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Anatomy and osmotic potential of the *Vitis* rootstock shoot tips recalcitrant to cryopreservation

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Abstract

This study was carried out on Kober 5BB (*Vitis Berlandieri* × *V. riparia*) grape rootstock shoot tips during the preparatory steps preceding the direct immersion in liquid nitrogen, in order to overcome until now unsuccessful cryopreservation with this species. The exposure of shoot tips to 0.3 - 0.4 M sucrose leads to a high cell solute concentration. The treatment with plant vitrification solution (PVS2) alone, *i.e.*, not followed by storage in liquid nitrogen, markedly affected shoot tip survival. After a 30 min exposure, regrowth percentage of shoot tips decreased from 94 % (control) to 57 %, and dropped to 15 % when the treatment was prolonged up to 60 min. After a 90 min exposure, no regrowth occurred. In addition, plantlets regenerated from shoot tips which underwent 60 min or more exposure to PVS2 showed signs of malformation. Microscope observations of shoot tips treated with 0.3 or 0.4 M sucrose and 30 min PVS2 showed the presence of cells starting to plasmolyze, localized in the area surrounding the apical meristem. A limited presence of starch grains in meristem and bract cells was also noted. However, the most conspicuous consequence of prolonged PVS2 treatment was convex plasmolysis. The phenomenon was dependent on the time of PVS2 exposure. Indeed, after a 30 min treatment, plasmolysis was minimal or absent, but it increased with longer exposure to PVS2 at 4 °C.

Additional key words: grapevine, micropropagation, plant vitrification solution, plasmolysis, sucrose.

Introduction

Cryopreservation is a valuable method for long term storage of plant germplasm, which allows the conservation of cells, tissues and organs from *in vitro* culture, such as shoot tips, callus cultures and somatic embryos in liquid nitrogen (LN) (*e.g.*, Reed 2008). The plant material is stored at low cost, for unlimited time and in absolute sanitary and genetic safety. In the last 20 years, innovative cryo-techniques, allowing the direct immersion of explants in LN, have been developed (Engelmann 2004, Panis and Lambardi 2006). Among them, those based on vitrification of shoot tips (excised from axillary and apical buds of shoot cultures) by means of their loading in plant vitrification solution No. 2 (PVS2; Sakai *et al.* 1990) paved the way to the optimization of effective cryopreservation protocols for various species (Lambardi and De Carlo 2003, Sakai and Engelmann 2007). Nevertheless, many species are still "recalcitrant" to the cryopreservation of shoot tips by PVS2-vitrification, as survival and regrowth of explants in post-thaw is low or even zero. For such a reason, a specific *EU COST Action* (*871 CryoPlanet*, in: http://www.biw.kuleuven.be/dtp/ tro/cost871/home.htm) is presently ongoing, aiming to improve the fundamental knowledge of cryoprotection through the determination of physico-biochemical changes associated with tolerance towards cryopreservation. Indeed, the success of a cryo-preservation procedure implies that the samples go through a series of successive steps (pregrowth, cryoprotective treatment, immersion in LN, thawing and plating), without losing their viability and the potential of regrowth in post-conservation.

Shoot tips vitrification-based techniques of simplified

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Abbreviations: BA - 6-benzyladenine; CP - cryoprotectant; MS - Murashige and Skoog medium; DMSO - dimethylsulfoxide; LN - liquid nitrogen; PVS2 - plant vitrification solution No. 2.
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cryogenic protocols eliminate the potential damage due to intracellular water crystallization and allow direct immersion of specimens in LN, inducing often high post-cryopreservation regeneration (*e.g.*, Engelmann 1997, Lambardi *et al.* 2000, Matsumoto and Sakai 2003, Sakai *et al.* 2008, Škrlep *et al.* 2008). Many cryopreservation protocols use solutes like glycerol, dimethylsulfoxide (DMSO), sucrose, alone or in combination, in order to decrease the free water content in cells and to limit ice-crystal formation during ultra-freezing. DMSO and glycerol (both components of PVS2) are cell wall and membrane penetrable and increase cellular osmolality. Sucrose has a larger molecule, and can penetrate the cell wall, but not easily the plasma membrane (Tao and Li 1986). When cells are frozen, sucrose is concentrated in the cell wall space. Here, it protects protoplasts from freeze-induced dehydration. It can also form a buffer layer between cell wall and the protoplast to protect the outer surface of the plasmalemma (Tao and Li 1986).

