient eradication of grapevine viruses (Wang *et al.* 2003b; Marković *et al.* 2015; Pathirana *et al.* 2015; Bettoni *et al.* 2016).

Pollen

Parfitt and Almehdi (1983) were the first to successfully cryopreserve the pollen of *V. vinifera* 'Black Champa' and 'Queen' by a two-step cooling process. Ganeshan (1985) also reported a two-step cooling process to cryopreserve grapevine pollen, in which pollen was precooled to −20 °C, followed by immersion in LN. Cryopreserved pollen was germinated on medium

containing 20% (*w/v*) sucrose. In this protocol, germination rates of cryopreserved pollen ranged from 54.7 to 77.3% in five *V. vinifera* cultivars. Further studies showed that pollen cryopreserved for 5 yr remained as viable as freshly collected pollen and could be used for pollination in field crosses (Parfitt and Almehdi 1983; Ganeshan and Alexander 1990).

Shoot Tips

Two-step cooling Earlier studies on shoot tip cryopreservation of grapevine used a two-step cooling process (Table 1). In the Ezawa *et al.* (1989) study, shoot tips of *V. labrusca* were collected from field-grown vines in different seasons and used for cryopreservation. Shoot tips (1–2 mm in size) were treated with a cryoprotectant solution containing 10% (*w/v*) dimethylsulphoxide (DMSO) and 60 g/L glucose for 2 h at room temperature. The samples were then prefrozen to −20, −30, and −40 °C, at a cooling rate of 0.5 °C/min, prior to immersion in LN for cryostorage. Frozen shoot tips were rapidly thawed in a water bath set at 30 °C and post-thaw cultured for recovery. When shoot tips collected in September were subject to cryopreservation, about half of 'Buffalo' and most of 'Campbell Early' shoot tips prefrozen to −30 °C did not survive, and shoot tips that survived prefreezing to −20 and −30 °C regrew very slow and eventually failed to regenerate shoots. When shoot tips collected in November were used for cryopreservation, many of them survived using prefreezing to −20, −30, and −40 °C in the three cultivars tested. Shoot tips prefrozen to −30 °C regrew rapidly into normal shoots, while those prefrozen to −40 °C survived for only a month and were unable to develop into shoots. In 'Campbell Early', most frozen shoot tips formed only callus, without any shoot elongation. When samples collected in December were used for cryopreservation, shoot tips of 'Delaware' produced similar recovery, regardless of the prefreezing temperature. Shoot tips of 'Buffalo' prefrozen to −20 °C grew slowly, compared with those prefrozen at −30 or −40 °C, and shoots failed to regrow. Most 'Campbell Early' shoot tips only formed callus in shoot tips that had been prefrozen to −20, −30, and −40 °C. The survival rate of cryopreserved 'Buffalo' shoot tips was about 80% after 6, 12, and 18 mo of cryostorage (Ezawa *et al.* 1989).

The use of explants such as shoot tips and dormant buds taken directly from greenhouse- or field-grown plants for cryopreservation would avoid *in vitro* tissue culture for the establishment of stock cultures before and/or plant regeneration following cryopreservation, thus simplifing cryopreservation procedures (Towill *et al.* 2004). Thus far, there have been two reports on the cryopreservation of grapevine using shoot tips or dormant buds that were sampled directly from greenhouse- or field-grown plants: one using a two-step cooling process, which was described in this section (Esensee and Stushnoff 1990), and another using droplet-vitrification (Fig. 2), which is described in a separate section below (Hassan and Haggag 2013). In the study of Esensee and Stushnoff (1990), dormant buds excised from field-grown vineyards were desiccated to 18 or 25% water content (on a fresh weight basis), prior to immersion in LN. With this protocol, some cryopreserved buds of *V. vinifera* 'Valiant' and a hybrid (*V. amurensis* × *V. riparia*. *V. riparia*) survived when the samples were desiccated to 25% water content, and all buds desiccated to 18% water content survived. *V. vinifera* 'Riesling' did not survive desiccation to 18–25% water content following cryopreservation.

Plessis *et al.* (1991) reported a two-step encapsulation-dehydration method for the cryopreservation of *V. vinifera* 'Chardonnay'. Shoot tips were encapsulated into beads, each containing 1–3 shoot tips, and then stepwise precultured with increasing sucrose concentrations. Precultured beads were dehydrated by air drying in a laminar airflow for 4 h to reduce water content to about 20% and then profrozen to −80 °C at 0.5 °C/min, prior to immersion in LN. After slowly rewarming at room temperature, beads with shoot tips were transferred to a basic medium (BM) containing 1% (*w/v*) bovine fetal serum for recovery. About 24% survival was obtained in cryopreserved shoot tips. The inclusion of 5% (*w/v*) DMSO into the preculture medium improved survival rates (Plessis *et al.* 1993). A similar two-step encapsulation-dehydration protocol was also tested for shoot tip cryopreservation of several *V. vinifera* cultivars, but shoots did not regrow, even though about 35–50% of them survived (Miaja *et al.* 2000).

Encapsulation-dehydration Wang *et al.* (2000) reported an encapsulation-dehydration protocol for the cryopreservation of shoot tips of the rootstock LN33 hybrid (Courderc 1613 × *V. vinifera* 'Thompson Seedless') and the scion cultivar 'Superior' (*V. vinifera*). Shoot tips (1 mm long) were excised from 4-wk-old *in vitro* stock shoots and encapsulated into beads (4–5 mm in diameter) using alginate solution (3% (*w/v*) Na-alginate, 2 M glycerol and 0.4 M sucrose) and a calcium chloride solution (0.1 M calcium chloride, 2 M glycerol, and 0.4 M sucrose). The beads, each containing a single shoot tip, were precultured stepwise with increasing sucrose concentrations of 0.25, 0.5, 0.75, and 1.0 M for 4 d, with 1 d for each step. Following preculture, encapsulated shoot tips were dehydrated by air drying in a laminar airflow to 15.6 and 17.6% water content for LN33 and 'Superior', respectively, prior to direct immersion in LN for 1 h. After rapidly thawing in a 40 °C water bath for 3 min, cryopreserved shoot tips were postcultured for recovery on a postculture medium composed of half-strength Murashige and Skoog (MS) (Murashige and Skoog 1962) medium supplemented with 1 mg/l 6-benzyladenine (BA) and 0.1 mg/l 1-naphthaleneacetic acid (NAA). With these optimized parameters, 60 and 40% shoot regrowth of cryopreserved shoot tips were obtained for the LN33 hybrid and 'Superior', respectively. Studies reported

Table 1. A list of successful cryopreservation of grapevine (*Vitis*)

