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ORIGINAL ARTICLE

Pre-treatment with salicylic acid improves plant regeneration after cryopreservation of grapevine (*Vitis* spp.) by droplet vitrification

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Abstract Despite many reports on regeneration of *Vitis* after cryopreservation, there is no cryopreserved collection of its germplasm. Some *Vitis* genotypes are reported to be recalcitrant to cryopreservation. Droplet vitrification, considered to be an emerging generic method of cryopreservation, has been applied only to a limited extent in *Vitis*. In the present study, we first tested the toxicity of plant vitrification solution in both axillary and apical buds in six diverse *Vitis* accessions. Droplet vitrification was then applied using 50 % predicted survival time of apical and axillary buds in vitrification solution after pre-treatment of donor plantlets with salicylic acid, a substance known to have a protective role in abiotic stress responses. Results showed that axillary buds are more tolerant of vitrification solution than apical buds and required longer treatment time. Pre-treatment of donor plantlets with 0.1 mM salicylic acid resulted in a significantly higher protection to cryopreserved buds, but serial dehydration in sucrose alone had little effect. Pre-treatment with salicylic acid enabled successful cryopreservation of previously recalcitrant rootstock 41B, albeit at a low regeneration rate. For other genotypes, cryopreservation of 6–11 explants will be sufficient to regenerate at least one plant at 95 % probability. This is the first report of successful cryopreservation of a set of diverse *Vitis* genotypes by droplet vitrification, and we show that pre-treatment of donor plantlets with salicylic acid is critical for the success. This research will contribute to conservation of *Vitis* germplasm in a cost-effective way avoiding the risks associated with field-based collections.

Keywords Abiotic stress · Conservation · Dehydration · Germplasm · In vitro culture · Sucrose

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Introduction

The common method for conserving germplasm of perennial fruit species, including grapevines, is as whole plants in the field. Field maintenance of nursery material carries the risk of not only infections with viral, fungal, bacterial diseases and insect pests, but also of loss due to environmental disasters such as floods, earthquakes, drought, fire, volcanic eruptions, etc. Duplicating material in different fields is an option but is expensive. The risks involved in field maintenance have led to the search for secure and low-cost alternatives. Cryopreservation has become the preferred option for the long-term conservation of clonally propagated plant germplasm to ensure the safe and cost-efficient long-term conservation (Keller et al. 2008; Nukari et al. 2009). Cryopreservation is the storage of viable cells, tissues, organs and organisms at ultra low temperatures, usually in liquid nitrogen (LN) and/or its vapour phase, at temperatures of c. −196 to −140 °C (Benson 2008).

Cryopreservation is now applied to a diverse range of horticultural species including banana (Panis et al. 2010), raspberry, hop (Häggman and Uosukainen 2010; Zamecnik

et al. 2007), potato (Gonzalez-Arnao et al. 2008; Keller 2007; Nukari et al. 2009; Zamecnik et al. 2007), garlic (Keller 2007; Kim et al. 2012), elm, mint (Keller 2007), apple (Forsline et al. 1998; Höfer 2015; Lambardi et al. 2011), pear, *Prunus* sp. (Zamecnik et al. 2007) and yam (Keller et al. 2008) in North America, Europe and Korea. In addition, Japan has a large collection of mulberry varieties, and India's National Bureau of Plant Genetic Resources (NBPGR) has six tropical fruit species and tea genetic resources in cryogenic storage (Reed 2001). Among the more than 9915 diverse accessions belonging to 729 species, NBPGR collection includes 32 species of *Citrus* germplasm (NBPGR 2015).

As an ancient crop, 10,000–14,000 different grapevine varieties are thought to be held in germplasm collections around the world (Alleweldt and Dettweiler 1994). The risk of pathogen transmission through vegetative multiplication and insects is high, and difficult to avoid in grapevine (Carimi et al. 2011). Maintenance of field collections is expensive, which has led to the erosion of valuable germplasm resources (Barba et al. 2008; Carimi et al. 2011). Despite its value as a crop, there is currently no collection of grapevine germplasm in cryostorage, partly because of recalcitrance of some genotypes to cryopreservation as shown by Ganino et al. (2012). Nevertheless, several approaches of cryopreservation have been tested in grapevine. For example, grapevine somatic embryos and embryogenic cultures have been cryopreserved by encapsulation dehydration and encapsulation vitrification (Wang et al. 2004; Gonzalez-Benito et al. 2009; Ben-Amar et al. 2013). Dussert et al. (1991) used a slow freezing technique to preserve embryogenic cell suspensions. Using encapsulation-based methods for cryopreservation of shoot tips, Wang et al. (2003) and Bayati et al. (2011) demonstrated the removal of *Grapevine Virus A* from infected vines. Encapsulation-based methods have also been used to preserve synchronised embryogenic cell cultures (Vasanth and Vivier 2011). Recently Shatnawi et al. (2011) reported the use of vitrification of shoot tips to cryopreserve *Vitis vinifera* cv. Salty Kodari. Marković et al. (2013) compared droplet vitrification with encapsulation–dehydration in *V. vinifera* cv Portan and subsequently showed that actively growing shoot tips sampled from microcuttings are better suited for cryopreservation than buds harvested from in vitro plantlets (Marković et al. 2014).

