Progress and Challenges in the Application of Synthetic Seed Technology for Ex Situ Germplasm Conservation in Grapevine (*Vitis* spp.)

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Abstract Although grapevine (*Vitis* spp.) is one of the most ancient and important fruit crops, there is no concerted international effort to conserve its genetic resources, which are estimated to consist of 10–14,000 cultivars. Synthetic seed technology offers opportunities to conserve clonal genetic resources either in the form of quiescent somatic embryos or as encapsulated regenerable somatic tissue. Since the first report of somatic embryogenesis in grapevine in 1976, much research has been conducted into synchronising the process, maturation, dehydration, encapsulation and testing longevity under cold storage. Since the development of vitrification-based cryopreservation methods, both somatic embryos and other somatic tissue with meristematic regions have been used in cryopreservation experiments, and methods have been optimised to reach post-thaw regeneration percentages that satisfy gene bank standards for implementing cryopreservation. Nevertheless, improved protocols for 'difficult' genotypes are still needed for induction of somatic embryos and synchronising their formation, maturation and germination, as well as cryopreservation. As a result of these difficulties, conservation by cryopreservation has progressed using encapsulated shoot tips or axillary buds of tissue culture plants. Some vitrification-based methods use a droplet of vitrification solution to protect the shoot tips on an aluminium strip allowing faster freezing of tissue, an important factor for post-cryo-survival. The novel V cryo-plate method combines the advantages of both encapsulating the shoot tips in alginate beads that then adhere to the aluminium of the V cryo-plate, meaning manipulations

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can be performed easily, and the high thermal conductivity of aluminium speeding processed of freezing and thawing. Cryopreservation of somatic embryos has been suggested as a way to conserve the diversity of wild *V. vinifera* ssp. *sylvestris*, and limited results obtained to date are promising.

Keywords Somatic embryogenesis · Non-zygotic embryo · Cryopreservation · Encapsulation · Vitrification · Embryo maturation · Tissue culture · Genetic resources

1 Introduction

Grapevine (*Vitis vinifera* L. ssp. *vinifera*) is a crop with great economic and cultural significance. There is 7.5 million ha under cultivation producing 75.8 million tons worldwide (OIV 2017). The genus *Vitis* belongs to the family Vitaceae and comprises about 60 inter-fertile species distributed through Europe, Asia and North America under subtropical, Mediterranean and continental-temperate climatic conditions (Carimi et al. 2011; Hancock 2004; Terral et al. 2010). While *V. vinifera* ssp. *vinifera* contributed almost entirely to the diversity of cultivars grown for fruit, juice and wine, other species such as the North American *V. rupestris*, *V. riparia, V. berlandieri or their hybrids* are used as rootstock for *V. vinifera* varieties, mainly due to their resistance to *Phylloxera* but also to other diseases such as *Oidium* and mildews, and for better tolerance to biotic stresses as well (Carimi et al. 2011; Terral et al. 2010). It is now established that grapevine as a crop originated independently in multiple regions, with evidence for West Asian and Caucasian centres of origin (Arroyo-Garcia et al. 2006; Imazio et al. 2013). It is also established that the cultivated form originated from the wild forms of *Vitis vinifera* L. ssp. *sylvestris* (Gmelin) Hegi (Bacilieri et al. 2013; Carimi et al. 2016; Myles et al. 2011).

Archaeobotanical and archaeological evidence for grape cultivation in the Caucasus region dates back to the sixth millennium BC (Imazio et al. 2013) and for the Aegean and Mesopotamian regions and in Egypt dates back to at least 4000 BC (Zohar and Horf 2000). Wild grapevine forms can be found from the South Atlantic coast of Europe through to the Western Himalayas and from sea level to 1000 m above sea level (Arnold et al. 1998). As grape cultivation spread to new areas, the cultivars probably hybridised with local wild and other cultivated genotypes resulting in selection of more adapted genotypes and cultivars with desirable fruit traits. Selection over several millennia has led to the development of an estimated 10,000–14,000 cultivars that are currently held in field collections (Alleweldt and Dettweiler 1994). Management of grapevine plants under field conditions is expensive and has resulted in loss of material in field gene banks (Maletić et al. 2008). Vegetative propagation adds another risk factor with potential for transfer of pathogens to new planting material. Therefore, development and application of plant tissue culture-based biotechnological approaches such as synthetic seeds and

cryopreservation are important for future conservation of *Vitis* germplasm. In this chapter, we present research on somatic embryogenesis in *Vitis* and its potential applications from an ex situ conservation perspective, with special reference to cryopreservation as a long-term conservation option.

2 Somatic Embryogenesis in Grapevine

Somatic embryogenesis (SEg) is the first step towards synthetic seed technology. The first successful induction of SEg was demonstrated in *V. vinifera* cv Cabernet sauvignon more than 40 years ago using unfertilised ovules (Mullins and Srinivasan 1976). Absence of a protective seed coat and lack of surrounding nutritive tissue make direct storage of somatic embryos (SE) difficult compared with zygotic embryos enclosed within seeds. Moreover, SE develop asynchronously and lack the quiescent resting stage of zygotic embryos (Gray et al. 1991). Therefore, research in synthetic seed technology has been directed not only at optimising induction of SE but also synchronising their induction (Jayasankar et al. 1999; Vasanth and Vivier 2011) and development, maturation (Vasanth and Vivier 2011), drying (McKersie et al. 1989; Senaratna et al. 1990; Madakadze and Senaratna 2000) and coating them to facilitate handling and supply of additional nutrients to the growing seedling (Senaratna 1992). Successful induction of SEg and maintenance of embryogenic cultures depend on a complex interaction between genotype, explant type used and culture conditions. Moreover, results are influenced by differential responses due to interaction of factors such as developmental stage of explants and nutrients and plant growth regulators (PGR) included in culture media (Carra et al. 2016; Prado et al. 2010b). Producing mature and well-developed somatic embryos that are able to grow into normal plants is challenging (Ji et al. 2017; Perrin et al. 2001).

2.1 Stages of Somatic Embryogenesis in Grapevine

SEg is the process by which somatic cells, under inductive conditions, generate embryogenic cells, which go through a series of morphological and biochemical changes that result in the formation of a SE. In grapevine, embryos pass through recognisable globular, heart, torpedo and early cotyledon stages, finally resulting in germinated embryos (Carimi et al. 2005).

Globular embryos usually appear on the surface of the embryogenic calli, and in this stage the young embryo is circular or slightly oblong and is still in close contact with the callus from which it was derived. Later, it detaches from the callus and the elongation of axial cells marks the beginning of tissue differentiation. At the end of the globular stage the two apical meristems are present and they persist through the heart-shaped to the torpedo stages. During the transition from the torpedo stage to the germinated embryo, grapevine somatic embryos undergo changes characteristic

of germination, e.g. radicle growth, tannin accumulation in the central cylinder and acquisition of an external suberin sheath (Faure et al. 1996; Ammirato 1987).