Explant		Cryogenic procedure	Species, no. genotypes tested	Recovery, viability or germination (%)[a]	Reference
Pollen		TSC	*V. vinifera*, 21	24.8 (7.4–53.9)	Parfitt and Almehdi 1983
			V. vinifera, 5	54.7–77.3	Ganeshan 1985
			V. vinifera, 2	Not specified	Ganeshan and Alexander 1990
Shoot tips		TSC	*V. labrusca*, 3	96.7 (90–100)	Ezawa *et al.* 1989
		DE (directly immersed in LN)	*V. vinifera*, 1	0	Esensee and Stushnoff 1990
			V. riparia, 2	Some (not specified)-100	
			V. amurensis × *V. riparia*, 1	Some (not specified)	
		En-Dehy + TSC	*V. vinifera*, 4	29 (15–40)	Zhao *et al.* 2001
			V. vinifera, 4	36	Zhai *et al.* 2003
			Data not available	Data not available	Plessis *et al.* 1991
			V. vinifera, 1	30	Plessis *et al.* 1993
		En-Dehy	*V. vinifera*, 2	49 (40–58)	Wang *et al.* 2000
			V. vinifera, 1	63	Wang *et al.* 2003a
			V. vinifera, 1	62	Wang *et al.* 2003b
			V. vinifera, 1	59	Bayati *et al.* 2011
			V. vinifera, 1	37	Marković *et al.* 2013b
		En-Vitri	*V. berlandieri* × *V. riparia*, 1	Low (not specified)	Benelli *et al.* 2003
		Vitri	*V. vinifera*, 7	65.5 (33.3–86.7)	Matsumoto and Sakai 2003
			V. berlandi × *V. riparia*, 2	46.7 (30.0–63.3)	
			V. mourvedre × *V. rupestris*, 1	75	
			V. coigneae, 1	75	
			V. vinifera, 1	45	Wang *et al.* 2003a
			V. vinifera, 1	50	Wang *et al.* 2003b
			V. vinifera, 1	55	Shatnawi *et al.* 2011
			V. berlandieri × *V. riparia*, 1	0	Ganino *et al.* 2012
			V. vinifera, 2	43 (40–46)	Hassan and Haggag 2013
			V. vinifera, 1	57	Lazo-Javalera *et al.* 2015
			V. vinifera, 1	55	Shatnawi *et al.* 2011
		Drop-Vitri	*V. vinifera*, 1	45	Marković *et al.* 2012
			V. vinifera, 1	50	Marković *et al.* 2013b
			V. vinifera, 1	30	Marković *et al.* 2013a
			V. vinifera, 1	46	Marković *et al.* 2014a, b
			V. vinifera, 12	Not specified	Toprak *et al.* 2014
			V. vinifera, 9	23.1 (0–70)	Marković *et al.* 2015
			V. vinifera, 4	34.8 (24–45)	Pathirana *et al.* 2016
			V. riperia × *V. rupestris*, 1	26	
			V. vinifera × *V. berlandieri*, 1	6	
			V. vinifera, 6	50 (40–76)	Bi 2017
			V. pseudoreticulata, 2	30 (10–50)	
SET	ECSs	TSC	*V. vinifera* × *V. berlandieri*, 1	60	Dussert *et al.* 1991
			V. vinifera × *V. berlandieri*, 1	58	Dussert *et al.* 1992
			V. vinifera, 1	50	
		En-Dehy + TSC	*V. berliandieri* × *V. rupestris*, 1	25	Ben-Amar *et al.* 2013
			V. vinifera, 2	17.5 (5–20)	
		En-Dehy	*V. vinifera*, 1	78	Wang *et al.* 2002
			V. vinifera, 2	23 (19–27)	González-Benito *et al.* 2009
			V. berliandieri × *V. rupestris*, 1	78	Ben-Amar *et al.* 2013
			V. vinifera, 2	51.5 (43–60)	
		EN-Vitri	*V. berlandieri* × *V. rupestris*, 1	76	Wang *et al.* 2004
			V. vinifera, 4	61.8 (46–82)	
			V. vinifera × *V. berlandieri*, 1	42	
			V. vinifera, 3	48 (44–52)	Vasanth and Vivier 2011
	SEs	Vitri	*V. vinifera*, 2	60 (41–79)	Miaja *et al.* 2004
		En-Dehy	*V. vinifera*, 2	55 (52–58)	Miaja *et al.* 2004
Seeds		DE (directly immersed in LN)	*V. vinifera*, 3	60 (50–70)	Hassan *et al.* 2013

[a] *Numbers* indicate means. *Numbers in parentheses* represent the lowest-highest results obtained in the study indicated. Recovery = survival or shoot regrowth in cryopreserved shoot tips; viability = recovery of cryopreserved somatic embryogenic tissues; germination = recovery of cryopreserved pollen or seeds

De desiccation, *Drop-Vitri* droplet-vitrification, *En-Dehy* encapsulation-dehydration, *TSC* two-step cooling, *Vitri* vitrification, *En-Vitri* encapsulation-vitrification, *SET* somatic embryogenic tissue, *ECS* embryogenic cell suspension, *SE* somatic embryo

thus far for grapevine cryopreservation by encapsulation-dehydration are listed in Table 1.

Vitrification Matsumoto and Sakai (2000, 2003) established a vitrification protocol for grapevine cryopreservation. In their

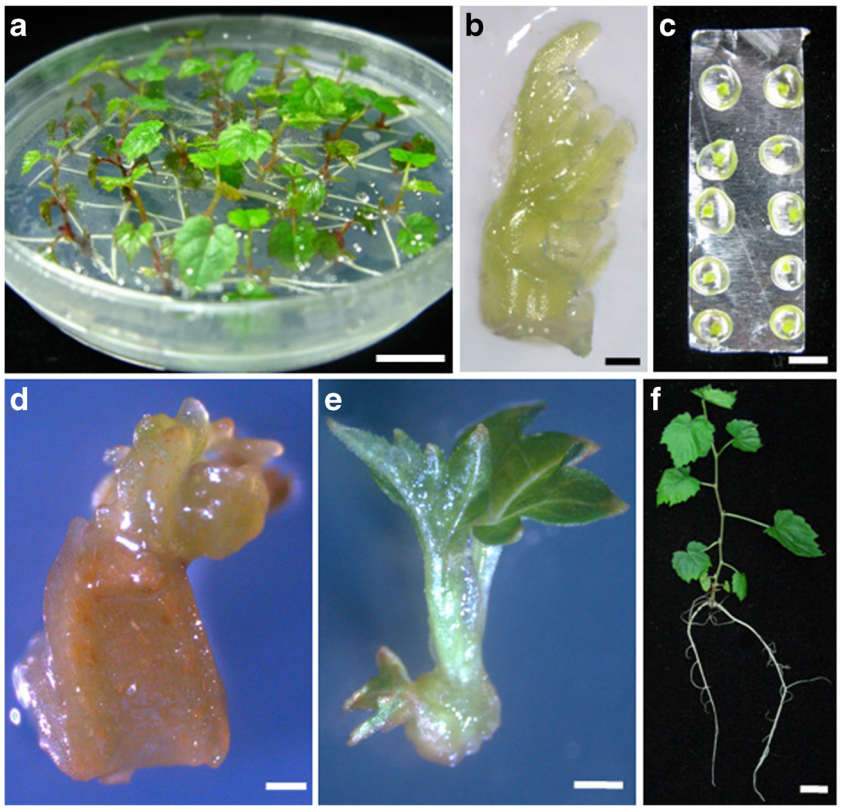

Fig. 2. Plant regeneration from cryopreserved shoot tip of *V. vinifera* 'Cabernet Sauvignon' by droplet-vitrification. (*a*) Two-wk-old nodal segments cultured on shoot multiplication medium to promote bud break. (*b*) Shoot tip excised from (*a*). (*c*) PVS2 droplets on an aluminum foil strip. (*d*) Surviving shoot tip after 7 d of post-thaw culture following cryopreservation. (*e*) Shoot regrowth after 6 wk of post-thaw culture following cryopreservation. (*f*) A whole plantlet with well-developed root system after 14 wk of post-thaw culture following cryopreservation. *Bars* in *a* and *f* 1.0 cm, in *b* and *d* 0.1 mm, and in *c* and *e* 0.2 cm. (Bi 2017).