Droplet vitrification was investigated in our research because it is proving applicable to many species, is relatively simple and the direct plunging of explants on aluminium foil strips allows one of the fastest rates of cooling achieved so far, which is a critical factor in vitrification (Panis et al. 2011; Yin et al. 2014). Furthermore, instant freezing would prevent DNA methylation (Fan et al. 2013) and lipid breakdown due to fatty acid peroxidation (Kaniuga et al. 1999) associated with slower methods of freezing. In order to vitrify tissues by rapid cooling in LN, without detrimental intracellular ice crystal formation, the explants must be sufficiently dehydrated prior to cooling. Plant vitrification solution 2 (PVS2) has been successfully used to prepare tissues of many different crop species for cryopreservation (Benelli et al. 2013; Benson 2008). Unfortunately, some grapevine genotypes can be extremely sensitive to the dehydration effect of PVS2 solution, as shown by Ganino et al. (2012). Therefore, the work reported here was aimed at comparing the tolerance of both apical and axillary buds in a range of *Vitis* genotypes to PVS2 solution. Then we tested the effect of treatment of the source plantlets with salicylic acid (SA), known to protect tissue from low temperature-induced oxidative damage in vivo (Mutlu et al. 2013; Chen et al. 2011; Sayyari 2012) as well as during cryopreservation (Bernard et al. 2002; Wang et al. 2009b).

Materials and methods

Plant material

Plant material of the wine grape (*V. vinifera*) cultivars Sauvignon blanc clone UCD1, Riesling clone 239-10, Grüner Veltliner clone UCD1, Gewürztraminer clone GM11, and two rootstocks—Schwarzmann (*V. riparia* × *V. rupestris*) and 41B (*V. vinifera Chasselas* × *V. berlandieri*) were used. The wine grape material was sourced from the collection of New Zealand Winegrowers in Marlborough, New Zealand, and the two rootstocks were sourced from the Wineworx Nurseries Ltd in Longburn, Manawatu, New Zealand.

Initiation of axenic cultures from green shoots of six genotypes

The buds of dormant cuttings were induced to produce new green shoots under greenhouse conditions by holding them in coarse sand in a mist bed with bottom heating to 28 °C. When the green shoots were 20–30 cm long, they were harvested for initiation of in vitro cultures as described by Pathirana and McKenzie (2005). The basal medium comprised half-strength Murashige and Skoog (1962) (MS) macronutrients, MS micronutrients, B5 vitamins (Gamborg et al. 1968) and 58.5 mM sucrose solidified by addition of 3 gl^{-1} Gelrite™. Multiplication medium consisted of basal medium supplemented with 2.22 µM 6-benzylaminopurine (BA) and was used to promote shoot growth from axillary buds.

For all experiments, the pH of culture media was adjusted to pH 5.8 using either NaOH or HCl before

autoclaving the medium for 20 min at 121 °C. In vitro growing plants were multiplied by shoot tip and nodal cuttings, comprising segments with two nodes, at 4-week intervals in basal medium. Cultures were initiated in 9-cm Petri plates holding 20 ml of medium and plantlets were multiplied in 290 ml clear wide-mouth disposable polystyrene tissue culture tubs holding 50 ml of medium. Culture rooms were maintained at 24 ± 1 °C with a 16 h photoperiod and a photosynthetic photon flux of 30 μmol m^{-2} s^{-1} at the top of the culture vessels provided by Phillips cool-white 18 W fluorescent lamps. Following initiation of in vitro cultures, work was carried out under aseptic conditions.

Testing tolerance of *Vitis* apical and axillary buds to vitrification solution

Shoots comprising 3–4 nodal sections were cultured on multiplication medium for 2 weeks. Apical and axillary bud explants from these shoots were harvested and held on sterile tissue paper (Whatman Qualitative Grade 2) laid on fresh plates of basal medium until processing. The explants were prepared by dissecting most of the protective scale leaves from the bud. Both apical and axillary buds were 1–1.5 mm in length. Once all the explant material was prepared, it was immersed in loading solution for 20 min at room temperature; the loading solution comprised a half-strength MS (macro and micro-nutrients) medium supplemented with 2 M glycerol and 0.4 M sucrose. The explants were then immersed in PVS2 solution (15 % w/v ethylene glycol, 15 % w/v DMSO, 30 % w/v glycerol, and 13.7 % w/v sucrose) (Sakai et al. 1990) in MS salts on ice for 20, 30, 40, 50 or 60 min (five treatments). Following PVS2 treatment, explants were removed to recovery solution (comprising 1.2 M sucrose in MS macro and micro salts) at room temperature for 20 min before removal to recovery medium (0.6 M sucrose in MS macro and micro salts solidified with agar) on sterile filter paper in Petri plates and maintained in the dark for 24 h at 24 ± 1 °C. After incubation on recovery medium, the filter papers with explants were removed to regeneration medium that comprised basal medium supplemented with 3 μM BA and 0.05 μM naphthaleneacetic acid. The cultures were maintained in darkness for 1 week before transfer to light. Filter papers with explants were removed to fresh regeneration medium plates at 4- to 6-week intervals. Regeneration was assessed after 16 weeks. Control treatments comprised apical buds and axillary buds not treated with PVS2 solution but maintained on basal medium on ice for the same periods as the material in the treatments and removed to regeneration medium.

