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Cryopreservation of grapevine (*Vitis vinifera* L.) *in vitro* shoot tips

Research Article

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Abstract: In this work, we compared the efficiency of encapsulation-dehydration and droplet-vitrification techniques for cryopreserving grapevine (*Vitis vinifera* L.) cv. Portan shoot tips. Recovery of cryopreserved samples was achieved with both techniques; however, droplet-vitrification, which was used for the first time with grapevine shoot tips, produced higher regrowth. With encapsulation-dehydration, encapsulated shoot tips were precultured in liquid medium with progressively increasing sucrose concentrations over a 2-day period (12 h in medium with 0.25, 0.5, 0.75 and 1.0 M sucrose), then dehydrated to 22.28% moisture content (fresh weight). After liquid nitrogen exposure 37.1% regrowth was achieved using 1 mm-long shoot tips and only 16.0% with 2 mm-long shoot tips. With droplet-vitrification, 50% regrowth was obtained following treatment of shoot tips with a loading solution containing 2 M glycerol + 0.4 M sucrose for 20 min, dehydration with half-strength PVS2 vitrification solution (30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% dimethylsulfoxide and 0.4 M sucrose in basal medium) at room temperature, then with full strength PVS2 solution at 0°C for 50 min before direct immersion in liquid nitrogen. No regrowth was achieved after cryopreservation when shoot tips were dehydrated with PVS3 vitrification solution (50% (w/v) glycerol and 50% (w/v) sucrose in basal medium).

Keywords: *Encapsulation-dehydration • Droplet-vitrification • Shoot tip size*

1. Introduction

Grapevine is one of the economically most important fruit worldwide [1]. Its long cultivation has resulted in the development of large numbers of cultivars adapted to a wide diversity of climates. Many grapevine cultivars are now endangered and international efforts aiming at preserving grapevine biodiversity have been undertaken. Although field collections play a preeminent role in grapevine conservation programmes, maintenance of plant genetic resources only in field collections is risky, as valuable germplasm can be lost because of pests, diseases and various calamities such as adverse weather conditions [2,3]. Today, biotechnology offers a broad range of techniques, which allow optimizing plant genetic resource conservation.

Among these techniques, cryopreservation (liquid nitrogen [LN], -196°C) is a highly suitable and efficient tool for long-term storage of plant germplasm, requiring minimum space and maintenance [2]. The cryopreservation protocols developed recently do not require controlled cooling, thereby allowing cells and shoot tips to be cryopreserved by direct transfer to LN. The development of simple and reliable cryopreservation methods should allow much borader use of cryopreserved cultured cells, shoot tips and somatic embryos [4]. Among the techniques developed, encapsulation-dehydration, vitrification and droplet-vitrification are the most frequently used for cryopreservation of shoot tips.

Encapsulation-dehydration is based on the technology developed for producing artificial seeds

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[5]. Explants are encapsulated in calcium alginate beads (usually 3% w/v), osmotically dehydrated in liquid medium enriched with sucrose at concentrations between 0.75 and 1.25 M for 1 to 7 days, then physically dehydrated to moisture contents (MC) around 20% (fresh weight basis) and cooled rapidly.

Vitrification involves osmotic dehydration of shoot tips with cryoprotectants. Shoot tips are treated with a loading solution with intermediate concentration (usually 2 M glycerol + 0.4 M sucrose, [6]), then dehydrated with highly concentrated vitrification solutions (total molarity around 5-7 M), cooled and rewarmed rapidly; cryoprotectants are removed by placing shoot tips in an unloading solution (containing 0.8-1.2 M sucrose) and apices are transferred on recovery medium for regrowth. The most commonly employed vitrification solutions are the so-called Plant Vitrification Solutions PVS2 [7] and PVS3 [8] developed by the group of the late Prof. Sakai in Japan.

Droplet-vitrification is the latest technique developed [9]. In a droplet-vitrification protocol, shoot tips are first treated with a loading solution, then with a vitrification solution, placed on aluminium foils in minute droplets of vitrification solution and cooled rapidly in LN. The high recovery percentages achieved with this technique are due to the very high cooling and rewarming rates achieved because of the direct contact between samples and LN during cooling, and between samples and the unloading solution during rewarming. There is an increasing number of species to which droplet-vitrification has been successfully applied [4].

The availability of an efficient and reliable cryogenic protocol yielding a high recovery percentage is a prerequisite for large scale, routine application of cryopreservation [10].