protocol, axillary shoot tips excised from 4–5 mo old *in vitro* stock plantlets were precultured with 0.3 M sucrose for 3 d and then loaded with a loading solution (LS) containing 2 M glycerol and 0.4 M sucrose for 20 min at 25 °C, followed by exposure to half-strength PVS2 (Sakai *et al.* 1990) at 0 °C for 30 min and then to full-strength PVS2 at 0 °C for 50 min. PVS2 contains (*w*/*v*): 30% glycerol, 15% DMSO, and 15% ethylene glycol in 0.4 M sucrose (Sakai *et al.* 1990). Dehydrated shoot tips were plunged directly into LN for cryostorage. Cryopreserved shoot tips were warmed rapidly in water at 40 °C, and post-thaw cultured for shoot regrowth on a recovery medium composed of half-strength MS supplemented with 1 mg/L BA. This cryoprocedure was applied to ten other species or cultivars of *Vitis*, with an average recovery of 64% obtained. Table 1 summarizes successful cryopreservation of grapevine by vitrification.

Encapsulation-vitrification Shoot tips of the rootstock Kober 5BB (*V. berlandieri* × *V. riparia*) were cryopreserved by encapsulation-vitrification (Benelli *et al.* 2003). In this protocol, *in vitro* stock shoots were hardened at 4 °C for 3 wk. Shoot tips (1–2 mm in size) excised from cold-hardened stock shoots were encapsulated in 3% (*w*/*w*) Na-alginate, according to Wang *et al.* (2000), followed by exposure to PVS2 for 90 min at 0 °C. After cryostorage, shoot tips contained in beads were thawed in a 40 °C water bath and post-thaw cultured for recovery. Although cryopreserved shoot tips were able to regenerate into shoots, shoot regrowth rates were low (Benelli *et al.* 2003). Further studies on increasing the tolerance of shoot tips to dehydration and subsequent freezing in LN were suggested, in order to achieve high rates of shoot regrowth (Benelli *et al.* 2003; Benelli 2016). Successful cryopreservation of grapevine shoot tips by encapsulation-vitrification is shown in Table 1.

Droplet-vitrification Droplet-vitrification, which combines advantages of droplet protocols with vitrification (Panis *et al.* 2005), has been demonstated to be the most applicable to diverse genotypes of a given species and considered the most promising solution to overcome species- or genotype-specific limitations, which is often a bottleneck for the establishment of cryo-banks (Panis *et al.* 2005; Reed 2008; Wang *et al.* 2014b). Hassan and Haggag (2013) reported a droplet-vitrification protocol for the cryopreservation of grapevine shoot tips. In their study, shoot tips that had been collected from greenhouse-grown plants were first surface-disinfected and then cultured for 3 d in the dark at 25 °C, to identify their sanitary status *in vitro*. Shoot tips were loaded with LS containing 0.4 M sucrose and 2 M glycerol for 20 min at 22 °C, followed by exposure at 0 °C to half-strength PVS2 for 10–15 min and then full-strength PVS2 for 10–20 min. After dehydration with PVS2, shoot tips were transferred onto 5 μl PVS2 droplets dotted on aluminum foil strips and placed directly in LN. Frozen foil strips containing shoot tips were

rewarmed by transfer to an unloading solution containing 1.2 M sucrose for 20 min at room temperature, and post-thaw cultured for shoot regrowth. About 47 and 40% shoot regrowth was obtained for *V. vinifera* 'Bez El-Anza' and 'Black Matrouh', respectively. To date, droplet-vitrification has been applied to a number of table and vine cultivars, and rootstocks (Marković *et al.* 2013a, b, 2014a, b, 2015; Pathirana *et al.* 2016; Bi 2017; Fig. 2), and wild grapevine germplasm (Bi 2017; Carimi *et al.* 2016), some of which, such as 'Baihe 35–1' and 'Hunan-1', are Chinese wild grapevine germplasm resistant to grapevine fungal diseases (Bi 2017). A list of successful cryopreservation protocols for grapevine shoot tips by droplet-vitrification can be found in Table 1.

Somatic Embryogenic Tissues

Two-step cooling Dussert *et al.* (1991, 1992) were the first to conduct studies on the cryopreservation of somatic embryogenic tissues in grapevine. Somatic embryogenic cell suspensions (ECSs) were established from embryogenic callus that had been induced from anthers of the rootstock 41 B (*V. vinifera* 'Chasselas' × *V. berlandieri*) (Dussert *et al.* 1991). Cell suspensions were mixed with a medium containing 0.25 M maltose and 5% (*w/v*) DMSO to reach 30% (*w/v*) cell volume and incubated at 0 °C for 1 h. In two-step cooling process, the treated ECSs were profrozen to −40 °C at 0.5 °C/min, followed by immersion in LN. After rapid thawing, cryopreserved ECSs were post-thaw cultured on a semi-solid medium containing activated charcoal (AC) for 18 d and then transferred to liquid medium for recovery. After one and a half months of post-thaw culture, the growth of cryopreserved cells was similar to that of the treated control. The two-step cooling resulted in 63% recovery, while rapid freezing failed to produce any recovery (Dussert *et al.* 1991). The addition of 1 mg/l NAA into the postculture medium improved the regrowth rates of cryopreserved ECSs and allowed the protocol to be applied to three other grapevine cultivars, including 41 BD1, CH 76, and 'Chardonnay' (Dussert *et al.* 1992). The application of two-step cooling to grapevine somatic embryogenic tissues can be found in Table 1.

Encapsulation-dehydration Wang *et al.* (2002) described encapsulation-dehydration for cryopreservation of *V. vinifera* 'Red Globe' ECSs. Somatic embryogenic callus was maintained on a solid ECS maintenance medium (ECS-MM) composed of Nitsch and Nitsch medium (NN; Nitsch and Nitsch 1969) supplemented with 18 g/L maltose, 1 g/L casein hydrolysate, 4.6 g/L glycerol, and 1 mg/L 2-naphthoxyacetic acid (NOA) (pH 6.0). ECSs were established by suspending the callus in liquid ECS-MM and placed on a gyratory shaker at 90 rpm and 25 °C in the dark. ECSs were stepwise precultured with 0.25, 0.5, 0.75, and 1.0 M sucrose, for 12 h in each sucrose concentration. Stepwise precultured ECSs were encapsulated into beads, each containing 25% (*w/v*) ECSs, according to Wang *et al.* (2000). The beads were further precultured with 1 M sucrose for 3 d, in liquid medium, and dehydrated by air drying in a laminar airflow to reduce water content to 20.6% of fresh weight, prior to direct immersion in LN for cryostorage. Following rapid thawing in a water bath set up at 38 °C, cryopreserved beads containing ECSs were post-thaw cultured for recovery on solid ECS-MM containing 0.25% (*w/v*) AC. ECSs were reestablished by suspending the beads in liquid ECS-MM maintained on a gyratory shaker (90 rpm) at 25 °C in the dark with weekly subcultures. Although cryopreserved cells showed a 5-d lag phase in regrowth, their growth pattern was the same as that of control cells after two subcultures. For plantlet regeneration, somatic embryos (3–4 mm long) at the torpedo-stage formed on the solid ECS-MM but lacking NOA were transferred onto Woody Plant Medium (WPM; Lloyd and McCown 1980) supplemented with 3% sucrose, 0.25% AC, and 0.2 mg/L NAA. Plantlets were obtained after 8–10 wk of culture. Interestingly, Wang *et al.* (2002) found that cryopreserved cells regenerated embryos much earlier and produced many more embryos at various developmental stages than control cells. Higher morphogenetic competence was also found in cryopreserved cell suspensions of *V. vinifera* 'Albariño' and 'Tempranillo' (González-Benito *et al.* 2009) and 'Riesling' (Ben-Amar *et al.* 2013). Similar results have also been observed in several other plant species such as *Citrus deliciosa* (Aguilar *et al.* 1993), *Pinus sylvestris* (Häggman *et al.* 1998), and *Gentiana cruciata* (Mikuła *et al.* 2011), indicating that exposure to LN may have selective effects on cells with a greater capacity for morphological differentiation (Aguilar *et al.* 1993; Häggman *et al.* 1998; Mikuła *et al.* 2011). Indeed, histological analysis demonstrated that only cells with meristematic and undifferentiated features were able to survive following cryopreservation, whereas all differentiated cells were killed or severely damaged after exposure to LN (Mikuła *et al.* 2011).