Pre-vitrification treatment of plantlets with SA and dehydration of their explants in sucrose

We tested the effect of pre-conditioning plants in SA (four treatments) and pre-treatment of the buds in sucrose prior to PVS2 treatment on plant regeneration after cryopreservation. Plantlets of six accessions were grown on multiplication medium supplemented with four concentrations of SA (0, 0.1, 0.5 and 1 mM) for 2 weeks. Axillary and apical bud explants were then excised and pre-cultured stepwise on basal MS medium supplemented with increasing sucrose concentrations of 0.25, 0.5, 0.75 and 1 M for 4 days.

Based on the results of the PVS2 tolerance assays, the treatment times that gave 50 % survival of both explant types (apical and axillary buds) across all the genotypes were used in droplet vitrification experiments to test the effect of pre-treatment with SA and sucrose. After treating with PVS2 solution in Petri plates on ice, the explants were placed on a drop of PVS2 solution on sterile aluminium foil (8 × 25 mm) and the foil was plunged into LN and transferred to 1.8-ml cryo tubes (Nunc, Roskilde, Denmark) filled with LN. Five explants were used per foil. Explants on aluminium foils were held in LN for a minimum of 60 min before the aluminium foils with explants were removed to recovery solution at room temperature. About 15 ml recovery solution was used for each aluminium foil with explants. After 20 min in recovery solution, individual buds were removed to plates with recovery medium on sterile filter paper in Petri plates and maintained in the dark for 24 h at 24 ± 1 °C. The filter papers with explants were removed to regeneration medium plates after 24 h and then to fresh regeneration medium plates at 4- to 6-week intervals. The cultures were maintained in darkness for 1 week before transfer to light. Regeneration percentages were recorded at 16 weeks. An explant was considered alive and capable of regenerating into a plantlet once it produced 3–4 small leaves.

Two replicates with a minimum of 20 explants per replicate were established over a time period of 8 weeks. The controls consisted of SA- (0.1 mM) and sucrose-treated explants maintained in PVS2 but not immersed in LN (LN control) and explants not treated with SA or sucrose but treated with PVS2 and cryopreserved by droplet vitrification (SA/sucrose control). In addition, in all cryopreservation experiments, a minimum of ten explants per replicate was directly transferred to regeneration medium as a third control to test the regeneration ability of explants in regeneration medium (regeneration control).

Statistical design and analysis

For the PVS2 tolerance assay of six genotypes, a minimum of 18 explants was used per replicate, and two replicates per treatment in a randomised block design were established and analysed as a binomial generalised linear model (GLM) (or logistic regression). The PVS2 exposure time was treated as a continuous variable. Axillary and apical bud explants were used as separate treatments.

Regeneration after cryopreservation of explants treated with four SA levels for six genotypes was also analysed using binomial GLM. Thereafter, modelling was carried out to estimate the number of explants that would be needed to be cryopreserved in each genotype to ensure that it would be reliable for long-term conservation of *Vitis* germplasm. Sample size calculations were made using binomial distribution, with a proportion obtained from optimal treatment (0.1 mM SA treatment), and searching for a sample size that gave at least one survivor 95 % of the time. Similar calculations were made for the sample size that gave at least one survivor 99 % of the time. Following the method of Dussert et al. (2003), the calculations were also made using the lower 95 % Wilson score continuity-corrected confidence interval limit (Pires and Amado 2008) for the proportion of surviving explants. The statistical software GenStat 17th edition (VSN International) was used for all analyses.

Results

Survival of apical and axillary buds of *Vitis* genotypes after PVS2 treatment

A direct relationship between plant regeneration and duration of exposure of explants to PVS2 solution ($P < 0.001$—Table 1) was observed; regeneration rates decreased with increasing time of exposure to PVS2 solution (Fig. 1) and there were highly significant differences between genotypes (Table 1). Axillary buds showed higher regeneration rates after PVS2 treatment than apical buds (Fig. 1; Table 2) and this difference was also highly significant (Explant, Table 1). All the interaction effects were not significant except the interaction of explant with duration in PVS2 solution that was highly significant (Table 1).

Across the range of genotypes tested, the PVS2 exposure time that gave 50 % regeneration was 42.6 min for axillary buds and 35.6 min for apical buds (Table 2). Considering the significance of differences in survival of the two explants after exposure to PVS2 solution (Table 1), these results were used as the basis for subsequent droplet vitrification experiments using pre-treatment of donor plantlets with SA.