Although cryopreservation research has been initiated over 20 years ago on grapevine, no such routinely applicable protocol is yet available. Different cryopreservation techniques have been tested with grapevine *in vitro* shoot tips. Grapevine apices sampled from *in vitro* plantlets have been cryopreserved using encapsulation-dehydration, with recovery ranging between 24 and 40% [11,12] or 40-60% [13]. Zhao *et al*. [14] cryopreserved shoot tips of three grape cultivars using the encapsulation-dehydration technique combined with a slow cooling-dehydration to -40ºC. Recovery ranged between 15% and 40%. The vitrification technique has been successfully employed with *in vitro* shoot tips of four grapevine cultivars [15]. In these experiments, the average recovery ranged from 47% to 85%. This vitrification protocol was improved by incorporating a two-step dehydration procedure and successfully applied to ten *Vitis* cultivars or species, with shoot recovery ranging between 60% and 80% [10].

The objective of this study was to compare the efficiency of two techniques (encapsulation-dehydration and droplet-vitrification) for cryopreservation of *in vitro* shoot tips of the grapevine cultivar Portan. This was the first time that droplet-vitrification was tested on grapevine shoot tips. The effect of several parameters of these protocols was also studied, including sucrose pretreatment, bead MC, and size of explants for encapsulation-dehydration and nature of the vitrification solution and duration of exposure to the vitrification solution for droplet-vitrification.

2. Experimental Procedures

2.1 Plant material

The plant material employed in this study consisted of *in vitro* plants of grapevine (*Vitis vinifera* L.) cultivar Portan. These cultures were initially established from field-grown plants in the grape germplasm collection of the Institut national de la recherche agronomique (INRA), Vassal (France).

2.2 *In vitro* culture

Grape *in vitro* plantlets were cultured on basal medium (BM) composed of half-strength MS [16] mineral elements with Morel's vitamins [17], 3% sucrose and 0.7% agar (Sigma) at pH 5.8. They were cultured at 24±2°C under a 12 h light/12 h dark photoperiod with a light intensity of 40 µE m^{-2} s^{-1} provided by cool white fluorescent tubes. *In vitro* mother-plants were kept without subculture for 2 months to reach a length of approximately 12 cm before use for cryopreservation experiments. *In vitro* plantlets were cut into single node microcuttings of approx. 1.5 cm in length (Figure 1), which were transferred to 9 cm Petri dishes (20 microcuttings/Petri dish) and placed on ½ MS medium containing 20 g/l sucrose, 7 g/l microagar and 1 µMol zeatin riboside (ZR). Petri dishes were placed in the environmental conditions described above. Shoot tips were excised from microcuttings after 2 weeks and used for cryopreservation experiments.

2.3 Cryopreservation

2.3.1 Encapsulation-dehydration (ED)

The standard ED protocol was the following. Excised shoot tips (1 mm long, Figure 1b) were suspended in half-strength MS medium devoid of calcium, supplemented with 3% (w/v) Na-alginate (low viscosity, Sigma A2158), 2 M glycerol and 0.4 M sucrose, according to Wang *et al*. [13]. The mixture with the shoot tips was dispensed dropwise with a sterile pipette into a 0.1 M $CaCl_2$ solution containing 2 M glycerol and 0.4 M sucrose at room temperature for 30 min, to form beads (about