Using a similar encapsulation-dehydration protocol described by Wang *et al.* (2002), Miaja *et al.* (2004) achieved 52.6 and 58.1% recovery for *V. vinifera* 'Brachetto' and 'Müller-Thurgau', respectively. Recently, encapsulation-dehydration, which was described by Wang *et al.* (2002), was successfully extended to the cryopreservation of ECSs of several *V. vinifera* cultivars including 'Albariño' and 'Tempranillo', with approximately 19 and 27% recovery (González-Benito *et al.* 2009), 'Tempranillo' and 'Riesling', with approximately 43 and 60% recovery (Ben-Amar *et al.* 2013), and *Vitis* rootstock '110 Richter', with 78% recovery (Ben-Amar *et al.* 2013). Somatic embryos developed in the cryopreserved ECSs were proliferated and were able to initiate secondary embryos after 4 wk of transfer onto the same fresh medium. These secondary embryos developed further,

matured, and eventually converted into whole plantlets, which were morphologically and structurally identical to those of the mother plantlets (Miaja *et al.* 2004).

Vitrification Miaja *et al.* (2004) reported a vitrification protocol for the cryopreservation of somatic embryos (Table 1). In their study, somatic embryos were first cultured for 1 d on NN medium (Nitsch and Nitsch 1969) with 0.4 M sucrose at 25 °C, followed by loading in LS containing 2 M glycerol and 0.4 M sucrose for 20 min at 25 °C. Loaded samples were exposed at 0 °C to half-strength PVS2 for 30 min, and then in full-strength PVS2 for an additional 30 min, prior to direct immersion in LN. After rapidly thawing, cryopreserved somatic embryos were post-thaw cultured on NN medium containing 0.3 M sucrose for 1 d at 25 °C and then transferred onto NN medium supplemented with 10 μM BA and 0.06 M sucrose. About 41 and 79% recovery was obtained for 'Brachetto' and 'Müller-Thurgau', respectively.

Encapsulation-vitrification When attempting to adopt the encapsulation-dehydration protocol to the cryopreservation of somatic embryogenic issues of other important *Vitis* species, low survival rates were observed (Wang *et al.* 2004). Therefore, Wang *et al.* (2004) described encapsulation-vitrification for the cryopreservation of ECSs. ECSs were established and maintained, as described by Wang *et al.* (2002). ECSs were precultured with increasing sucrose concentrations of 0.25, 0.5, and 0.75 M for 3 d, with each concentration for 1 d. Precultured cell suspensions were suspended in ECS-MM containing 2.5% (*w*/*v*) Na-alginate and 0.4 M sucrose, and encapsulated into beads (about 4 mm in diameter) by dripping in 0.1 M $CaCl_2$ solution containing 0.4 M sucrose, followed by exposure of the beads to PVS2 for 270 min at 0 °C, prior to direct immersion in LN. Thaw and postculture were the same as described by Wang *et al.* (2002). This encapsulation-vitrification protocol was successfully applied to two rootstocks (110 Ritcher, *V. berlandieri* × *V. rupestris* and 41B, *V. vinifera* × *V. berlandieri*), and four *V. vinifera* cultivars, including table and vine grapes, with recovery rates ranging from 42 to 82% obtained across the species/genotypes tested. Further information on encapsulation-vitrification for cryopreservation of grapevine somatic embryogenic tissues can be found in Table 1.

Seeds

Grapevine seeds belong to the orthodox group and their storage by traditional strategies and cryopreservation are relatively easy. Hassan *et al.* (2013) reported seed cryopreservation of grapevine. In their study, seeds were harvested from mature berries and stored at 4 °C for 10–12 wk to break dormancy. Thereafter, the seeds were dehydrated by air drying in a laminar airflow for 6 h, prior to direct immersion in LN. Cryopreserved seeds were thawed in a 40 °C water bath for 1 min, sown in a mixed substrate (peat moss: sand = 1:1) and maintained in a greenhouse (22 °C ± 1 °C) for germination. Cryopreserved seeds germinated into seedlings after 4 wk of culture, with 50–70% germination obtained across the three local *V. vinifera* cultivars tested (Table 1).

Cryotherapy for Virus Eradication

Wang *et al.* (2003b) were the first to report cryotherapy for the efficient eradication of a grapevine virus. In their study, the effects of two cryogenic procedures, including encapsulation-dehydration and vitrification, were tested for the elimination of *Grapevine virus A* (GVA). They found that these two cryoprocedures produced similarly high frequencies of virus-free *V. vinifera* 'Bruti' plantlets, as detected by Western blotting for GVA movement protein. They also found that only the plantlets recovered after freezing in LN were GVA-free, while no other steps before freezing were able to produce GVA-free plants. These results indicate that virus elimination occurs through freezing in LN. Furthermore, they demonstrated that shoot tip size (0.5 to 2.0 mm) affected shoot regrowth rates, but not the frequency of virus elimination, and cryotherapy was much more efficient for virus eradication than meristem culture. The study of Wang *et al.* (2003b) provided a fundamental basis for grapevine virus eradication by cryotherapy.

Using a slightly modified encapsulation-dehydration protocol, as described by Wang *et al.* (2000), Bayati *et al.* (2011) obtained 42% GVA-free plantlets, detected by reverse transcription PCR (RT-PCR), of *V. vinifera* 'Black', and further confirmed that virus eradication was only possible by freezing shoot tips in LN during cryotherapy. Marković *et al.* (2015) applied droplet-vitrification to eradicate *Grapevine fanleaf virus* (GFLV) and GLRaV-3 and obtained 78% of GFLV-free plantlets *in V. vinifera* 'Chardonnay' and 100% of GLRaV-3-free plantlets in 'Cabernet Sauvignon'. Notably, the treated control, which received all treatments but was not frozen in LN, showed a fairly similar virus-free status as plantlets derived from cryotherapy-treated samples. As explained by Marković *et al.* (2015), in their study, shoot tips (1 mm in size) were used for cryotherapy. Such small shoot tips may already be free of virus infection before cryotherapy, thus resulting in a high percentage of virus-free plantlets, even without exposure to LN. In the Marković *et al.* (2015) study, the sanitary status of tissue was detected by enzyme-linked immunosorbent assay (ELISA) in plantlets 2 mo after plantlet regeneration. ELISA is much less sensitive than molecular methods such as RT-PCR or Western blotting or next generation sequencing, a more recently developed method for virus detection (Boonham *et al.* 2014). The sanitary status of plants