As for the *Vitis* genus, few reports of cryopreservation are available, and all dealing with the cryopreservation of germplasm, using non vitrification-based techniques, such as encapsulation-dehydration (Plessis *et al.* 1991, 1993) and encapsulation-dehydration combined with slow cooling to -40° C of explants (Zhao *et al.* 2001). On the other hand, our previous investigations (Fabbri *et al.* 2007) showed that shoot tips, excised from the rootstock Kober 5BB and treated following a classic multi-step vitrification protocol, survived but never regrew in post-cryopreservation (Fabbri *et al.* 2007). Hence, working with this system, recalcitrant to cryopreservation by the PVS2-vitrification approach, an osmometrical and histological study was carried out at each successive step of the vitrification-based cryopreservation protocol, in order to understand the mechanisms of adaptation to low temperatures, as well as to improve the knowledge on the effects of the preparatory steps on which successful regeneration in post-cryopreservation strictly depends.

Materials and methods

An *in vitro* shoot culture of the grape rootstock Kober 5BB (*Vitis Berlandieri* × *V. riparia*) was maintained on Murashige and Skoog (1962; MS) medium, containing 3 % sucrose, 0.8 % agar and 5 μM benzyladenine (BA), the latter added after autoclaving by cold filtration. Medium pH was stabilized at 5.8 before autoclaving. Shoots were maintained at 25 °C, under a 16-h photoperiod (irradiance of 90 μmol m^{-2} s^{-1}), and monthly subcultured. For cryopreservation, shoot tips (2 ± 1 mm, on average) were obtained from axillary buds excised from not less then 10 cm long shoots.

Sixty shoot tips were used for each treatment. They were excised after the shoots had been cold-hardened for two weeks at 4 °C, and pre-cultured on solidified MS medium supplemented with different sucrose concentrations (0.2, 0.3 or 0.4 M) for two days at 4 °C. Pre-cultured shoot tips were transferred in 2 cm^3 cryovials (10 shoot tips per cryovial), treated with a cryoprotective (CP) solution (2 M glycerol and 0.4 M sucrose, pH 5.8; from Matsumoto *et al.* 1995) for 30 min at 4 °C and then loaded at 0 °C in PVS2 solution (30 % glycerol, 15 % ethylene glycol, 15 % DMSO, all m/v, in 0.4 M sucrose; from Sakai *et al.* 1990) for different periods of time (30, 60 or 90 min). Afterwards, the PVS2 was replaced with 1 cm^3 of fresh solution. The cryovials were then directly plunged into LN and maintained there for at least 1 h. For thawing, cryovials were rapidly warmed in a water bath at 40 °C for 1 min. Excess PVS2 was drained and shoot tips washed with 1.2 M sucrose in liquid MS medium for 20 min. After both PVS2 and LN treatments, regeneration tests were performed by transferring the shoot tips onto solidified ½ MS medium supplemented with 5 μM BA. The experiment, for each protocol tested, was performed at least three times.

Throughout the cryopreservation procedures the osmotic potential of the cell sap was evaluated. Measurements were performed using a vapour pressure osmometer *Wescor 5500* (Logan, UT, USA). The cell sap solutions were pipetted onto a paper disc in the instrument sample holder and the sample was conveyed into the instrument sample chamber. Samples of at least 15 shoot tips for each protocol were collected at each step of the vitrification procedure (see below). The samples were put in a 1 cm^3 syringe without needle. Cell disruption was obtained by 10 repeated freeze-thaw cycles. The cell sap obtained by syringe squeezing (0.05 cm^3) was then collected in 1.5 cm^3 vials. Cell debris was separated through centrifugation (Torelli *et al.* 1999). Samples of 0.01 cm^3 of supernatant were utilized for each measurement. The analysis was performed at least three times.

Samples were collected at the end of each step of vitrification procedure and shoot tips were fixed in FAA solution, and after at least 24 h they were dehydrated with gradual alcohol concentrations. Embedding was made in a methacrylate resin (*Technovit 7100*, Wehrheim, Germany), and the resulting blocks were sectioned at 3 μm thickness (longitudinal sections) using a *Leitz 1512* (Wetzlar, Germany) microtome. The sections were treated with periodic acid-Schiff reagent and Amido Black (Ruzin 1999). Ten shoot tips were sampled for each step of the vitrification.

Shoot-tip regrowth percentages and osmotic potential values were independently determined. Statistical differences among data were analyzed by *ANOVA*, followed by the Tukey's HSD test at $P \leq 0.05$ using *SPSS 17.0* software (SPSS, Chicago, USA).

Results and discussion

PVS2 toxicity was determined by evaluating shoot-tip regrowth after loading in PVS2 and washing (non-frozen control). A high regrowth percentage was obtained with non-PVS2 treated shoot tips (94 ± 2 %). Regrowth percentage was lower (57 ± 3.6 %) after shoot tips were loaded in PVS2 for 30 min. When loading was prolonged to 60 min the value dropped to 14 ± 3 %, while no regrowth was obtained from 90 min loaded shoot tips. It was also observed that, following a 60 min PVS2 treatment, the regrowing shoot tips showed a high percentage of malformations (44 %). Indeed, young leaflets displayed quite different structures if compared to control ones (data not shown). Shoots regenerated from 30 min PVS2 treated shoot tips showed no malformation.