Table 1 Analysis of deviance of regeneration data of apical and axillary buds (explants) of six grape genotypes after five PVS2 treatment periods fitted to a binomial generalised linear model (or logistic regression)

Factor	*P* value
Duration[a]	<0.001
Genotype	0.008
Explant	<0.001
Genotype × explant	0.759
Duration × genotype	0.427
Duration × explant	0.006
Duration × genotype × explant	0.737

The PVS2 exposure duration (from 20 to 60 min at 10-min intervals) was treated as a continuous variable

[a] Linear trend on the logit scale

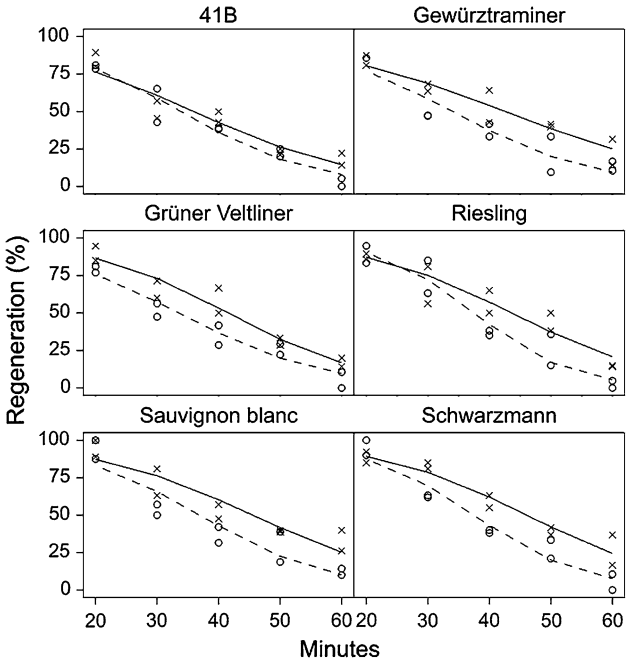

Fig. 1 Percentage of grapevine apical (*circles*, *broken line*) and axillary buds (*crosses*, *solid line*) regenerated after treatment with PVS2 solution for different periods. The buds were immersed in loading solution for 20 min and removed to PVS2 solution and maintained for 20, 30, 40, 50 or 60 min before removal to recovery solution (20 min) and then to recovery plates (24 h) and regeneration media. Regeneration percentages were recorded after 16 weeks

Effect of salicylic acid and sucrose pre-treatments on plant regeneration after cryopreservation of *Vitis*

Survival of buds from the SA-treated plantlets following cryopreservation was significantly influenced by genotype and SA concentration (both $P < 0.001$) with a highly significant genotype × SA interaction also apparent (Table 3). There was no significant effect of explant (i.e.,

Table 2 Estimated duration (min) in PVS2 solution for 50 % regeneration of apical and axillary buds of six grape genotypes

Genotype	Apical buds		Axillary buds	
	Estimated min	95 % CI[a]	Estimated min	95 % CI
41B	33.9	(29.7, 37.5)	36.0	(30.6, 40.5)
Gewürztraminer	34.0	(29.4, 37.9)	42.6	(37.5, 47.7)
Grüner Veltliner	33.5	(28.6, 37.5)	41.5	(37.6, 45.6)
Riesling	37.6	(34.4, 40.6)	43.6	(39.5, 47.8)
Sauvignon blanc	36.9	(32.9, 40.8)	45.5	(41.0, 51.3)
Schwarzmann	37.5	(34.1, 40.8)	46.1	(41.9, 51.2)
Mean	35.6		42.6	
Standard error	0.7		0.8	

The buds were immersed in loading solution for 20 min and removed to PVS2 solution and maintained for 20, 30, 40, 50 or 60 min before removal to recovery solution (20 min) and then to recovery plates (24 h) and regeneration media

Regeneration percentages were recorded after 16 weeks

[a] *CI* confidence interval

Table 3 Analysis of deviance results from a binomial generalised linear model on survival of apical and axillary buds (explant) of six grapevine genotypes cryopreserved by droplet vitrification using PVS2 treatment period corresponding to the predicted 50 % regeneration

Factor	*P* value
Genotype	<0.001
Explant	0.77
Salicylic_acid	<0.001
Genotype × explant	0.28
Genotype × salicylic_acid	0.013
Explant × salicylic_acid	0.62
Genotype × explant × salicylic_acid	0.31