should be measured at least 6 mo after regeneration from cryotherapy. Therefore, we assume that the use of ELISA rather than molecular-based methods for virus detection and the use of 2-mo-old plantlets rather than at least 6-mo-old ones may also be reasons responsible for the high frequencies of virus-free plantlets recovered from cryotherapy and even from the treated samples reported in the Marković *et al.* (2015) study. Pathirana *et al.* (2015) reported the successful eradication of GLRaV-1, -2, and -3 by droplet-vitrification cryotherapy. The sanitary status of the plantlets recovered from cryotherapy was confirmed by DAS-ELISA. Their results showed that all plantlets recovered from cryotherapy were free of GLRaV-3 in *V. vinifera* 'Chardonnay' and 'Lakemont Seedless', of PLRaV-2 in 'Pinot gris' and 'Sauvignon blanc 316', and of GLRaV-1 and -3 in'Sauvignon blanc'. In contrast, none of these viruses could be eradicated from the treated control (Pathirana *et al.* 2015). These results again support the notion that virus elimination occurs only in the freezing step of cryotherapy (Wang *et al.* 2003b). Additional information on cryotherapy for eradication of grapevine viruses can be found in a recent review (Bettoni *et al.* 2016).

Genetic Stability

Genetic stability in regenerants recovered from cryopreservation is the issue of the greatest concern in terms of preservation of plant germplasm (Harding 2004; Benson 2008; Wang *et al.* 2014b). Although plant cell division and metabolism are arrested when stored in LN, thus limiting the chances of genetic alternations in cryostored materials, cryopreservation techniques involve not only storage in LN but also other steps such as preculture, dehydration by either air drying or exposure to PVS, and *in vitro* tissue culture for maintenance of stock cultures before cryopreservation and for plant regeneration after cryopreservation. All these steps may result in genetic variations in the regenerants recovered from cryopreservation (Benson 2008; Harding 2004, 2010; Wang *et al.* 2014a, b). Over the last few decades, numerous studies have been conducted on the assessment of genetic stability in regenerants recovered from plant cryopreservation, including grapevine (Harding 2004; Benson 2008; Wang *et al.* 2014a, b).

In grapevine, genetic stability has been assessed in regenerants recovered from cryopreserved shoot tips by encapsulation-dehydration using random amplified polymorphic DNA (RAPD; Zhai *et al.* 2003), by vitrification using flow cytometry (FCM; Toprak *et al.* 2014), and by droplet-vitrification using amplified fragment length polymorphism (AFLP; Marković *et al.* 2015). Overall, the results obtained thus far are promising. However, it is worth noting that after using droplet-vitrification, Marković *et al.* (2015) reported that no significant variations in AFLP profiles were observed in the samples after sucrose preculture, loading and exposure to half-strength PVS2, even though polymorphic fragments were observed in samples treated with full-strength PVS2, and the number of polymorphic fragments increased as the exposure time to PVS2 increased to 50 min. These results provide some valuable information on controlling excessive exposure time to PVS, in order to ensure genetic stability in regenerants following cryopreservation.

Major Factors Affecting Successful Cryopreservation of Grapevine

Species and genotypes Like other plants, the need for species- and genotype-specific protocols has been a bottleneck constraining the establishment of grapevine cryo-banks (Matsumoto and Sakai 2003; Wang *et al.* 2014b). Applying an optimized vitrification protocol for different *Vitis* species and genotypes, Matsumoto and Sakai (2003) found shoot regrowth rates varied from 30% in Teleki 5BB (*V. berlandi* × *V. riparia*) to 86.7% in 'Merlot' (*V. vinifera*). Species- and genotype-specific responses have been consistently observed in almost all studies on the cryopreservation of grapevine, including shoot tips (Wang *et al.* 2000; Zhao *et al.* 2001; Zhai *et al.* 2003; Marković *et al.* 2015; Pathirana *et al.* 2016) and somatic embryogenic tissues (Wang *et al.* 2004; González-Benito *et al.* 2009).

***In vitro* stock cultures and explants** The physiological status of stock cultures influences the success of plant cryopreservation (Engelmann 1997). In grapevine, the preparation of shoot buds used for cryopreservation significantly influences the recovery of cryopreserved shoot tips. In the stuides of Marković *et al.* (2012, 2013b, 2014a), *in vitro* stock shoots of *V. vinifera* 'Portan' were maintained on a BM composed of half-strength MS mineral elements with Morel's vitamins (Morel 1948), 30 g/L sucrose and 7 g/L agar and grown without subculture for 2 mo. Shoot segments (1.5 cm in length) were excised from the *in vitro* stock shoots and cultured on shoot-induction medium consisting of half-strength MS medium containing 20 g/L sucrose, 7 g/L agar and 1 μM zeatin riboside (ZR), BA or no plant growth regulators, to promote bud elongation within 2 wk. Following cryopreservation, shoot regrowth rate was much higher in buds produced by shoot segments than in those taken directly from *in vitro* stock shoots. Buds produced in shoot segments cultured on medium containing ZR or BA gave similarly higher shoot regrowth rates than those cultured on plant growth regulator-free medium.

Working on encapsulation-dehydration cryopreservation of *V. vinifera* 'Bruti', Wang *et al.* (2003b) found the highest shoot recovery from 1–1.5 mm-long shoot tips, while larger or

smaller shoot tips produced lower shoot recovery. Similar results were also obtained in shoot tips (*V. vinifera* 'Portan') cryopreserved by encapsulation-dehydration (Marković *et al.* 2013b). While reviewing the literature relevant to grapevine cryopreservation, we discovered that 1.0–1.5 mm shoot tips were most frequently used for cryopreservation (Wang *et al.* 2000; Benelli *et al.* 2003; Matsumoto and Sakai 2003; Bayati *et al.* 2011; Marković *et al.* 2012, 2013b, 2014a, b, 2015; Pathirana *et al.* 2016). In addition, axillary buds were found to be more tolerant to PVS2 than apical buds and required a longer period of exposure (Pathirana *et al.* 2016).

Preculture Like other plant species, explant preculture is necessary to achieve high recovery of cryopreserved samples. Sucrose was the most often used sugar for preculture. Grapevine was sensitive to high sucrose concentrations (Plessis *et al.* 1991), and therefore, stepwise preculture with increasing sucrose concentrations from 0.25 to 1.0 M for 2–7 d was usually used (Plessis *et al.* 1991; Wang *et al.* 2000, 2002, 2004; Marković *et al.* 2012, 2013b, 2015; Pathirana *et al.* 2016). More recently, Pathirana *et al.* (2016) reported that inclusion of 0.1 mM salicylic acid in the stock culture maintenance medium significantly enhanced shoot regrowth in cryopreserved shoot tips of grapevine.

Dehydration In vitrification-based cryoprocedures, dehydration can be performed, usually by exposure of samples either to PVS or to air drying. In air drying, the water content of beads is usually reduced to about 20%, prior to freezing in LN (Engelmann 1997). This value may vary depending on the species and explant type. In grapevine, optimal water content of the beads was 20% in the cryopreserved shoot tips of *V. vinifera* 'Chardonnay' (Plessis *et al.* 1991, 1993), 15.6% in the LN33 hybrid, 17.6% in *V. vinifera* 'Superior' (Wang *et al.* 2000), 21% in *V. vinifera* 'Cabernet Franc' (Zhao *et al.* 2001), and 22.3% in *V. vinifera* 'Portan' (Marković *et al.* 2013b).