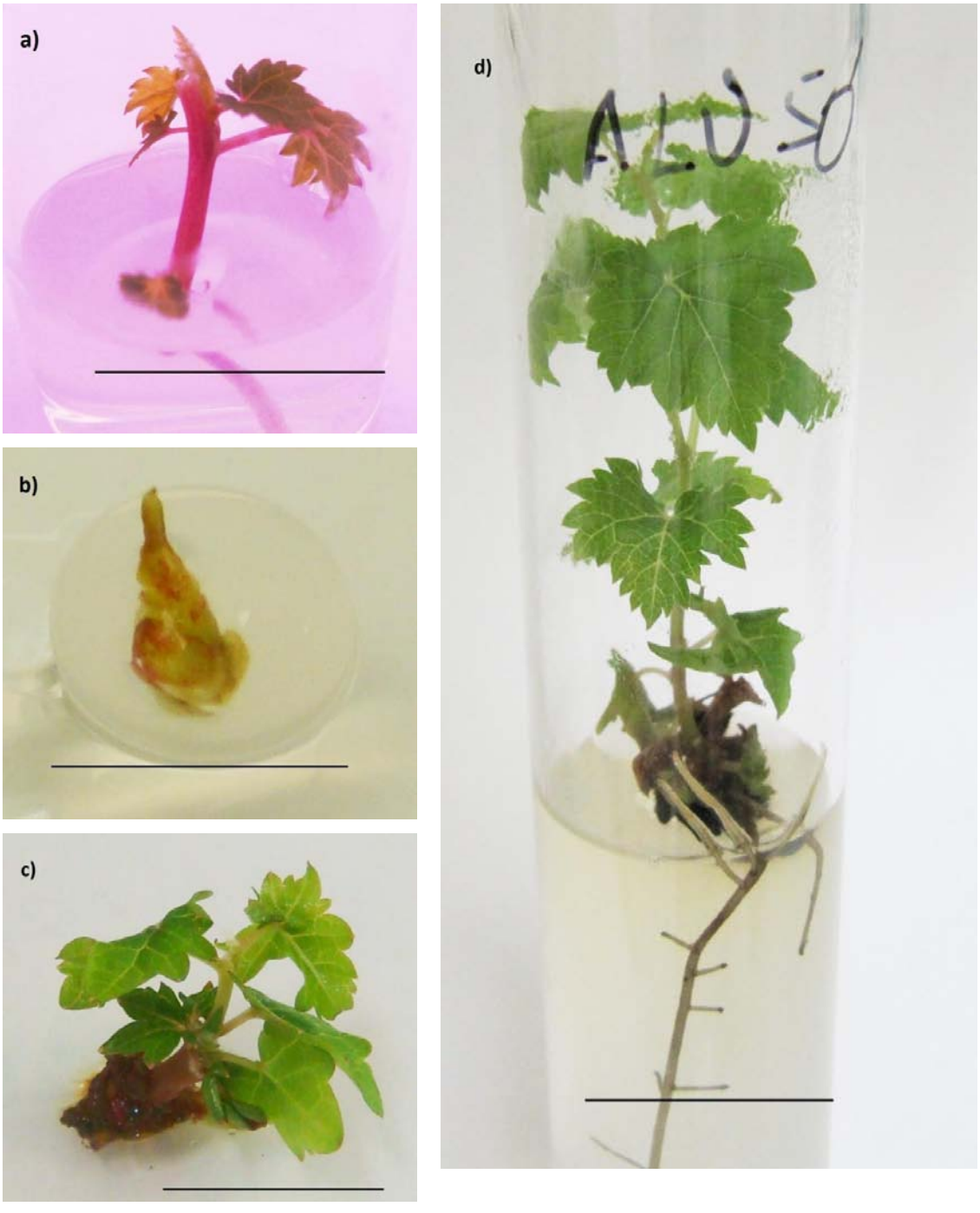

Figure 1. Successive steps of grape shoot tip cryopreservation using ED and DV. (a) One node microcutting before dissection of shoot tip (bar 10.0 mm). (b) Surviving shoot tip cryopreserved by ED (dehydration to 22.3% MC) 2 weeks after rewarming (bar 4.0 mm) (c) Recovery of shoot tip cryopreserved by DV (75 min PVS2 exposure) 6 weeks after rewarming (bar 10.0 mm). (d) Regenerated plant eight months after cryopreservation by DV within (50 min PVS2 exposure) (bar 10.0 mm).

4 mm in diameter, Figure 1c), each bead containing one shoot tip. The beads were precultured over a period of 2 days in 250 ml Erlenmayer flasks in liquid (100 ml) BM with progressively increasing (every 12 h) sucrose concentrations (0.25 M, 0.5 M, 0.75 M and 1.0 M sucrose). Beads were afterwards dehydrated with silica gel (10 beads above 80 g silica gel in 120 ml flasks) to 22.28% MC (fresh weight basis), which was achieved after 4 h. Dehydrated beads were placed in cryotubes and directly immersed in LN. Encapsulated shoot tips

subjected to dehydration alone or to dehydration and LN exposure were post-cultured on BM containing 1 µM benzyladenine (BA), maintained in the dark at 26°C for 7 days and then transferred to the conditions described for stock cultures.

Two experiments were performed with ED. In the first experiment, we studied the effect of bead MC on recovery of grapevine shoot tips. After preculture as described above, encapsulated shoot tips were dehydrated to 16.16%, 20.26% and 22.28% MC (achieved after 4, 5 and 6 h, respectively), immersed, or not, in LN and transferred to recovery medium. In the second experiment, we investigated the effect of explant size on recovery. The standard ED protocol was thus applied to 1 and 2 mm long shoot tips.