2.2 *Explant, Genotype and Growth Regulator Effects on Somatic Embryogenesis of Grapevine*

The influence of genotype, explant and PGR on somatic embryogenesis in grapevine including significant interactions between those factors have been demonstrated by several authors (Table 1).

Genotype is considered to be one of the most important factors affecting plant regeneration in vitro (Conde et al. 2008; Landi and Mezzetti 2006; Rodríguez et al. 2008). In grapevine, genotypes vary relatively widely in their embryogenic potential, and although several protocols have been published, methods still need improvement to optimise media and explant combinations to initiate embryogenic cultures from recalcitrant genotypes (Carra et al. 2016; Gambino et al. 2011a). SEg has been successfully achieved from different genotypes of *Vitis latifolia*, *V. longifolia*, *V. rupestris*, *V. rotundifolia, V. vinifera* L. ssp. *vinifera* and *V. vinifera* L. ssp. *sylvestris* starting from different explants (Carimi et al. 2013, 2016). Anthers have been widely used and embryogenic callus has been obtained for a remarkable number of genotypes (Martinelli and Gribaudo 2009). SEg has also been achieved from different reproductive organs like ovaries (López-Pérez et al. 2005), stigmas and styles (Carimi et al. 2005; Carra et al. 2016; Morgana et al. 2004), anthers (Gribaudo et al. 2004; Kikkert et al. 2005), anther filaments (Acanda et al. 2013; Perrin et al. 2004) and whole flowers (Gambino et al. 2007). Even if less common, SE can be induced from tissues derived from vegetative structures like tendrils (Salunkhe et al. 1997), leaf discs (Das et al. 2002), leaves and petioles (Martinelli et al. 1993) and stem nodal explants (Maillot et al. 2006, 2016).

The most common tissue culture media for inducing SE are based on MS (Murashige and Skoog 1962), NN (Nitsch and Nitsch 1969) or B5 (Gamborg et al. 1968) salts supplemented with different PGR. Usually auxins are the most important PGR to induce SE, and the auxin most frequently used is 2,4-dichlorophenoxyacetic acid (2,4-D). Indole-3-acetic acid (IAA), 2-naphthoxyacetic acid (NOA) and naphthalene acetic acid (NAA) are also used. When cytokinins are used in SEg induction media, they are added to the culture medium together with auxins. The most used cytokinin, 6-benzylaminopurine (BAP), supplemented at different concentrations depending on the type of explant and genotype, is used to initiate embryogenic cultures in combination with 2,4-D. Urea derivatives like thidiazuron (TDZ) or N-(2-chloro-4-pyridyl)-N-phenylurea (4-CPPU) in combination with auxins were effective when used in the induction phase in anther, pistil and ovary culture of *V. vinifera* (Acanda et al. 2013; Bouamama-Gzara et al. 2017; Carra et al. 2016; Kikkert et al. 2005). After embryogenic callus has been induced, in some cases development of somatic embryos is achieved by reducing or removing auxin from the culture medium (Coutos-Thevenot et al. 1992).

Table 1 Successful somatic embryogenesis protocols in *Vitis*

Explant	Species (cultivar/genotype)	PGR	References
Anther filaments	*Vitis vinifera* (Hencha)	2,4-D 9 μM + TDZ 10 μM	Bouamama-Gzara et al. (2017)
Anther filaments	Rootstock (6 genotypes); *V. vinifera* (13 cvs)	2,4-D 4.5 μM + BAP 9 μM	Perrin et al. (2004)
Anthers	*Vitis vinifera* (6 cvs)	NOA 5 μM + BAP 2 μM	Vasanth and Vivier (2011)
Anthers	*Vitis berlandieri* × *Vitis rupestris; V. vinifera* (2 cvs)	NOA 5 μM + BAP 1 μM	Ben-Amar et al. (2013)
Anthers	*V. berlandieri x V. rupestris* (110 Richter)	2,4-D + TDZ combinations	Forgács et al. (2017)
Anthers	*V. vinifera x V. rupestris* (Gloryvine and other cvs)	2,4-D 5 μM + BAP 1 μM	Rajasekaran and Mullins (1979)
Anthers	*V. rupestris, V. longii, V. vinifera* (Grenache)	2,4-D 5 μM + BAP 1 μM	Mullins and Rajasekaran (1980)
Anthers	*V. vinifera* (Cabernet Sauvignon)	2,4-D 4.5 μM + BAP 1.1 μM	Mauro et al. (1986)
Anthers	*V. riparia*	2,4-D 5 μM + BAP 0.9 μM	Mozsar and Sule (1994)
Anthers	*V. vinifera* (4 cvs)	2,4-D 9 μM + BAP 0.9 μM	Perl et al. (1995)
Anthers	*V. vinifera* (Grenache Noir)	2,4-D 4.5 μM + BAP 1.1 μM	Faure et al. (1996)
Anthers	*Vitis* ssp. (10 cvs)	2,4-D 5 μM + BAP 1 μM	Torregrosa (1998)
Anthers	*V. vinifera* (Sultana)	2,4-D 4.5 μM + BAP 9 μM	Franks et al. (1998)
Anthers	*V. latifolia*	2,4-D 20 μM + BAP 9 μM	Salunkhe et al. (1999)
Anthers	*V. vinifera* (15 cvs)	2,4-D 2.5 μM + BAP 0.8 μM	Perrin et al. (2001)
Anthers	*V. vinifera* (9 cvs)	2,4-D 9 μM + TDZ 11.35 μM	Bouamama et al. (2007)
Anthers	*V. vinifera* (Macabeo and Tempranillo)	2,4-D 5 μM + BAP 1 μM	Cutanda et al. (2008)
Anthers	*V. vinifera* (16 cvs); *Vitis* hybrids (11 cvs)	2,4-D 5 μM + TDZ 0.2 μM; 2,4-D 5 μM + BAP 0.4 μM; 2,4-D 2.5 μM + NOA 2.5 μM + 4-CPPU 5 μM	Oláh et al. (2009)
Anthers	*Vitis vinifera* (Pinot noir)	2,4-D 4.4 μM + BAP 4.4 μM	Larrouy et al. (2017)
Anthers and gynoecia	*Vitis vinifera* (Manicure Finger)	2,4-D 4.5 μM + BAP 4.4 μM	Xu et al. (2014)

(continued)

Table 1 (continued)