Plantlets were grown on basal media supplemented with four salicylic acid concentrations for 2 weeks before harvest of explants for the experiment

apical and axillary buds) either alone or in interaction with the other treatments (all $P \geq 0.28$, Table 3)—we used 50 % survival data from PVS2 tolerance assay of the two explants for PVS2 treatment before cryopreservation. Therefore, the data for the two explant types were pooled for further analysis and results for each genotype and SA concentration are shown in Fig. 2. Regeneration of untreated explants of each genotype ranged between 67 and 100 %, the lowest being the rootstock 41B (Fig. 2). Treatment with SA (0.1 mM), sucrose and PVS2, without cryopreservation in LN, significantly reduced regeneration of explants (from 8 % in 41B to 60.81 % in Riesling). Cryopreservation following that treatment further reduced regeneration of each genotype (from 7 % in 41B to 45 % in Riesling). Among SA treatments tested, 0.1 mM SA provided the highest regeneration of cryopreserved explants and further increases in SA concentration were accompanied by diminishing regeneration. This effect was especially evident in Riesling and Gewürztraminer (Fig. 2); however, it was apparent in other varieties as well. Omission of SA, or SA plus sucrose treatment before cryopreservation resulted in the lowest regenerations of each genotype (from 0 % in 41B to 13 % in Schwarzmann). Over all genotypes, regeneration of cryopreserved explants treated with sucrose alone was little different from those without both SA and sucrose treatment; thus indicating that sucrose had little influence on regeneration on its own. The cryopreserved buds of rootstock 41B could be regenerated, albeit at low frequency, only after SA and sucrose pre-treatments (Fig. 2). The differences in regeneration after PVS2 treatment (LN control) were significant only for genotypes ($P < 0.001$), but not for explant type ($P = 0.97$) or the interaction of genotype × explant type ($P = 0.40$).

Several days after cryopreservation, surviving apical and axillary buds were green, whereas those that did not survive had a bleached appearance (Fig. 3). After cryopreservation, regenerating explants were chlorotic and the bases were blackened in contrast to LN control material (Fig. 4). Regrowth of the plantlets regenerated after cryopreservation was slower than the plantlets from control cultures. After subculture they grew normally.

Modelling of cryopreservation reliability

Assuming a binomial distribution of plant survival, the number of explants that need to be cryopreserved to achieve a 95 % probability of at least one surviving plant varied from 6 to 43 (Table 4). When the most sensitive to PVS2 rootstock 41B is excluded this probability ranges from 6 to 11. Using lower 95 % confidence interval, these probabilities range from 8 to 20 (Table 4).

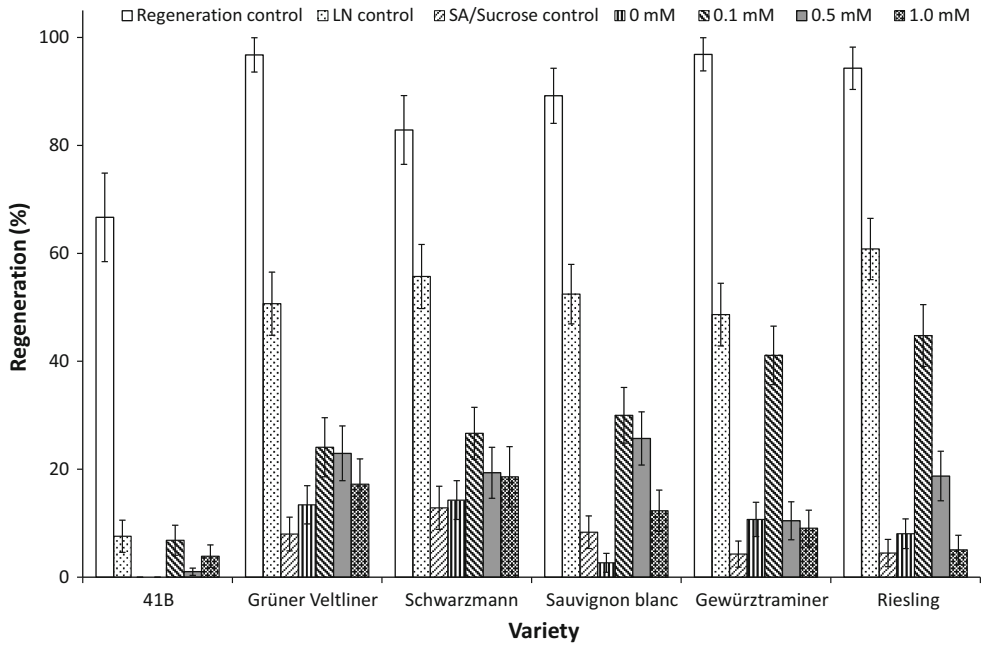

Fig. 2 Plant regeneration (%) of six grapevine genotypes cryopreserved by droplet vitrification after pre-treatment of donor plants for 2 weeks with 0, 0.1, 0.5 or 1.0 mM salicylic acid (SA) followed by stepwise pre-culture of explants on basal MS media supplemented with increasing sucrose concentrations of 0.25, 0.5, 075 and 1 M for 4 days. Regeneration control represents regeneration (%) of explants plated directly on regeneration media without any treatment. LN control represents regeneration (%) of explants from plantlets grown on MS media supplemented with 0.1 mM SA for 2 weeks followed by stepwise pre-culture of explants on basal MS media supplemented with increasing sucrose concentrations of 0.25, 0.5, 075 and 1 M for 4 days, treated with PVS2 and transferred to recovery media, recovery plates and regeneration media without freezing in LN. SA/Sucrose control represents regeneration (%) of explants cryopreserved without pre-treatment in SA or sucrose. Data were pooled for explant type (apical and axillary buds). Apical buds were treated in PVS2 for 36 min and axillary buds for 43 min. Bars represent standard error of the mean. For 41B, 0 mM SA and SA/Sucrose Control have 0 values