2.3.2 Droplet-vitrification

Excised shoot tips (1 mm long, Figure 1b) were precultured on solid ½ MS medium with 0.1 M sucrose for 24 h. Precultured shoot tips were then treated with a loading solution (LS) containing 2 M glycerol + 0.4 M sucrose in MS medium [18] for 20 min at room temperature. Shoot tips were dehydrated with half-strength PVS2 or PVS3 at room temperature for 30 min, then with full strength PVS2 at 0°C or PVS3 at room temperature. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), 15% dimethylsulfoxide (DMSO) and 0.4 M sucrose in MS medium [7] and PVS3 50% (w/v) glycerol and 50% (w/v) sucrose in basal medium [8]. Shoot tips were then placed on aluminium foils in 5 µl droplets of PVS2/PVS3 and immersed in LN for at least 1 h. For rewarming, the aluminium foils with the shoot tips were immersed for 20 min in unloading solution containing 1.2 M sucrose at room temperature [19], then transferred to recovery medium, as described for ED.

Two experiments were performed with DV. In the first experiment, we studied the effect of duration of exposure to PVS2 on recovery of grapevine shoot tips. Shoot tips were treated with PVS2 for 25, 50 and 75 min, immersed, or not, in LN, treated with unloading solution and transferred to recovery medium. In the second experiment, we compared the effect of treatment with PVS2 and PVS3 on recovery of grapevine shoot tips. Shoot tips were treated with PVS2 at 0°C for 40, 80 and 120 min or with PVS3 at room temperature for 40, 80 and 120 min immersed, or not, in LN, treated with unloading solution and transferred to recovery medium.

2.4 Assessment of survival and regrowth and statistical analyses

Survival was evaluated 2 weeks after cryopreservation by counting the number of shoots that showed any type of growth, while regrowth was defined as the development of apices into shoots with expanded leaves 8 weeks after rewarming. Both survival and regrowth percentages were expressed relative to the total number of shoot tips treated. Experiments were replicated twice, with 10 shoot tips per experimental condition. Survival and regrowth data, presented as mean percentages with standard error of the mean (SD) were subjected to arcsine transformation. Statistical differences between mean values of all parameters were assessed by analysis of variance (ANOVA) and Duncan's Multiple Range Test for mean separation.

3. Results

3.1 Encapsulation-dehydration

In the first experiment, we studied the effect of bead MC on survival and recovery of grapevine shoot tips cryopreserved using the standard ED protocol. Survival and regrowth of shoot tips were high after sucrose pretreatment (Table 1). Survival and regrowth of dehydrated shoot tips decreased in line with decreasing MCs, from 90.0% (survival) and 40.0% (regrowth) at 22.28% MC to 30.0% (survival) and 10% (regrowth) at 16.16% MC. After cryopreservation, survival and regrowth were low, reaching similar values for the three MCs tested, *i.e.* 20-22% for survival and 10.0% for regrowth.

In the second experiment, we studied the effect of explant size on survival and recovery of grapevine shoot tips. After sucrose pretreatment, survival and regrowth of smaller (1 mm) and larger (2 mm) shoot tips were comparable (Table 2). After dehydration, survival of larger shoot tips was higher compared to smaller explants; however, regrowth reached similar values for explants of both sizes. After cryopreservation, survival and regrowth were higher for smaller explants, reaching 45.5% (survival) and 37.1% (regrowth) for 1 mm-long shoot tips, against 24.0% (survival) and 16.0% (regrowth) for 2 mm-long shoot tips.

3.2 Droplet-vitrification

Loading and treatment of shoot tips for 30 min with half-strength PVS2 had no significant effect on their survival and regrowth (Table 3). Increasing the duration of dehydration with PVS2 decreased survival and regrowth from 60% for 25 min treatment to 30% after 75 min. After LN exposure, survival decreased from 70% for 25 min treatment to 30% after 75 min, while regrowth was 40% for 25 min treatment, 50% for 50 min and 30% for 75 min treatment.

In the second experiment, we compared the effect of shoot tip dehydration with PVS2 and PVS3 vitrification

Treatment	Survival (%±SD)		Regrowth (%±SD)	
	-LN	+LN	-LN	+LN
Sucrose pretreatment	100.0±0a	-	75.0±35.3a	-
Dehydration to 22.28% MC	90.0±14.1ab	20.0±0c	40.0±0ab	10.0±14.1b
Dehydration to 20.26% MC	70.8±5.9b	32.5±10.6c	29.2±5.9b	10.0±14.1b
Dehydration to 16.16% MC	30.0±14.1c	20.0±0c	10.0±14.1b	10.0±14.1b

Table 1. Effect of bead MC (%, FWB) on survival and regrowth (%) of grapevine cv. Portan shoot tips after sucrose pretreatment, dehydration (-LN) and cryopreservation (+LN) using the standard ED protocol.