Explant	Species (cultivar/genotype)	PGR	References
Anthers and ovaries	*V. longii* (microsperma)	2,4-D 5 μM + BAP 1 μM	Gray and Mortensen (1987)
Anthers and ovaries	*V. vinifera* (2 cvs); *V. berlandieri* x *V. rupestris* (110 Richter); *V. berlandieri* x *V. riparia* (5BB)	2,4-D 9 μM + BAP 4.4 μM	Martinelli et al. (2001)
Anthers and ovaries	*V. vinifera* (6 cvs); *Vitis* hybrid (Chancellor); *V. labruscana* (Concord and Niagara)	2,4-D 2.5 μM + NOA 2.5 μM + 4-CPPU 5 μM	Kikkert et al. (2005)
Anthers and ovaries	*V. vinifera* (7 cvs)	2,4-D 4.5 μM + BAP 8.9 μM	Croce et al. (2005)
Anthers and ovaries	*V. vinifera* (Touriga Nacional)	2,4-D 4.5 μM + BAP 8.9 μM	Pinto-Sintra (2007)
Anthers and pistils	*Vitis vinifera* L. ssp. *sylvestris*	NOA 5 μM + BAP 4.4 μM	Carimi et al. (2016)
Anthers, ovaries and flower buds	*Vitis vinifera* (Chardonnay)	2,4-D + BAP + picloram several concentrations	Dai et al. (2015)
Filaments	*V. vinifera*; *V. labruscana* (Bailey)	2,4-D 1 μM + TDZ 1 μM	Nakajima and Matsuta (2003)
Floral explants	*V. vinifera* (Albariño)	2,4-D 4.52 μM + BAP 4.4 μM; 2,4-D 4.52 μM + NOA 2.5 μM+ 4-CPPU 5 μM	Saporta et al. (2014)
Immature anthers	*Vitis vinifera* (Mencía)	2,4-D 4.5 μM + BAP 9 μM	Prado et al. (2014)
Immature anthers and ovaries	*V. vinifera* (8 cvs)	2,4-D 4.52 μM + BAP 4.4 μM; 2,4-D 4.52 μM + NOA 2.5 μM+ 4-CPPU 5 μM	Vidal et al. (2009)
Immature anthers and ovaries	*V. vinifera* (6 cvs)	2,4-D 4.5-9 μM + BAP 4.5-9 μM	Prado et al. (2010a)
Immature leaves	*V. vinifera* (Thompson Seedless)	NOA 2.5 μM + BAP 5 μM+ 2,4-D 2.5 μM	Tapia et al. (2009)
Immature leaves and stamens	*V. rotundifolia* (2 cvs); *V. vinifera* (4 cvs)	2,4-D + BAP + NOA several combinations	Li et al. (2014)
Immature seeds	*Vitis vinifera* L (14 cvs)	TDZ 0.90 μM	San Pedro et al. (2017)
Leaves regenerated in vitro	*V. vinifera* (Crimson Seedless)	NOA 4.95 μM+ BAP 4.44 μM+ phenylalanine 5.0 mM	Nookaraju and Agrawal (2012)

(continued)

Table 1 (continued)

Explant	Species (cultivar/genotype)	PGR	References
Leaves regenerated in vitro	*Vitis vinifera* (Crimson Seedless)	NOA 5 μM+ BAP 4.5 μM + several amino acids	Nookaraju and Agrawal (2013)
Leaves regenerated in vitro	*V. vinifera* (Velika)	2,4-D 9 μM+ IAA 6 μM + BAP 4.4 μM + GA_3 1.8 μM	Tsvetkov et al. (2014)
Leaves	*V. vinifera* (Koshusanjaku)	2,4-D 5-10 μM + TDZ or 4-CPPU 5-10 μM	Matsuta and Hirabayashi (1989)
Leaves	*Vitis* hybrids (Seyval Blanc and Chancellor); *V. thunbergii*	NOA 20 μM + BAP 40 μM or TDZ 4 μM	Harst (1995)
Leaves	*V. rupestris* (du Lot)	2,4-D 9 μM + BAP 9 μM	Tsolova and Atanassov (1996)
Leaves	*V. vinifera* (Podarok Magaracha)	2,4-D 9 μM + BAP 4.4 μM then NAA 5.4 μM + BAP 4.4 μM	Kuksova et al. (1997)
Leaves	*V. vinifera* (Pusa Seedless, Beauty Seedless, Perlette and Nashik)	2,4-D 0.45 μM + BAP 4.5 μM	Das et al. (2002)
Leaves and anthers	*V. vinifera*; *V. rupestris* (several cvs)	NOA 5 μM + BAP 0.9–4.5 μM	Stamp and Meredith (1988b)
Leaves and petioles	*V. rupestris*	IAA 5.7 μM or IBA 0.5 μM	Martinelli et al. (1993)
Leaves and petioles	*V. rotundifolia* (Regale and Fry)	2,4-D 9 μM + BAP 4.4 μM, then NAA 10.7 μM + BAP 0.9 μM	Robacker (1993)
Ovaries	*V. labruscana* (Fredonia and Niagara)	2,4-D 9 μM + BAP 1 μM + IASP 17 μM then 2,4-D 2 μM or 2,4-D 2 μM + IASP 4 μM	Motoike et al. (2001)
Ovaries, anther filaments, stigmas and styles	*Vitis vinifera* (8 cvs)	2,4-D 5 μM + CPPU 5 μM; NOA 20 μM + TDZ 4 μM; NOA 5 μM + BAP 4.4 μM; NOA 10 μM + BAP 4.4 μM	Carra et al. (2016)
Ovules	*V. vinifera* (Cabernet Sauvignon and Grenache), *Vitis* hybrid (Gloryvine)	2,4-D 5 μM + BAP 1 μM	Srinivasan and Mullins (1980)
Ovules	*V. labruscana* (Kyoto)	2,4-D 1 μM + TDZ 0.2 μM	Nakajima et al. (2000)
PEM from anthers and ovaries	*V. vinifera* (Chardonnay)	2,4-D 2 μM	Jayasankar et al. (2001)
Petioles	*V. vinifera*; *Vitis* hybrid	BAP 2.2 μM	Zlenko et al. (2002)

(continued)

Table 1 (continued)