In PVS-mediated cryoprocedures, the type of PVS and duration and temperature of exposure need to be defined. Notably in grapevine, Marković *et al.* (2013b) found that shoot regrowth of the treated control (without freezing in LN) was only 10% after 40 min treatment and was nil after longer than 40 min treatment by PVS3 (Nishizawa *et al.* 1993). PVS3 contains (*w*/*v*) 50% glycerol and 50% sucrose in a basal medium. No recovery was achieved after LN exposure following PVS3 treatment. With dehydration by exposure to PVS2, shoot regrowth of the treated control was 30% after 40 min treatment and 10.0% even after 120 min treatment. After cryopreservation, shoot regrowth was obtained in shoot tips that had been exposed to PVS2 for 40 min. These data indicate that grapevine is more sensitive to PVS3 than to PVS2. Therefore, PVS2 has been applied more frequently than PVS3 in the cryopreservation of *Vitis*. Ganino *et al.* (2012) found that 30 min of exposure to PVS2 was optimal for obtaining the highest shoot regrowth in shoot tips of Kober 5BB (*V. berlandieri* × *V. riparia*) following cryopreservation by vitrification.

Cryogenic procedures Comparing encapsulation-dehydration and droplet-vitrification, Marković *et al.* (2013b) found much higher shoot regrowth by the latter than the former. As shown in Fig. 1, processess involved in cryopreservation differ between cryoprotocols. Stress caused by these processes to cells differs largely and can thus lead to differences in cryo-injury or cause damage to cells in different cryoprocedures (Wang *et al.* 2014a). These differences in resulting patterns of variations in survival and shoot regrowth eventually result in different levels of recovery (Wang *et al.* 2014a).

Time duration of cryostorage Once protocols are developed that allow samples to survive following immersion in LN, they can theoretically be cryostored in LN for an indefinite period of time (Engelmann 1997). Ezawa *et al.* (1989) tested the effects of cryostorage period on recovery and found similar survival rates (78.6–86.7%) in shoot tips of 'Buffalo' (*V. vinifera*) cryostored from 24 h to 18 mo. Pollen cryopreserved for 5 yr. remained as viable as freshly collected pollen (Parfitt and Almehdi 1983; Ganeshan and Alexander 1990).

Post-thaw culture medium Post-thaw culture medium significantly affects the recovery of shoot tips and somatic embryogenic tissues following cryopreservation. Cytokinins have been shown to play an important role in the regulation of shoot regrowth of cryopreserved shoot tips of various plant species such as citrange (Wang *et al.* 2003a), *Solanum tuberosum* (Wang *et al.* 2014a), *Chrysanthemum* (Wang *et al.* 2014c), and *Vitis* (Wang *et al.* 2003a). In *Vitis*, Wang *et al.* (2003a) found that the addition of BA into the post-thaw culture medium increased the survival of cryopreserved shoot tips of the hybrid LN 33. The optimal BA concentration was 3–4 μM for encapsulation-vitrification and 2 μM for encapsulation-dehydration. A higher BA concentration induced callus in cryopreserved shoot tips. Working on encapsulation-dehydration, Wang *et al.* (2002) found that the viability of cryopreserved ECSs of *V. vinifera* 'Red Globe' was much higher when post-thaw cultured on solid medium than in liquid medium. The addition of AC to the solid post-thaw culture medium promoted the viability of cryopreserved ECSs. Similar results of positive effects of the use of solid medium and AC on improvement of viability of cryopreserved ECSs were also observed by Vasanth and Vivier (2011) for other *V. vinifera* cultivars.

Conclusion and Future Perspectives

Grapevine, a high-value fruit crop that can be consumed as wine, table grapes, raisins, juice, and spirits, consists of about 60 species. *V. vinifera* is the most widely cultivated grape species and other species or hybrids can be used as rootstocks. In addition, some wild species contain valuable genes that are resistant or tolerant to abiotic or biotic stresses and can further be used for the genetic improvement of grapevine (Wan *et al.* 2013). The availability of and easy access to these diverse genetic resources are necessary for genetic improvement by both traditional and biotechnological strategies in grapevine. Cryopreservation has long been considered as an ideal means for the long-term preservation of plant germplasm and recently cryo-banks have been established for several plants, including *Solanum tuberosum* (Keller and Dreiling 2003), *Malus* genus (Towill *et al.* 2004) and *Musa* genus (Panis 2009). Although some progress has been achieved over the last 3 decades, studies on the cryopreservation of grapevine are far less advanced than in tuber crops (Wang *et al.* 2009; Feng *et al.* 2011), other fruit crops (Benelli *et al.* 2013; Wang *et al.* 2014b), and ornamental plants (Wang and Perl 2006; Kulus and Zalewska 2014). The lack of species- or genotype-independent cryopreservation protocols has been a bottleneck constraining the establishment of grapevine cryo-banks. Thus, a key objective in grapevine cryopreservation is to break down this bottleneck. The establishment of somatic embryogenic tissues of grapevine is difficult and dependent on several factors. Once established, somatic embryogenic tissues need periodic subculture to maintain their morphogenic capability (Lambardi *et al.* 2008; Wang *et al.* 2014b). Subculture is labor intensive with high costs and has risks of contamination that can result in the loss of established cultures. In addition, the morphogenic potential of embryogenic tissues decreases as subculture time increases (Lambardi *et al.* 2008; Wang *et al.* 2014b). The cryopreservation of embryogenic tissues provides a solution to these problems (Wang *et al.* 2002, 2014b). Thus far, cryoprocedures are available only for a limited number of grapevine cultivars, and further studies are clearly needed to expand this knowledge base. Cryotherapy has proven to be a novel and efficient biotechnology for the eradication of grapevine viruses. To date, about 47 virus species have been reported to attack grapevine, among which three virus diseases caused by at least 13 viruses cause serious damage to the viticulture industry (Martelli and Walter 1998). Existing studies have been limited to only GVA, GLRV, and GFLV. The evaluation of field performance of cryo-derived virus-free plants is necessary before they enter commercial field production, which would help the sustainable development of viticulture.

Acknowledgement The authors thank Dr. Jean Carlos Bettoni (University of the State of Santa Catarina, UDESC/CAV, Lages, Brazil) for providing some difficult-to-access literature.