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

Size of explants	Survival (%±SD)				Regrowth (%±SD)			
	1 mm		2 mm		1 mm		2 mm	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
Sucrose pretreatment	100.0±0a	-	95.8±5.9a	-	75.0±35.4a	-	66.7±23.6a	-
22,3% beads MC	30.0±14.1b	45.5±5.4ab	73.3±18.9ab	24.0±1.4b	16.7±4.7a	37.1±4.2a	11.7±2.4a	16.0±0.9a

Table 2. Effect of explant size on survival and regrowth (%) of grapevine cv. Portan shoot tips after sucrose pretreatment, dehydration (-LN) and cryopreservation (+LN) using the standard ED protocol.

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

Treatment	Survival (%±SD)		Regrowth (%±SD)	
	-LN	+LN	-LN	+LN
Loading	100.0±0a	-	100.0±0a	-
½ PVS2 /30 min	100.0±0a	-	83.03±23.6ab	-
PVS2 /25 min	60.0±0b	70.0±14.1b	60.0±28.3bc	40.0±0d
PVS2 /50 min	50.0±14.1bc	50.0±14.1bc	40.0±0cd	50.0±14.1bcd
PVS2 /75 min	30.0±14.1c	30.0±14.1c	30.0±14.1cd	30.0±14.1cd

Table 3 Effect of duration of dehydration with PVS2 solution on survival and regrowth (%) of non-cryopreserved (-LN) and cryopreserved (+LN) grapevine cv. Portan shoot tips.

In columns, mean values for survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

solutions. PVS3 was highly toxic since survival and regrowth were only 10% after 40 min treatment and were nil for longer durations (Table 3). No survival was achieved after LN exposure following PVS3 treatment. By contrast, with the PVS2 vitrification solution, survival and regrowth were 30% after 40 min treatment and low survival and regrowth (10.0%) were still noted after 120 min treatment. After cryopreservation, survival and regrowth were achieved only following a 40 min PVS2 treatment, with values reaching 40 % for both survival and regrowth.

4. Discussion

In the present work, we compared the efficiency of ED and DV techniques for cryopreserving shoot tips of grapevine cv. Portan. Recovery of cryopreserved samples was achieved with both techniques; however, DV, which was used for the first time with grapevine shoot tips, produced higher regrowth. Fifty percent regrowth was obtained with DV, following 50 min exposure of shoot tips to PVS2. No regrowth was achieved following treatment of shoot tips with PVS3 vitrification solution.

	Survival (%±SD)				Regrowth (%±SD)			
	PVS2		PVS3		PVS2		PVS3	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
Loading control	70.0±14.1b	-	70.0±14.1a	-	70.0±14.1a	-	70.0±14.1a	-
½ PVS/30 min	100.0±0a	-	60.0±0a	-	90.0±14.1a	-	50.0±14.1b	-
PVS/40 min	30.0±14.1cd	40.0±0c	10.0±14.1b	0.0b	30.0±14.1bc	40.0±0b	10.0±14.1c	0.0c
PVS/80 min	8.3±11.8de	0.0e	0.0b	0.0b	8.3±11.8cd	0.0e	00.0c	0.0c
PVS/120 min	10.0±14.1de	10.0±14.1de	0.0b	0.0b	10.0±14.1cd	0.0e	00.0c	0.0c

Table 4. Effect of duration of dehydration with PVS2 and PVS3 solution on survival (%) and regrowth (%) of non-cryopreserved (-LN) and cryopreserved (+LN) grapevine cv. Portan shoot tips.

In columns, mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test.

By comparison, with ED, 37.1% regrowth after LN exposure was achieved using 1 mm-long shoot tips and only 16.0% with 2 mm-long shoot tips.