Explant	Species (cultivar/genotype)	PGR	References
Protoplasts	*Vitis* hybrids (Seyval Blanc)	NOA 20 μM + TDZ 4 μM	Reustle et al. (1995)
Protoplasts	*V. vinifera* (Koshusanjaku)	NAA 10.7 μM + BAP 2.2 μM	Zhu et al. (1997)
Seed integuments	*V. vinifera* (Autumn Royal Seedless)	2,4-D 9 μM + BAP 4 μM	Xu and Lu (2009)
Stamen filaments	*Vitis vinifera* (Mencía)	2,4-D 1 μM+ TDZ 4.5 μM	Acanda et al. (2013)
Stamen filaments	*Vitis vinifera* (Mencía)	2,4-D 1 μM+ TDZ 4.5 μM	Acanda et al. (2015)
Stamens	*V. vinifera* L. (Thompson Seedless)	2,4-D 2.25 μM + BAP 18 μM	Zhou et al. (2014)
Stamens and pistils	*Vitis vinifera* L. (4 cvs)	2,4-D 4.5 μM + BAP 8.9 μM	Gambino et al. (2011b)
Stem segments with a unique axillary bud	*Vitis vinifera* (Chardonnay)	2,4-D + BAP at several concentrations	Maillot et al. (2016)
Styles and stigmas	*V. vinifera* (Sugraone)	NOA 5 μM + BAP 9 μM	Morgana et al. (2004)
Styles and stigmas	*V. vinifera* (4 cvs)	NOA 9.9 μM+ BAP 4.5 μM; BAP 9 μM	Carimi et al. (2005)
Tender stems	*V. amurensis* Rupr	NAA 0.5 μM + BAP 2.2 μM	Sun et al. (2016)
Tendrils	*V. vinifera* (3 cvs)	NAA 0.4 μM + BAP 10 μM + GA_3 2.8 μM	Salunkhe et al. (1997)
Unopened leaves, petioles and fully opened leaves	*Vitis rotundifolia* (5 cvs)	2,4-D 9 μM + BAP 4.4 μM	Dhekney et al. (2011)
Whole flower bud	*Vitis vinifera* (Thompson Seedless)	NOA 2.5 μM + 2,4-D 2.3 μM + 4-CPPU 4 μM	Ji et al. (2017)
Whole flowers, anthers and ovaries	*V. vinifera* (8 cvs); *V. berlandieri x V. rupestris* (110R)	2,4-D 4.5 μM + BAP 9 μM	Martinelli et al. (2003)
Whole flowers, anthers and ovaries	*V. vinifera* (8 cvs); *V. berlandieri x V. rupestris* (110R)	2,4-D 4.5 μM + BAP 9 μM	Gribaudo et al. (2004)
Whole flowers, anthers and ovaries	*V. vinifera* (8 cvs); *V. berlandieri x V. rupestris* (110R)	2,4-D 4.5 μM + BAP 9 μM	Gambino et al. (2007)
Whole flowers, anthers and ovaries	*V. vinifera* (8 cvs); *V. berlandieri x V. rupestris* (110R)	2,4-D 4.5 μM + BAP 9 μM	Cadavid-Labrada et al. (2008)
Zygotic embryos	*V. vinifera* (4 cvs); *V. longii*	NOA 5 μM + BAP 0.9 μM	Stamp and Meredith (1988a)

(continued)

Table 1 (continued)

Explant	Species (cultivar/genotype)	PGR	References
Zygotic embryos	*V. rotundifolia* (5 cvs)	NOA 5 μM + BAP 0.9 μM	Gray (1992)

2,4-D 2,4-dichlorophenoxyacetic acid, *BAP* 6-benzyladenine, *4-CPPU* N-(2-chloro-4-pyridyl)-N-′-phenylurea, *cvs* cultivars, *IAA* indole-3-acetic acid, *IBA* indole-3-butyric acid, *IASP* indole-3-acetyl-L-aspartic acid, *NAA* 2-naphthaleneacetic acid, *NOA* 2-naphthoxyacetic acid, *PEM* proembryogenic masses, *PGR* plant growth regulators, *TDZ* N-(1,2,3-thiadiazol-5-yl)-N-′-phenylurea (thidiazuron)

Physical culture conditions significantly influence the embryogenic response. By manipulating light intensity and temperature, Das et al. (2002) developed an efficient leaf-disc method for the regeneration of plants via SEg. Other workers incubate cultures in total darkness until embryogenic callus is formed and then transfer cultures to the light (Oláh et al. 2009; Prado et al. 2010a). Several parameters such as composition of basal medium, medium pH, type of gelling agent, presence of activated charcoal, carbohydrate source and light intensity and spectral composition can influence culture success (Jittayasothorn et al. 2007). Type of culture, liquid or solid, influences the final result, and the liquid culture is to be preferred in the induction phase while structured embryogenic callus grows better on solid media (Jayasankar et al. 2003; Mullins and Srinivasan 1976). Moreover, quality of SE affects regeneration frequency, which varies depending on type of culture: SE cultured on solid media often show dormancy, whereas in liquid media SE were not dormant and showed higher regeneration efficiency (Jayasankar et al. 2003; Mullins and Srinivasan 1976).

SEg is significantly influenced by the developmental stage of explants, and preconditioning treatments have been proved to be crucial in determining the final result. Gribaudo et al. (2004) screened six different developmental stages of anthers to initiate SEg cultures and identified a correlation between anther stage and SEg efficiency. The optimal developmental stage is related to genotype, and for *V. vinifera* 'Chardonnay' and 'Barbera', higher rates of SEg had been obtained when explants were collected at early stages, while in the rootstock '110R', later stages proved to be more efficient. In *V. lambruscana*, the best results were obtained with anthers collected 20 days before anthesis (Nakajima et al. 2000), while for eight Tunisian cultivars of *V. vinifera*, the best performance was achieved with anthers at the tetrad stage of microspore development (Bouamama et al. 2007). Similar results were reported more recently also for *V. vinifera* 'Sultana', 'Red Globe' and 'Merlot' (Vasanth and Vivier 2017).

2.3 *Synchronisation of Somatic Embryo Production and Their Germination*

The synchronisation of SE development is a critical step for taking advantage of SEg for applications, such as micropropagation, germplasm conservation and genetic

transformation, and for gene expression studies. Anatomical and developmental studies towards this goal have been made, comparing SE development in solid media with those in suspension cultures (Jayasankar et al. 2003). Developing SEs on solid media had large cotyledons, little or no visible suspensor structure and a relatively undeveloped concave shoot apical meristem, whereas those developing in liquid media had smaller cotyledons, a distinct suspensor and a flat-to-convex shoot apical meristem. Also, SEs derived from solid media exhibited physiological dormancy and did not germinate without a dormancy-breaking treatment (Jayasankar et al. 2003). Faure et al. (1996) observed asynchronous development of SEs when embryogenic callus cultured in liquid media supplemented with 2,4-D and BAP was transferred to the same liquid media devoid of PGR. They described developmental stages of proembryos in PGR supplemented media, followed by development of globular stage embryos in PGR-free media (Faure et al. 1996).