Discussion

Significantly higher tolerance of axillary buds to PVS2-induced dehydration compared with apical buds in our study is interesting and intriguing, and is the first such report to our knowledge. Similar to grapevines growing in the field, tissue culture-established plantlets have an apical dominance with axillary buds showing signs of dormancy. Multiple growth factors have been implicated in the control of bud dormancy status. Localised biochemical and physiological changes within vascular and meristematic tissues around the bud underlie bud dormancy development in woody plants and the differences in tolerance to PVS2 observed in the two types of explants in the present study could be partly explained by these differences. Wang et al. (2000) showed that water content of grapevine buds at the time of cryopreservation by encapsulation method is critical for their survival. Axillary buds can be different from apical buds not only in their metabolic profiles, but also in water content. Fennell and Line (2001) determined that water content rapidly decreases in the axillary bud and in vascular stem tissues associated with it during dormancy induction in *Vitis riparia*. Using magnetic resonance microimaging, Kalcsits et al. (2009) showed that apparent water diffusion coefficient measurements in axillary buds of *Populus* spp. had a higher correlation with dormancy induction than with vascular tissue measurements, indicative of greater water movement in the buds.

While plant hormones are involved directly in the control of dormancy, limiting sucrose availability to axillary buds has been shown to be central to the maintenance of apical dominance (Mason et al. 2014). Therefore, higher sucrose content in the actively growing apical buds could be another reason for the shorter period of dehydration in PVS2 required by them compared with axillary buds, to achieve the same degree of dehydration and thus survival in PVS2.

Significant genotypic differences in response to dehydration in grapevine buds similar to that found in the present research has been observed by Wang et al. (2000) in the two cultivars they studied. These differences observed in the sensitivity of genotypes and two types of explants to dehydration in our research suggested a need to work with a range of genotypes to understand the response of this diverse genus to dehydration and cryopreservation. In addition to wine grape varieties, two rootstocks were included in trials, including 41B, which is considered generally difficult to culture in vitro (Goebel-Tourand et al.

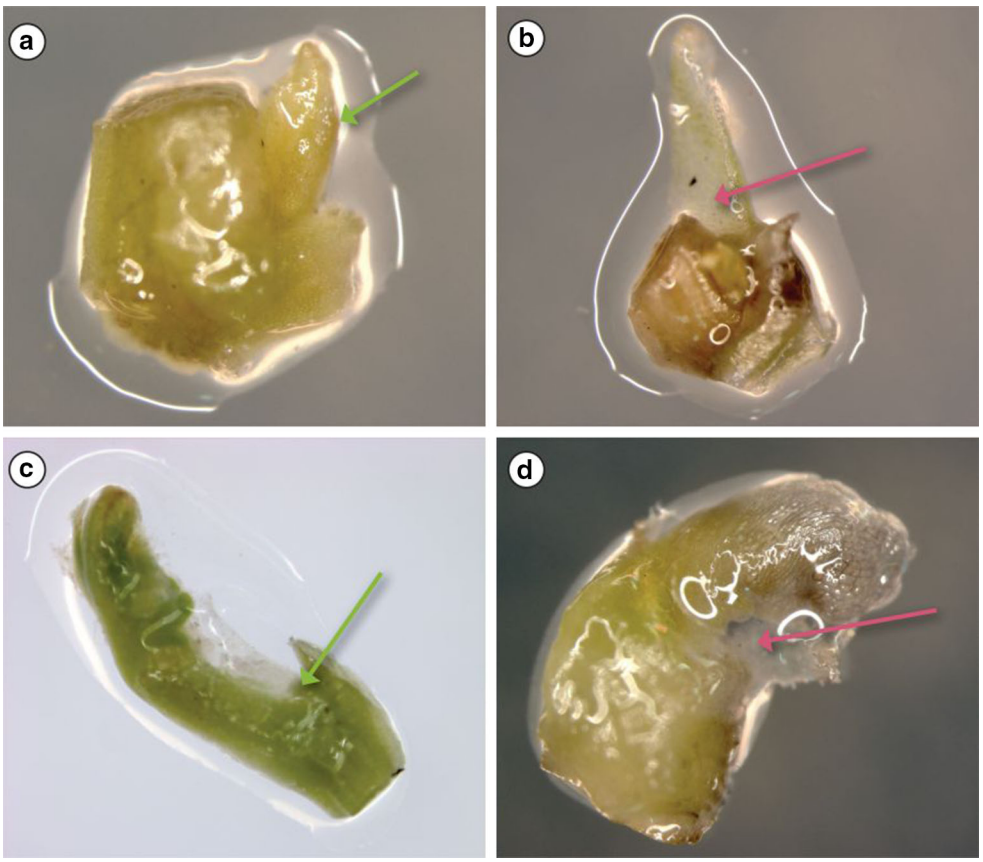

Fig. 3 Surviving grapevine axillary buds (**a**) and apical buds (**c**) after droplet vitrification were more solid and *greener* at the meristem (*green arrows*), whereas dead tissue was either transparent or *white* (*red arrows*), axillary bud (**b**), apical bud (**d**)