The ED technique comprises several successive steps, the conditions of which must be optimized to achieve maximal recovery after cryopreservation. Bead preculture with progressive sucrose concentration has been shown to improve recovery [12,13,20]. In our case, sucrose preculture allowed high survival (95.8-100.0) and regrowth (66.7-75.0) percentages. Explant dehydration is one of the crucial steps in this protocol. In general, the bead MC, which ensures highest regrowth after cooling in LN is approx. 20%, which corresponds to the amount of unfreezable water in the samples [21]. This value may vary depending on the species and explant type. In the case of grapevine, Plessis *et al*. [12] achieved 20% survival of cryopreserved shoot tips following dehydration to 25% MC, Wang *et al*. [13] achieved 59.3% survival after dehydration to 16.1% MC, and Zhao *et al*. [14] noted 40% survival after dehydration to 21% MC. In our work, we did not define an optimal MC for cryopreserving grape shoot tips and regrowth after cryopreservation was lower, compared with the results of Plessis *et al*. [12] and of Wang *et al*. [13]. Indeed, we obtained a similar, low regrowth of 10% after LN exposure following dehydration to three MCs, 22.28%, 20.26% and 16.1%. These results may be due to the high sensitivity of cv. Portan shoot tips to the ED technique, and/or to the fact that the preculture and dehydration conditions selected were not optimal.

An important parameter of cryopreservation protocols is the size of shoot tips employed as experimental materials. Explants must be of a sufficiently small size to avoid the occurrence of gradients between external and more internal cell layers during cryoprotection and cooling and warming, which would be detrimental to survival. However, at the same time, shoot tips must be of a sufficiently large size for rapid and direct regrowth to take place after cryopreservation [5]. When cryopreserving pear shoot apices using the ED technique, Scottez [22] showed that smaller shoot tips, of a size comprised between 1-2 mm, produced higher recovery compared to those of the two other categories tested, which had a size of 3-4 and 5-6 mm. In the case of grape, Wang *et al*. [20] compared the survival after cryopreservation of shoot tips of four different sizes (0.5, 1.0, 1.5 and 2.0 mm). The highest survival (65%) was obtained with 1-1.5 mm-long shoot tips, while larger or smaller shoot tips produced lower survival. We obtained comparable results in our experiments, with the smaller (1 mm long) shoot tips employed producing significantly higher regrowth, compared with larger ones (2 mm long).

In the DV technique, explants are rapidly dehydrated with highly concentrated vitrification solutions, resulting in the vitrification of internal solutes upon cooling in LN [4]. Because of their high concentration in cryoprotective agents, these vitrification solutions are often highly toxic. Optimizing the duration of exposure of samples to these solutions is therefore of paramount importance to achieve high regrowth after cryopreservation [23]. The optimal duration of exposure to vitrification solutions is associated with the size and structure of the excised shoot tips and appears to be highly species-specific at the same temperature. Ganino *et al.* [24] showed that exposure of grapevine shoot tips to vitrification solutions markedly affected shoot tip survival. Regrowth of shoot tips decreased from an initial value of 94% to 57% after a 30 min exposure and to 15% after 60 min. Optimal treatment durations with PVS2, which contains the penetrating cryoprotectants DMSO and EG, are in the magnitude of 30-60 min, while longer exposures are generally required with PVS3, which contains only the non-penetrating cryoprotectants sucrose and

glycerol [4]. In our experiments, the optimal PVS2 treatment duration was 50 min, which was comparable to the results obtained by Matsumoto and Sakai [6] with grapevine shoot tips cryopreserved with the vitrification technique.

For plant materials, which are highly sensitive to PVS2, dehydration with the PVS3 vitrification solution, which does not contain penetrating cryoprotectants and is generally less toxic, often produces high regrowth after cryopreservation. This was the case with chrysanthemum shoot tips, which showed significantly higher regrowth after cryopreservation following PVS3 dehydration, compared with PVS2 dehydration [25]. By contrast, shoot tips of other plant species such as garlic and sugarcane proved to be equally tolerant to dehydration with PVS2 and PVS3 [25,26]. Our experiments showed that grapevine shoot tips had an original behaviour, as they were extremely sensitive to PVS3 exposure. It can be hypothesized that grapevine shoot tips are highly sensitive to the high concentrations of sucrose and/or glycerol present in the PVS3 vitrification solution.

In conclusion, we showed for the first time that the DV technique could be successfully applied for cryopreservation of grapevine shoot tips. Recovery was significantly higher with DV, in comparison with the ED technique. The current results might be improved by optimizing various steps of the DV protocol, including conditioning *in vitro* plants or microcuttings on medium enriched with growth regulators such as BAP or zeatine riboside [27], or using alternative loading and vitrification solutions [28,29]. Before its large scale application can be envisaged, the optimized protocol should be validated through its application to a range of grapevine cultivars.

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