Jayasankar et al. (1999) reported a high degree of synchronisation of somatic embryo production by alternating solid and liquid media for culture. However, further development of somatic embryos was better achieved in semi-solid media. Embryo germination was influenced by genotype and culture conditions. SEs derived from suspension cultures of 'Chardonnay' did not have a dormant phase and germinated precociously, whereas 'Thompson Seedless' SEs did not develop beyond the heart stage in liquid medium (Jayasankar et al. 1999). In contrast, Zlenko et al. (2002, 2005) successfully converted somatic embryos developed on liquid induction media by subculturing them on liquid media supplemented with BAP and GA_3, or GA_3 alone. Plant regeneration appears to be easier for SEs of Muscadine grapes (*Muscadinia rotundifolia*), as Lu et al. (2007) were able to germinate more than 95% of synchronously produced SEs using suspension cultures established in woody plant medium (Lloyd and McCown 1980). In contrast, in some cultivars of *V. vinifera* such as 'Grenache noir', the germination of somatic embryos is very poor due to their inability to utilise the starch and lipids accumulated in cotyledons at the torpedo stage (Faure and Aarrouf 1994). Another problem with suspension cultures of grapevine is the browning of the suspension of cells and medium, due to production of phenolic compounds. Jayasankar et al. (1999) overcame this problem by sieving the larger cell masses, which contained differentiated somatic embryos. After three subcultures, they managed to produce large numbers of cytoplasm-rich proembryonic masses (PEM). Two cultivars used behaved quite differently: 'Chardonnay' produced SEs from PEMs directly upon subculture onto hormone-free media, whereas 'Thompson Seedless' did not advance beyond the heart stage. The use of conditioned medium has been reported to facilitate embryo proliferation and conversion. Supplementing liquid culture media with arabinogalactan-proteins has been shown to facilitate cell proliferation of grapevine embryogenic cultures (Ben Amar et al. 2007). Recently, an improved protocol based on the dynamic maintenance of culture medium has been proposed. Forgács et al. (2017) stated that culture density affects both the amount of differentiating embryos and their stage of development in 'Richter 110'. Results show that to achieve full synchrony, it is essential to use low cell density obtained through readjusting it to the initial value every week.

2.4 Applications of Somatic Embryogenesis in Grapevine

SEg is the preferred method for cell to plant regeneration in *V. vinifera* L. and has been reported for several important *Vitis* species. SEg has been widely applied in crop genetic improvement and it was suggested as a specific tool to induce somaclonal variation in grapevine and specifically to amplify clonal variability (Acanda et al. 2015; Desperrier et al. 2003; Kuksova et al. 1997; Torregrosa et al. 2001). SEs have proven to be an excellent resource for mutations (Pathirana 2011) even if this approach does not guarantee a good rate of SE induction and conversion into plantlets, which is strictly genotype dependent. For this reason, mutagenesis has not been extensively used for grapevine improvement even if physical and chemical mutagens have been investigated. Recently, production of colchicine- and oryzalin-induced polyploid mutants has been described starting from SEs of 'Crimson seedless', 'BRS Clara' and 'Mencia' (Acanda et al. 2015; Sinski et al. 2014). Currently, SEg is the most suitable tool for in vitro manipulation of the *Vitis* genus (Kikkert et al. 2001; Martinelli and Mandolino 2001). For this reason, mutagenesis of somatic embryos has become an interesting tool in genomics programs to assign gene function, particularly since the availability of the draft genome sequence of grapevine (Jaillon et al. 2007; Velasco et al. 2007).

Virus infections cause severe economic losses in grapevine with several viruses known to negatively influence grape quality and yield. SEg has been proved to be highly effective in eliminating some viruses, either alone or in combination with thermotherapy (Gambino et al. 2006, 2009). Bouamama-Gzara et al. (2017) reported 100% elimination of *Grapevine leafroll-associated virus 3*, *Grapevine stem pitting-associated virus* and *Grapevine virus A* from the Tunisian cultivar Hencha through SEg.

Other applications include the isolation of natural somatic mutants (Boss and Thomas 2002; Franks et al. 2002), and germplasm conservation through synthetic seeds technology and cryopreservation (Brambilla 1999; Carimi et al. 2016; Gray and Compton 1993; Gray et al. 1991; Jayasankar et al. 2005).

SEs have been tested as a resource for conservation of grapevine germplasm. Jayasankar et al. (2005) cultured mature somatic embryos at low density (250 embryos/40 ml liquid media) for 2 weeks, dried them in the laminar flow for ~4 h and sealed in Petri dishes. Ninety percent of these dehydrated SEs produced plants after 42 months of storage at 4 °C in *V. vinifera* 'Chardonnay'. *V. vinifera* 'Autumn Seedless' SEs were generally poor in germination from the beginning but did not show any deterioration during the month storage trial, rather their conversion rate increased from 30 to 40% over the storage period, a statistically significant difference (Jayasankar et al. 2005).

Possible use of SEs for clonal propagation was demonstrated by Jayasankar et al. (2001) using *V. vinifera* 'Chardonnay'. They blot-dried mature SEs and germinated directly in different sterilised agar-free potting media under aseptic conditions. Commercial potting mixture overlaid by sand produced the best results with 32% of the SEs growing into normal plantlets ready for greenhouse acclimation. They proposed encapsulating SEs for further improvements to the propagation methodology.

2.5 *Genetic Stability of Somatic Embryo-Derived Grapevine Plants*

Plantlets derived from an in vitro culture may exhibit somaclonal variations (Larkin and Scowcroft 1981). There are two types of somaclonal variations: heritable and epigenetic (Skirvin et al. 1994). Heritable changes in in vitro cultures occur at higher frequency than occurs spontaneously in seeds or grafted plants (Prado et al. 2010b; Sahijram et al. 2003). Therefore, somaclonal variations may constitute a serious problem in clonal propagation systems aimed at the preservation of plant genetic integrity (Sahijram et al. 2003). However, somaclonal variations may also be exploited as a source of new genetic variability for crop improvement, especially in trees and long-lived perennial species and vegetatively propagated plants (Karp 1995). Methods for detecting somaclonal variation were extensively reviewed by Bairu et al. (2011). Somaclonal variants can be detected using various techniques that are broadly categorised as morphological and molecular detection techniques.

Morphological variants can be easily detected based on characters such as differences in plant stature, leaf morphology or pigmentation abnormalities (Israeli et al. 1991). In grapevine, variation in leaf shape was reported for several somaclones of 'Grenache' (Martinez et al. 1997). Variability in cropping level, berry weight and vigour are also reported (Torregrosa et al. 2011). DNA-based techniques, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), microsatellite or inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers, are valuable tools for analysing the genetic fidelity of in vitro propagated plants. Variations in tissue culture-derived plants can also be generated by changes in methylation (Schellenbaum et al. 2008) and ploidy (Martinelli and Gribaudo 2001). Genetic profiles determined by DNA markers (ISSR, AFLP, RAPD, SSR) have shown that genetic fidelity is not compromised during SE, except in very rare instances (Prado et al. 2010b; Yang et al. 2006). Flow cytometry has been used in *V. vinifera* to verify the ploidy level and ploidy stability of SE-derived plants (Leal et al. 2006; Prado et al. 2010b). Researchers reported a low percentage of somaclonal variation, taking into account that all the embryos were produced from callus tissue, which is prone to genetic variability (Sato et al. 2011; Smulders and De Klerk 2011).