Authors' contributions W-L. Bi: data collection and analysis, preparation of the text, Figures and Tables; C. Pan, X-Y. Hao, Z-H. Cui, M.M. Kher, Z. Marković: assistance with data collection and discussion; Q-C. Wang: data collection and analysis, preparation of the text, Figures and Tables; JA Teixeira da Silva: project proposal and development, data collection and analysis, preparation of the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

References

Aguilar ME, Engelmann F, Michaux-Ferriere N (1993) Cryopreservation of cell suspensions of *Citrus deliciosa* Tan. and histological study. Cryo Letters 14:217–228

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

Ben-Amar A, Daldoul S, Allel D, Reustle G, Mliki A (2013) Reliable encapsulation-based cryopreservation protocol for safe storage and recovery of grapevine embryogenic cell cultures. Sci Hortic 157:32–38

Benelli C (2016) Encapsulation of shoot tips and nodal segments for *in vitro* storage of "Kober 5BB" grapevine rootstock. Horticulturae 2:10

Benelli C, De Carlo A, 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*. Biotech Adv 31:175–185

Benelli C, Lambardi M, Fabbri A (2003) Low temperature storage and cryopreservation of the grape rootstock "Kober 5BB.". Acta Hortic 623:249–253

Benson EE (2008) Cryopreservation of phytodiversity: a critical appraisal of theory and practice. Crit Rev Plant Sci 27:141–219

Bettoni JC, Costa MD, Gardin JPP, Kretzschmar AA, Pathirana R (2016) Cryotherapy: a new technique to obtain grapevine plants free of viruses. Rev Bras Frutic 38:e-833

Bi W-L (2017) Cryopreservation of *Vitis* spp. and cryotherapy for eradication of grapevine viruses. PhD thesis, Northwest A&F University, Yangling, China

Boonham N, Kreuze J, Winter S, van der Vlugt R, Bergervoet J, Tomlinson J, Mumford R (2014) Methods in virus diagnostics: from ELISA to next generation sequencing. Virus Res 186:20–31

Carimi F, Carra A, Panis B, Pathirana R (2016) Strategies for conservation of endangered wild grapevine (*Vitis vinifera* L. subsp. *sylvestris* (C.C. Gmel.) Hegi). Acta Hortic 1115:81–86

Dussert S, Mauro MC, Deloire A, Hamon A, Engelmann F (1991) Cryopreservation of grape embryogenic cell suspensions 1: influence of pretreatment, freezing and thawing conditions. Cryo Letters 12:287–298

Dussert S, Mauro MC, Engelmann F (1992) Cryopreservation of grape embryogenic cell suspensions 2: influence of post-culture conditions and application to different strains. Cryo Letters 13:15–22

Engelmann F (1997) *In vitro* conservation methods. In: Callow JA, Ford-Lloyd BV, Newbury HJ (eds) Biotechnology and plant genetic resources: conservation and use, Biotechnology in Agriculture Series: 19. CAB International, Wallingford, pp 119–161

Esensee V, Stushnoff C (1990) Cryoconservation of dormant grape (*Vitis* sp.) buds. Contributed papers (oral and poster) 87th Annual Meeting of the American Society for Horticultural Science 25: Abstract #190

Ezawa T, Harada T, Yakawa T (1989) Studies on freeze-preservation of fruit tree germplasm: III Freeze-preservation of grape shoot tips. J Fac Agric, Hokkaido Univ 64:51–55

Feng C, Yin Z, Ma Y, Zhang Z, Chen L, Wang B, Li B, Huang Y, Wang QC (2011) Cryopreservation of sweetpotato (*Ipomoea batatas*) and its pathogen eradication by cryotherapy. Biotech Adv 29:84–93

Ganeshan S (1985) Cryogenic preservation of grape, *Vitis vinifera* L., pollen. Vitis 173:169–173

Ganeshan S, Alexander M (1990) Fertilizing ability of cryopreserved grape, *Vitis vinifera* L. pollen. Vitis 29:145–150

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

Gardiman M, Bavaresco L (2015) The *Vitis* germplasm repository at the CRA-VIT, Conegliano (Italy): conservation, characterization and valorisation of grapevine genetic resources. Acta Hortic 1082:239–244

González-Benito ME, Martín C, Vidal JR (2009) Cryopreservation of embryogenic cell suspensions of the Spanish grapevine cultivars "Albarino" and "Tempranillo". Vitis 48:131–136

Häggman HM, Ryynänen LA, Aronen TS, Krajnakova J (1998) Cryopreservation of embryogenic cultures of Scots pine. Plant Cell Tissue Organ Cult 54:45–53

Harding K (2004) Genetic integrity of cryopreserved plant cells: a review. Cryo Letters 25:3–22

Harding K (2010) Plant and algal cryopreservation: issues in genetic integrity, concepts in cryobionomics and current applications in cryobiology. Asia-Pacific J Mol Biol Biotech 18:151–154

Hassan NA, Gomaa AH, Shahin MA, El Homosany AA (2013) *In vitro* storage and cryopreservation of some grape varieties. J Hortic Sci Ornamental Plants 5:183–193

Hassan NA, Haggag AM (2013) Cryopreservation of two Egyptian grape (*Vitis vinifera*) cultivars using two steps vitrification protocol. World Applied Sci J 28:254–258

Keller ERJ, Dreiling M (2003) Potato cryopreservation in Germany – using the droplet method for the establishment of a new large collection. Acta Hortic 623:193–200

Kulus D, Zalewska M (2014) Cryopreservation as a tool used in long-term storage of ornamental species—a review. Sci Hortic 168:88–107

Lambardi M, Ozudogru AE, Benelli C (2008) Cryopreservation of embryogenic callus. In: Reed B (ed) Plant cryopreservation: a practical guide. Springer, Berlin, pp 177–210

Lazo-Javalera MF, Tiznado-Hernández ME, Vargas-Arispuro I, Valenzuela-Soto E, Rocha-Granados M del C, Martínez-Montero ME, Rivera-Domínguez M (2015) Data on antioxidant activity in grapevine (*Vitis vinifera* L.) following cryopreservation by vitrification. Data in Brief 5:549–555

Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Int Plant Prop Soc Proc 30:421–427

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

Marković Z, Chatelet P, Preiner D, Sylvestre I, Kontić JK, Engelmann F (2014a) Effect of shooting medium and source of material on grapevine (*Vitis vinifera* L.) shoot tip recovery after cryopreservation. Cryo Letters 35:40–47

Marković Z, Chatelet P, Sylvestre I, Karoglan-Kontić J, Engelmann F (2012) Duration of culture of grapevine (*Vitis vinifera*) microcuttings on medium with zeatin riboside affects shoot tip recovery after cryopreservation. Paper presented at: Cryopreservation of crop species in Europe. In: Cryopreservation of crop species in Europe, Proceedings of the final meeting, pp 145–147

Marković Z, Chatelet P, Sylvestre I, Kontić J, Engelmann F (2013b) Cryopreservation of grapevine (*Vitis vinifera* L.) *in vitro* shoot tips. Central Eur J Biol 8:993–1000

Marković Z, Preiner D, Bošnjak A, Safner T, Stupić D, Andabaka Ž, Maletić E, Chatelet P, Engelmann F, Kontić J (2014b) *In vitro* introduction of healthy and virus-infected genotypes of native Croatian grapevine cultivars. Central Eur J Biol 9:1087–1098

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

Martelli GP, Walter B (1998) Virus certification of grapevines. In: Hadid A, Khetarpal RK, Koganezawa H (eds) Plant virus disease control. American Phytopathological Society (APS) Press, St Paul, pp 150–166

Martinelli L, Gribaudo I (2009) Strategies for effective somatic embryogenesis in grapevine: an appraisal. In: Roubelakis-Angelakis KA (ed) Grapevine molecular physiology & biotechnology. Springer Netherlands, Dordrecht, pp 461–493

Matsumoto T, Sakai A (2000) Cryopreservation of *in vitro*-cultured axillary shoot tips of *Vitis* by vitrification. Acta Hortic 538:177–181

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

McGovern PE, Glusker DL, Exner LJ, Voigt MM (1996) Neolithic resinated wine. Nature 381:480–481