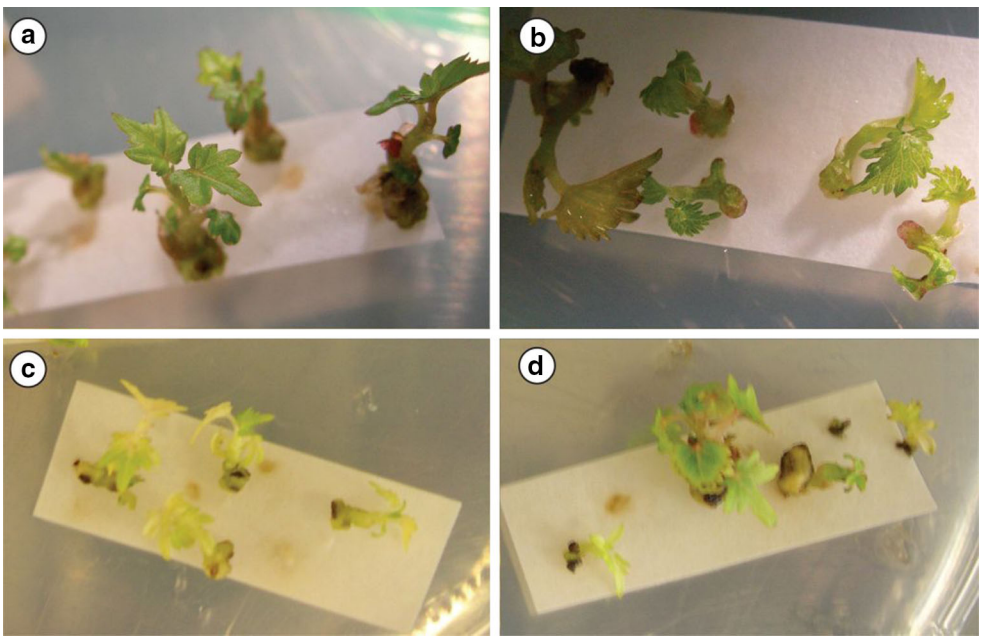

Fig. 4 Plant regeneration in LN control (Grüner Veltliner—**a**, Sauvignon blanc—**b**) and after droplet vitrification (Grüner Veltliner—**c**, Sauvignon blanc—**d**). Photos taken after 12 weeks on regeneration media

Table 4 Mean regeneration percentages of six grapevine accessions after droplet vitrification and predicted numbers of explants to be cryopreserved to ensure a 95 or 99 % reliability of recovering at least one plant

Genotype	Regeneration (%)	Probability	
		95 %	99 %
41B	6.8 (2.5)	43 (117)	66 (179)
Grüner Veltliner	24.0 (13.9)	11 (20)	17 (31)
Schwarzmann	26.7 (17.4)	10 (16)	15 (25)
Sauvignon blanc	30.0 (19.9)	9 (14)	13 (21)
Gewürztraminer	41.3 (29.9)	6 (9)	9 (13)
Riesling	44.7 (32.4)	6 (8)	8 (12)

Calculations were based on the treatment giving the highest regeneration percentage (0.1 mM salicylic acid)

Values in brackets are the lower 95 % CI limit for the percentage regeneration and the corresponding predicted numbers of required explants at 95 and 99 % probability

1993). We used the predicted duration in PVS2 solution that resulted in 50 % regeneration to treat apical (36 min) and axillary (43 min) buds for the cryopreservation experiments. We assumed that a sample that results in 50 % regeneration of explants received enough dehydration in PVS2 in order to vitrify upon rapid cooling and used these predicted times for PVS2 treatment. As a result, the effect of explant could be disregarded, indicating that it is possible to optimise the treatment times for axillary and apical buds. Since we used 50 % regeneration from PVS2 toxicity assay as the basis for PVS2 treatment in cryopreservation experiment, none of the accessions was expected to exceed 50 % regeneration after LN treatment.

The physiological condition of the explants at the time of cryopreservation is also crucial for regeneration (Johnston et al. 2007; Marković et al. 2014). Some *Vitis* genotypes have proven recalcitrant to cryopreservation (Ganino et al. 2012). The introduction of SA pre-treatment of plantlets and high sucrose prior to PVS2 treatment of the buds was designed to increase tolerance to desiccation imparted by PVS2 solution as well as freezing tolerance. High sucrose concentrations in media have proven useful when explants have been sensitive to different methods of desiccation before cryopreservation (Benelli et al. 2013; Johnston et al. 2007). Lynch et al. (2011) suggested that oxidative processes may influence regrowth after cryopreservation and that optimal pre-treatments could, in part, increase tolerance by an overall enhancement of endogenous antioxidants, particularly glutathione reductase, proline and sugars. In *Ribes* spp. tolerance imparted by sucrose-simulated cold acclimation was associated with greater increases in hydroxyl radical activity, antioxidant status, phenolic accumulation, anthocyanin pigmentation, and protein SH group status (Johnston et al. 2007).