3 Conservation of High-Health Germplasm Through Cryopreservation

Plant cryopreservation is the storage of structurally intact cells, tissues or organs in liquid nitrogen (LN) or its vapour phase at ultra-low temperatures, mainly for conservation of genetic resources. The temperature in LN (−196 °C) or its vapour phase ensures the cellular activity is slowed to the point of cessation where vital functions such as enzymatic activity, gene function and respiration cease, thus

arresting the cell aging. Hence in theory, cryopreserved cells cannot age beyond the physiological point at which they were placed in cryo storage (Benson 2008). Thus, cryopreservation provides a means to conserve plant genetic resources in gene banks that are otherwise under threat when maintained in the field, particularly in the case of clonally propagated species such as grapevine that are expensive to duplicate in different locations or to maintain as in vitro cultures.

3.1 Developing High-Health Plants for Industry and Conservation

In addition to storage of genetic resources for future use, cryopreservation has been used in recent years to eradicate infecting microorganisms in many horticultural species including grapevine (Bettoni et al. 2016; Wang and Valkonen 2007, 2009; Bi et al. 2018a; Wang et al. 2003). Table 2 gives details of the grapevine cultivars from which different viruses were eradicated using cryopreservation techniques.

There are a number of advantages of using cryotherapy to obtain high-health plants for the industry compared with traditional methods of virus eradication. Among these advantages, the ability to treat a large number of samples and genotypes simultaneously, higher frequency of virus-free plants and cost-effectiveness are the major benefits of cryo-based technologies (Bettoni et al. 2016).

4 Methods Used for Grapevine Cryopreservation

Although the first method of cryopreservation of plant material developed using winter dormant twigs of *Salix*, mulberry and poplar (Sakai 1960) may apply to frost hardy grapevine, its wider application may be limited. Further manipulation of freezing tolerance, for example, through exogenous abscisic acid (Rubio et al. 2018), may enable the use of the method in grapevine. Currently, the dormant bud method is successfully used mainly in apple for long-term preservation (Höfer 2015; Pathirana et al. 2018; Towill and Bonnart 2005). On the other hand, development of vitrification-based methods applied to embryogenic masses, SEs, shoot tips and axillary buds from tissue cultured plants has progressed rapidly.

Vitrification is freezing of a solution without crystallisation. The water molecules are sparsely distributed in highly concentrated solutions, including cytoplasm of highly dehydrated plant tissue, particularly in the non-vacuolar, highly cytoplasmic meristematic tissue. Snap freezing of such tissue in LN will result in the solution transitioning to a vitreous (glassy) state. In this state, the metabolic activity in cells ceases and cells survive without aging, theoretically for eternity.

The vitrification method of cryopreservation involves dehydration of cells/tissues in glycerol-based cryoprotective solutions such as plant vitrification solution

Table 2 Efficiency of plant regeneration and virus eradication in cryopreserved grapevine

Grapevine cultivar	Method of cryopreservation	Efficiency (% explants regenerated)	Virus	Efficiency of eradication (% clean)	References
Bruti	Vitrification	50	*Grapevine virus A*	97	Wang et al. (2003)
Bruti	Encapsulation-dehydration	62	*Grapevine virus A*	97	Wang et al. (2003)
Black	Encapsulation-dehydration	59	*Grapevine virus A*	42	Bayati et al. (2011)
Chardonnay	Droplet-vitrification	30.7	*Grapevine fanleaf virus*	77.8	Markovic et al. (2015)
Cabernet sauvignon	Droplet-vitrification	41.6	*GLRaV-3*[a]	100	Markovic et al. (2015)
Chardonnay	Droplet-vitrification	13.0	*GLRaV-3*	100	Pathirana et al. (2015)
Pinot gris	Droplet-vitrification	13.6	*GLRaV-2*[b]	100	Pathirana et al. (2015)
Sauvignon blanc 316	Droplet-vitrification	15.7	*GLRaV-2*	100	Pathirana et al. (2015)
Lakemont seedless	Droplet-vitrification	16.2	*GLRaV-3*	100	Pathirana et al. (2015)
Sauvignon blanc	Droplet-vitrification	30.0	*GLRaV-1*[c] and *GLRaV-3*	100 (both viruses)	Pathirana et al. (2015)
Cabernet sauvignon	Droplet-vitrification	23–59[c]	*GLRaV-3*	100	Bi et al. (2018a)
Chardonnay	Droplet-vitrification	47	*GLRaV-3*	100	Bi et al. (2018a)
Kyoho (*V. vinifera* x *V. labrusca*)	Droplet-vitrification	51	*GLRaV-3*	100	Bi et al. (2018a)
Hunan-1 (*V. pseudoreticulata*)	Droplet-vitrification	43	*GLRaV-3*	100	Bi et al. (2018a)

[a]*Grapevine leafroll-associated virus 3*
[b]*Grapevine leafroll-associated virus 2*
[c]*Grapevine leafroll-associated virus 1*

2 (PVS2) (Sakai et al. 1990). Penetrative cryoprotectants such as ethylene glycol and dimethyl sulphoxide (DMSO) in vitrification solutions are believed to prevent ice formation by interfering with hydrogen bonding between water molecules (Best 2015), and they also displace water within the cytoplasm, thus supporting dehydration of tissue.

There are several methods of vitrification described for plant tissue:

(a) Two-Step Freezing

This older method was mainly used for 'unprotected' (Ezawa et al. 1989) and protected shoot tips (Plessis et al. 1991, 1993) and axillary buds through encapsulation in alginate beads (Miaja et al. 2000; Zhao et al. 2001), as well as for embryogenic cell suspensions (Dussert et al. 1991, 1992; Ben-Amar et al. 2013). Ezawa et al. (1989) used a freezing solution containing 10% DMSO and 60 g/L sucrose to cryopreserve *Vitis labrusca* shoot tips (1–2 mm). After holding shoot tips for 2 h in this solution at room temperature, they cooled the shoot tips in the same solution at a rate of −0.5 °C per minute using a programmable freezer and found that freezing to −30 °C followed by immersion in LN gave better regeneration than those frozen to −20 °C or − 40 °C and immersed in LN. Also, shoot tips from twigs harvested from the field in November and December from Hokkaido Research Station fields in Japan responded better to freezing in LN than those harvested in October (Ezawa et al. 1989).

Zhao et al. (2001) encapsulated axillary buds of four *V. vinifera* accessions and treated them in increasing sucrose concentrations from 0.1 to 1 M and desiccated with silica gel to 21% moisture content then slowly cooled to −40 °C at a rate of −0.2 °C/min before immersion in LN. They found that plants maintained without subculture for 3 or 4 months and those that received a cold-acclimation of 1 month at 5 °C were amenable to cryopreservation. Shoot tips from younger plants in tissue culture did not regenerate after cryopreservation by this method.