Miaja ML, Gambino G, Vallania R, Gribaudo I (2004) Cryopreservation of *Vitis vinifera* L. somatic embryos by vitrification or encapsulation-dehydration. Acta Hortic 663:599–604

Miaja ML, Gribaudo I, Vallania R, Fernandez LF (2000) Low temperature storage and cryopreservation of a *Vitis vinifera* L. germplasm collection: first results. Acta Hortic 538:177–181

Mikuła A, Tomiczak K, Rybczyński JJ (2011) Cryopreservation enhances embryogenic capacity of *Gentiana cruciata* (L.) suspension culture and maintains (epi)genetic uniformity of regenerants. Plant Cell Rep 30:565–574

Morel G (1948) Recherches sur la culture associée des parasites obligatoires et des tissues végétaux. In: Annales des Épiphyties, SÉrie Pathologie VÉgÉtale, p 123–234

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

Naidu R, Rowhani A, Fuchs M, Golino D, Martelli GP (2014) Grapevine leafroll: a complex viral disease affecting a high-value fruit crop. Plant Dis 98:1172–1185

Nishizawa S, Sakai A, Amano Y, Matsuzawa T (1993) Cryopreservation of asparagus (*Asparagus officinalis*) embryogenic suspension cells and subsequent plant regeneration by vitrification. Plant Sci 91:67–73

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Panis B (2009) Cryopreservation of *Musa* germplasm. In: Engelmann F, Benson E (eds) Technical Guidelines No. 9, 2nd edn. Bioversity International

Panis B, Piette B, Swennen R (2005) Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae. Plant Sci 168:45–55

Parfitt DE, Almehdi AA (1983) Cryogenic storage of grape pollen. Amer J Enol Viticulture 34:227–228

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

Plessis P, Leddet C, Collas A, Dereuddre J (1993) Cryopreservation of *Vitis vinifera* L. cv. Chardonnay shoot tips by encapsulation-dehydration: effect of pretreatment, cooling and postculture conditions. Cryo Letters 14:309–320

Plessis P, Leddet C, Dereuddre J (1991) Resistance to dehydration and to freezing in liquid nitrogen of alginate coated shoot tips of grapevine (*Vitis vinifera* L. cv. Chardonnay). Comptes Rendus Acad Sci Paris Ser III Sci Vie 313:373–380

Reed BM (ed) (2008) Plant cryopreservation: a practical guide. Springer, New York

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

Shatnawi M, Anfoka G, Shibli R, Almazra'Awi M, Shahrour W, Arebiat A (2011) Clonal propagation and cryogenic storage of virus-free grapevine (*Vitis vinifera* L.) via meristem culture. Turkish J Agric For 35:173–184

This P, Lacombe T, Thomas MR (2006) Historical origins and genetic diversity of wine grapes. Trends Genet 22:511–519

Toprak FC, Kayhan F, Alan AR (2014) *In vitro* propagation and cryopreservation of important grape cultivars (*Vitis vinifera* L.) and rootstocks. Int J Sec Metab 1:75 (Special issue Abstract)

Towill LE, Forsline PL, Walters C, Waddell JW, Laufmann J (2004) Cryopreservation of *Malus* germplasm using a winter vegetative buds method: results from 1915 accessions. Cryo Letters 25:323–334

Vasanth K, Vivier MA (2011) Improved cryopreservation procedure for long term storage of synchronised culture of grapevine. Biol Plant 55:365–369

Wan Y, Schwaninger HR, Baldo AM, Labate JA, Zhong GY, Simon CJ (2013) A phylogenetic analysis of the grape genus (*Vitis* L.) reveals broad reticulation and concurrent diversification during neogene and quaternary climate change. BMC Evol Biol 13:141

Wang QC, Perl A (2006) Cryopreservation in floricultural crops. In: Teixeira da Silva JA (ed) Floriculture, ornamental and plant biotechnology: advances and topical issues. Global Science Books Inc., Isleworth, UK, pp 523–539

Wang QC, Valkonen JPT (2009) Cryotherapy of shoot tips: novel pathogen eradication method. Trends Plant Sci 14:119–122

Wang QC, Tanne E, Arav A, Gafny R (2000) Cryopreservation of *in vitro*-grown shoot tips of grapevine by encapsulation-dehydration. Plant Cell Tissue Organ Cult 63:41–46

Wang QC, Gafny R, Sahar N, Sela I, Mawassi M, Tanne E, Perl A (2002) Cryopreservation of grapevine (*Vitis vinifera* L.) embryogenic cell suspensions by encapsulation-dehydration and subsequent plant regeneration. Plant Sci 162:551–558

Wang QC, Li P, Batuman Ö, Gafny R, Mawassi M (2003a) Effect of benzyladenine on recovery of cryopreserved shoot tips of grapevine and citrus cultured *in vitro*. Cryo Letters 24:293–302

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

Wang QC, Mawassi M, Sahar N, Li P, Violeta CT, Gafny R, Sela I, Tanne E, Perl A (2004) Cryopreservation of grapevine (*Vitis* spp.) embryogenic cell suspensions by encapsulation-vitrification. Plant Cell Tissue Organ Cult 77:267–275

Wang QC, Li P, Hanania U, Sahar N, Mawassi M, Gafny R, Sela I, Tanne E, Perl A (2005) Improvement of *Agrobacterium*-mediated transformation efficiency and transgenic plant regeneration of *Vitis vinifera* L. by optimizing selection regimes and utilizing cryopreserved cell suspensions. Plant Sci 168:565–571

Wang QC, Panis B, Engelmann F, Lambardi M, Valkonen JPT (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 B, Ma Y, Zhang Z, Wu Z, Wu Y, Wang QC, Li M (2011) Potato viruses in China. Crop Prot 30:1117–1123

Wang B, Li JW, Zhang ZB, Wang RR, Ma YL, Blystad DR, Keller ERJ, Wang QC (2014a) Three vitrification-based cryopreservation procedures cause different cryo-injuries to potato shoot tips while all maintain genetic integrity in regenerants. J Biotech 184:47–55

Wang B, Wang RR, Cui ZH, Bi WL, Li JW, Li BQ, Ozudogru EA, Volk GM, Wang QC (2014b) Potential applications of cryogenic technologies to plant genetic improvement and pathogen eradication. Biotech Adv 32:583–595

Wang RR, Gao XX, Chen L, Huo LQ, Li MF, Wang QC (2014c) Shoot recovery and genetic integrity of *Chrysanthemum morifolium* shoot tips following cryopreservation by droplet-vitrification. Sci Hortic 176:330–339

Yin ZF, Chen L, Zhao B, Zhu YX, Wang QC (2012) Cryopreservation of embryogenic cell suspensions by encapsulation–vitrification and encapsulation–dehydration. In: Loyola-Vargas VM, Ochoa-Alejo N (eds) Plant cell culture protocols Volume 877 of the series Methods in Molecular Biology. Springer Science+Business Media, LLC, pp 81–93

Zhai ZY, Wu YJ, Engelmann F, Chen RZ, Zhao YH (2003) Genetic stability assessments of plantlets regenerated from cryopreserved *in vitro* cultured grape and kiwi shoot tips using RAPD. Cryo Letters 24:315–322

Zhao YH, Wu YJ, Engelmann F, Zhou M (2001) Cryopreservation of axillary buds of grape (*Vitis vinifera*) *in vitro* plantlets. Cryo Letters 22:321–328