SA is a known elicitor of defence proteins in plants in response to both abiotic (Senaratna et al. 2000; Stevens et al. 2006) and biotic (Repka 2001) stresses and is a central component in growth signalling pathways (Taşgın et al. 2006; Li et al. 2011). It was the best elicitor of defence responses among 14 elicitors tested by Repka (2001). There is increasing evidence that SA can enhance tolerance to chilling (Chen et al. 2011; Sayyari 2012; Wang et al. 2009b) and freezing (Li et al. 2011) in plants. SA-induced tolerance to chilling and freezing stress in plants is achieved through the increased activity of anti-oxidative enzymes such as superoxide dismutase (Li et al. 2011), catalase, peroxidase, polyphenol oxidase (Taşgın et al. 2006; Mutlu et al. 2013), ascorbate peroxidase (Chen et al. 2011) as well as phenylalanine ammonia-lyase (Cao et al. 2009) through increased transcript levels of stress-responsive genes (Chen et al. 2011; Dong et al. 2014). The activity of these enzymes results in the inhibition of lipid peroxidation (Sayyari 2012), decreased levels of malondialdehyde (an oxidative damage marker) and electrolyte leakage (Chen et al. 2011; Li et al. 2011). In the current study, 2 weeks of culture in SA-supplemented media, particularly at 0.1 mM, considerably improved regeneration after cryopreservation of grapevine buds. Bernard et al. (2002) tested the effect of incorporating 0, 0.05 and 0.2 mM SA in alginate beads before cryopreservation of *Melia azedarach* embryonic axes. They reported a significant increase in plant regeneration in the treatment with 0.2 mM SA, but not with 0.05 mM SA. Exogenous application of 0.1 mM SA to 7-day-old seedlings of barley before applying cold stress increased the activity of apoplastic antioxidative enzymes, de novo synthesis of proteins and ice nucleation resulting in improved protection from cold stress (Mutlu et al. 2013). The higher concentrations of SA (0.5 and 1 mM) tested in the present research resulted in lower regeneration than 0.1 mM concentration. Plants' reaction to cold stress seems to differ according to the level of exogenous SA applied. For example, application of 0.01 mM SA reduced apoplastic catalase activity in winter wheat under cold stress (Taşgın et al. 2006), whereas it increased in barley when applied at 0.1 mM (Mutlu et al. 2013). These results are indicative of a presence of a dose-dependent response in signalling pathways to exogenously applied SA in plants.

Sucrose pre-treatment of explants without growing source plantlets in SA-supplemented media did not result in an increase in plant regeneration after cryopreservation in our research. Plant regeneration after droplet vitrification increased significantly in all genotypes only when the buds from plantlets cultured in SA-supplemented media (0.1 mM SA in particular) were subjected to serial dehydration in sucrose. The slower re-growth after cryopreservation observed in our study conforms to the observations by Zhao et al. (2001). The slower recovery of plantlets after cryopreservation can be attributed to the fact that only the apical

dome with cytoplasmic cells can survive the dehydration treatment and subsequent freezing (Wang et al. 2009a).

In our research, a strong genotype effect in response to droplet vitrification was noted, with rootstock 41B showing the least regeneration potential. It is interesting that a rootstock with a similar genetic background to 41B, Kober 5BB (both have *V. berlandieri* as a parent), was shown to be recalcitrant to vitrification-based cryopreservation because a 30-min exposure of shoot tips to PVS2 was not optimal for shoot tip dehydration, and longer exposure was toxic (Ganino et al. 2012). In our research, the combination of SA with sucrose pre-treatment resulted in regeneration of 41B rootstock after droplet vitrification, albeit at low rates, whereas previous attempts failed. This gives confidence that the SA pre-treatment of donor plants could be a critical step in droplet vitrification of *Vitis* and it would be interesting to test this with Kober 5BB, which has so far been recalcitrant to cryopreservation (Ganino et al. 2012).

We applied probabilistic tools to ensure a minimum regeneration rate, giving confidence that post-storage regeneration can be accurately predicted. We predict that in *V. vinifera* genotypes studied, storage of 20 explants cryopreserved by droplet vitrification will ensure regeneration of at least one plant at 95 % probability, and 31 explants would increase that to 99 %. We used the lower confidence interval to predict these values as suggested by Dussert et al. (2003) for use in cryobanking. However, further research is required to improve plant regeneration in genotypes that have lower regeneration levels such as 41B rootstock.

Author contribution statement RP, BP and FC planned and conducted the research, DH and AM analysed the data. All authors contributed to the writing of manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interests.

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