Dussert et al. (1991) used anthers of rootstock '41B' (*V. vinifera* 'Chasselas' x *V. berlandieri*) to induce embryogenic callus from which they produced embryogenic cell cultures. These were used in their cryopreservation experiments. They demonstrated that fast freezing is not effective for cryopreservation of embryogenic cell suspensions and only two-step freezing allowed successful cryopreservation. DMSO (5% w/v) and 0.25 M maltose in the freezing solution gave the highest (>60%) regeneration rates after slow cooling to −40 °C at the rate of −0.5 °C/min followed by immersion in LN. Another important step is the incubation of the cells in the medium for 1 h at 0 °C as a pretreatment step before slow freezing. The optimum post-thaw culture medium consisted of a semi-solid medium supplemented with 2 mg/L naphthoxyacetic acid (Dussert et al. 1992). Activated charcoal (0.1%) helped prevent browning of the cells after thawing, but regrowth was reduced in its presence. A minimum of 6 days in the semi-solid medium was essential for recovery of cells after cryopreservation (Dussert et al. 1992).

Ben-Amar et al. (2013) compared a two-step freezing method with direct freezing for cryopreservation of embryogenic cell lines of three grapevine accessions: Rootstock 110 Richter (*V. berlandieri* x *V. rupestris*) and *V. vinifera* cv. Riesling and

cv. Tempranillo. For both procedures, they employed pre-culture of embryogenic cell masses in increasing sucrose concentrations from 0.25, 0.5, 0.75 M through to 1 M over 4 days either before encapsulating in alginate beads (dehydration-encapsulation) or after (encapsulation-dehydration). Then, the cells in beads were treated for 3 days in a mixture of 2 M glycerol and 0.4 M sucrose before either being dehydrated in the laminar flow hood airflow before direct immersion in LN or maintained at 0 °C for 30 min, for 45 min at −20 °C or for 45 min at −80 °C before transfer to LN. Direct freezing recorded significantly higher regeneration rates (43.3–78%) than slow freezing (15.3–25.3%) in all three accessions. However, the slow freezing method employed was different from that of previous workers who demonstrated better results when a programmed freezer was used (Dussert et al. 1991, 1992; Zhao et al. 2001) to control the rate of temperature drop.

The method used by Plessis et al. (1993) and Miaja et al. (2000) involved sucrose pre-culture of shoot tips in alginate beads followed by dehydration for 4 h in the laminar hood airflow that resulted in 30% moisture content (Miaja et al. 2000), followed by slow freezing at 0.5 °C/min to −80 °C and transfer to LN. While Plessis et al. (1993) recorded 24% shoot tip survival in *V. vinifera* 'Chardonnay' by this two-step encapsulation-dehydration procedure, Miaja et al. (2000) did not recover whole plants in three cultivars ('Nebbiolo', 'Barbera' and 'Brachetto'), although cells of some explants showed viability when tested using fluorescent microscopy.

In the three-step vitrification method, explants are stepwise pre-cultured in tissue culture media enriched with increasing sucrose concentrations over several days followed by osmoprotection using a glycerol and sucrose mixture and finally PVS2 dehydration. In this method, after the final treatment in vitrification solution, explants are held in a cryotube with vitrification solution and transferred directly to LN. Researchers describe such vitrification methods as two-step or three-step vitrification methods; however, this should not be confused with two-step freezing method where the explants are first cooled slowly at a selected rate and then transferred to LN after reaching a designated temperature such as −30 °C.

(b) Encapsulation-Dehydration

This method was first reported by Fabre and Dereuddre (1990) for cryopreservation of *Solanum* shoot tips and is based on the technology developed for the production of artificial seeds. Encapsulation-dehydration has been experimented with in well over 70 different plant species (Engelmann et al. 2008). Plessis et al. (1991) were the first to report this method to cryopreserve grapevine shoot tips. In this method, explants are placed into alginate solution (3% Na-alginate (w/v), 2 M glycerol and 0.4 M sucrose in MS liquid medium with no $CaCl_2$), and then individual explants in alginate solution are transferred by pipette to a calcium chloride solution (0.1 M calcium chloride, 2 M glycerol and 0.4 M sucrose in MS liquid medium) in a droplet. The explant gets entrapped in beads by ionotropic gelation, with $CaCl_2$ acting as the crosslinking agent. Crosslinking is complete in about 30 min and produces beads of 4–5 mm diameter containing an explant. Then the beads are pre-cultured on basal MS media supplemented with increasing sucrose concentrations of 0.25, 0.5, 0.75 and 1 M for 4 days, a step per day, before partial desiccation in the air current of a

laminar flow hood or on silica gel to desiccate the beads to about 16 to 20% moisture content (Markovic et al. 2013; Wang et al. 2000). The dehydration period can vary depending on the ambient temperature and humidity, especially for air-drying in a laminar flow hood. The two desiccation methods produce similar results with respect to *Vitis* shoot regrowth as demonstrated in rootstock LN33 (Couderc 1613 × *Vitis vinifera* 'Thompson Seedless'). The highest survival (60%) was achieved when beads were dehydrated for 7 h in laminar flow hood airflow or for 4.5 h on silica gel, with a final bead moisture content of about 16% (Wang et al. 2000). However, desiccation on silica gel is easier to reproduce than air-drying, since the room conditions are variable among labs or even at different periods of the year within the same lab. After desiccation, the beads are transferred to cryovials (~10 beads per cryovial) and plunged into LN. For testing viability, cryovials are warmed in a 40 °C water bath for 3 min and encapsulated shoot tips are cultured on recovery medium. Encapsulation-dehydration procedures for different *Vitis* genotypes have been tested using both direct immersion in LN after osmoprotection (Bayati et al. 2011; Bi et al. 2018a; Carimi et al. 2016; Dussert et al. 1991; Wang et al. 2000, 2002, 2004) and by two-step freezing (Dussert et al. 1991, 1992; Ezawa et al. 1989; Miaja et al. 2000, 2004; Plessis et al. 1991, 1993; Zhao et al. 2001) focusing on several *Vitis* species.

(c) Encapsulation-Vitrification

This method is a combination of encapsulation-dehydration and vitrification procedures, where explants are encapsulated in alginate beads and dehydrated chemically using vitrification solutions; it combines the advantages of ease of manipulation of encapsulated explants and the fast dehydration by vitrification (Matsumoto and Niino 2017; Sakai and Engelmann 2007). The encapsulation-vitrification method has been studied less than other cryopreservation procedures for grapevine (Bettoni et al. 2016). In the study of Benelli et al. (2003), shoot tips from rootstock Kober 5BB (*Vitis berlandieri* x *Vitis riparia*) were excised from cold acclimated cultures (3-week at 4 °C) and encapsulated in 3% calcium alginate. The beads were placed in cryovials and exposed to PVS2 at 0 °C for 90 min. Cryovials containing shoot tips and PVS2 were then immersed in LN and stored. For recovery, LN-stored cryovials were warmed in a 40 °C water bath and encapsulated shoot tips were cultured on recovery medium. This protocol resulted in low regrowth levels, not specified by the authors (Benelli et al. 2003).

(d) Droplet-Vitrification

This technique is a variant of vitrification-based cryopreservation and was derived from the droplet-freezing technique developed by Kartha et al. (1982) for freezing cassava shoot tips using slow cooling and then modified by Schafermenuhr et al. (1994) for potato shoot tip cryopreservation. In this method, explants are osmoprotected, exposed to vitrification solution, placed onto individual droplets or a thin layer of PVS2 on aluminium foil strips and then transferred to LN (Bi et al. 2018b; Pathirana et al. 2016). The main advantage of this technique over the traditional vitrification procedures is the possibility of achieving faster freezing and warming rates due to the direct contact of explants with LN (Panis et al. 2011; Sakai and Engelmann 2007). Aluminium, with its high thermal conductivity, further

facilitates fast freezing of the shoot tips. Droplet-vitrification has been applied successfully to in vitro grown shoot tips of diverse plant species, including grapes (Bi et al. 2018b; Carimi et al. 2016; Hassan and Haggag 2013; Markovic et al. 2013; Pathirana et al. 2016; Volk et al. 2018). So far, droplet-vitrification appears to be a promising method to overcome species- and genotype-specific responses that have been bottlenecks for the widespread use of *Vitis* cryopreservation (Bi et al. 2018b; Volk et al. 2018). Recently, improvements in droplet-vitrification protocols for cryopreservation of *Vitis* have been reported, and these are associated with improving the explant quality (Markovic et al. 2013) and pretreatment conditions, adding antioxidants (Bi et al. 2018a) and elicitors of defence proteins such as salicylic acid (Bi et al. 2018b; Pathirana et al. 2016; Volk et al. 2018). Volk et al. (2018) reported a widely applicable *Vitis* droplet-vitrification method and applied it to nine species. In their protocol, shoot tips were excised from nodal sections that were grown on MS medium containing 0.2 mg L^{-1} N^6-benzyladenine, 1 mM salicylic acid, 1 mM glutathione (reduced form) and 1 mM ascorbic acid for 2 weeks. Then, the shoot tips were pre-cultured on half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid and 1 mM glutathione (reduced form) for 3 days, treated with loading solution (half-strength MS + 2 M glycerol +0.4 M sucrose) for 20 min and then with half-strength PVS2 for 30 min at 22 °C followed by full-strength PVS2 treatment for 90 min at 0 °C prior to immersion in LN. Shoot tips were warmed in unloading solution (half-strength MS + 1.2 M sucrose) for 20 min and post-thaw cultured for shoot regrowth. This cryo-protocol resulted in 24–43% shoot regrowth and averaged 35 ± 2% across nine *Vitis* species. Bettoni et al. (2018) reported a similar *Vitis* droplet-vitrification method to that described by Volk et al. (2018); in addition, they showed the possibility of cryopreserving *Vitis vinifera* 'Chardonnay' shoot tips without first introducing the accession into tissue culture, using plants from a growth chamber. Nodal sections were harvested from the growth chamber plants, surface sterilised and plated on pretreatment medium for 2 weeks, and then shoot tips (1 mm) were dissected and pre-cultured for 3 days. The pretreatment and pre-culture medium were those described above by Volk et al. (2018), with addition of the plant preservation mixture (PPM®, 1.5% v/v) to reduce microbial contamination. Pre-cultured shoot tips were treated with loading solution for 20 min, followed by half-strength PVS2 for 30 min at 22 °C and then full-strength PVS2 treatment for 30 min at 0 °C prior to immersion in LN. Following LN, the shoot tips were warmed in unloading solution for 20 min and post-thaw cultured for shoot regrowth. About 43% shoot regrowth was obtained for *V. vinifera* 'Chardonnay'.

(e) V Cryo-plate Method

The recently developed V cryo-plate technique is an optimisation of the encapsulation and droplet-vitrification methods and combines advantages of both (Yamamoto et al. 2011). Explant handling is facilitated by droplet encapsulated plant tissues adhering to the wells of aluminium cryo-plates, and at the same time, this process aids fast cooling and warming rates, an important requirement for successful cryopreservation protocols (Niino et al. 2013; Panis et al. 2005; Yamamoto et al. 2011).

The main advantages of the cryo-plate method, over the other vitrification-based cryopreservation techniques, are simplicity and user-friendliness; samples can be easily transferred between solutions with minimal risk of mechanical injuries because all treatments can be carried out on cryo-plates with shoot tips attached, i.e. the cryo-plate is manipulated only after the shoot tips have adhered onto it (Rafique et al. 2015). The V cryo-plate method has been successfully applied to a diverse range of species (Matsumoto and Niino 2017). Bettoni et al. (2019) reported a practical and promising cryopreservation protocol for in vitro grapes shoot tips using the V cryo-plate method. In this protocol, microcuttings were grown on MS pretreatment medium supplemented with 0.2 mg L^{-1} N^6-benzyladenine, 1 mM salicylic acid, 1 mM glutathione (reduced form) and 1 mM ascorbic acid for 2 weeks, and then shoot tips (1 mm) were dissected from the shoots and pre-cultured on half-strength MS medium containing 0.3 M sucrose, 0.1 mM salicylic acid, 1 mM ascorbic acid and 1 mM glutathione (reduced form) for 3 days. Pre-cultured shoot tips were attached to wells of a cryo-plate with alginate (2% Na-alginate (w/v)) and calcium (0.1 M calcium chloride) beads and treated with loading solution for 30 min and then exposed to PVS2 at 22 °C for 40 min. The cryo-plate was then immersed in LN for 1 h and warmed into unloading solution for 20 min, and the alginate beads were transferred onto the recovery medium. This protocol resulted in 68–70% shoot regrowth in *Vitis* accessions *V. aestivalis* (DVIT 1408) and *Vitis jacquemontii* (PI 135726).

5 Conclusions and Future Perspectives

Since the first report of SEg in grapevine, much research has been conducted on improving the conditions for inducing embryogenic cultures and on manipulating those to obtain mature SEs synchronously. Lack of synchrony and consistency in SE formation, poor maturation, lack of a quiescent stage resulting in autonomous germination and inability to dehydrate them unlike zygotic embryos are problems. There are only a few reports of attempts to store dehydrated SEs of grapevine, but some accessions have stored well up to 2 years at 4 °C. Variability in response in different genotypes requires optimising media and conditions for recalcitrant genotypes. As a result of these difficulties, conservation by cryopreservation has progressed using encapsulated shoot tips or axillary buds of tissue culture plants. One vitrification-based method uses a droplet of vitrification solution to protect the shoot tips on an aluminium strip allowing faster freezing of tissue, an important factor for post-cryo-survival. The novel V cryo-plate method combines the advantage of both encapsulation in alginate beads to adhere the explants, so manipulations can be performed easily, and the high thermal conductivity of aluminium. Cryopreservation of SEs has been suggested as a way to conserve the diversity of wild *V. vinifera* ssp. *sylvestris*, and the limited results are promising. Further research towards incorporating dehydration tolerance in SEs would allow further progress in using SEs as the clonal unit of conservation.

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