Confirming our previous investigations (Fabbri *et al.* 2007), none of the PVS2 treatments was able to induce tolerance to ultra-rapid freezing, as no shoot-tip regrowth after direct immersion in LN, thawing and plating was observed. The study was eventually focused on concentration of cell sap and on anatomical changes occurring in the explants during the whole process. Results indicate the importance of pre-culture treatment on cell sap concentration prior to ultra-rapid freezing of explants in LN (Table 1). The lowest sucrose dose (0.2 M) produced a slight and constant solute concentration increase, reaching a maximum osmotic potential of -3.99 MPa. However, sucrose pre-culturing at 0.3 and 0.4 M induced more negative osmotic potential, as the cells concentrated their cell sap by more than twice (Table 1).

Table 1. Shoot tip osmotic potential [-MPa] after pre-culture with three sucrose concentrations (2 d at 4 °C) and treatment with CP. Osmotic potential was measured before treatment, after 2 d of pre-culture and 30 min after CP treatment. Means ± SE, n = 3. Statistically significant differences within each column determined by Tukey test are marked by different letters.

Sucrose	Untreated	After pre culture	After CP treatment
0.2 M	1.03 ± 0.016	1.97 ± 0.039^{a}	3.99 ± 0.159^{c}
0.3 M	1.03 ± 0.016	1.98 ± 0.013^{a}	7.75 ± 0.216^{a}
0.4 M	1.03 ± 0.016	1.08 ± 0.026^{b}	6.15 ± 0.238^{b}

Considering the three different PVS2 exposure times for each sucrose concentration (Table 2), a different behaviour for a 30 min PVS2 exposure time was clearly evidenced. The two higher exposure times seemed to follow the same trend, with the exception of the strongest osmotic treatment (0.4 M sucrose in preculture, followed by a 90 min PVS2 loading of shoot tips, Table 2), where osmotic potential showed a decrease after LN and explant thawing, probably caused by irreversible cell damage. The anatomical observations showed that, following pre-culture at 0.2 M sucrose, cells are still turgid and cytoplasm is not significantly dehydrated; hence in this case the onset of dehydration occurs after CP treatment. In the other treatments (0.3 and 0.4 M sucrose) dehydration already started at the end of sucrose pre-culturing (Fig. 1*a*). Differentiated cells appeared slightly dehydrated after CP treatment and the first signs of plasmolysis were detected in the shoot-tip parenchyma (Fig. 1*a*). During all the cryoprotectant treatments, cell dehydration was linked with the appearance of starch granules, initially present in the subapical zone, but which eventually spread to all the apex tissues, including the apical meristem (Fig. 1*b*). In shoot tips pre-treated with 0.3 and 0.4 M sucrose, osmotic potential increased immediately after pre-culturing; apparently, this range of sucrose concentration, associated with a 30 min PVS2 loading, represents the best condition for a slow and steady dehydration (Table 2). Indeed, 60 and 90 min PVS2 treatments, besides being toxic for shoot tips, were associated with low dehydration when pre-culture was made with 0.3 M sucrose, and with strong dehydration when sucrose pre-culture was raised to 0.4 M. In shoot tips pre-cultured in 0.3 and 0.4 M sucrose followed by a 30-min PVS2 treatment, anatomical observation also shows that non-meristematic cells started to plasmolyse after sucrose pre-culture and had scant presence of starch. Starch grains can be detected in the subapical cell zone, in apical meristem and in bracts (Fig. 1*b*). At all sucrose concentrations, explants appear extremely dehydrated after 30 min of PVS2 treatment. The following immersion in LN and thawing caused degeneration of differentiated shoot-tip cells, but not

Table 2. Shoot tip osmotic potential [-MPa] after pre-culture with three sucrose concentrations (2 d at 4 °C) followed by additional steps of the cryopreservation procedure. Means ± SE, n = 3. Statistically significant differences within each column determined by Tukey test are marked by different letters.

Sucrose [M]	Exposure to PVS2 [min]	After PVS2 treatment	After thawing
0.2	30	8.48 ± 1.728^{b}	15.46 ± 0.696^{b}
	60	16.78 ± 1.927^{a}	21.01 ± 0.198^{a}
	90	15.02 ± 3.001^{a}	18.82 ± 2.402^{a}
0.3	30	16.81 ± 2.462^{a}	20.75 ± 0.279^{a}
	60	10.98 ± 0.521^{b}	19.33 ± 0.424^{b}
	90	10.61 ± 0.247^{b}	17.60 ± 2.470^{b}
0.4	30	14.77 ± 0.663^{b}	22.50 ± 0.259^{b}
	60	21.30 ± 3.992^{a}	25.63 ± 0.331^{a}
	90	21.21 ± 3.450^{a}	15.19 ± 0.962^{c}

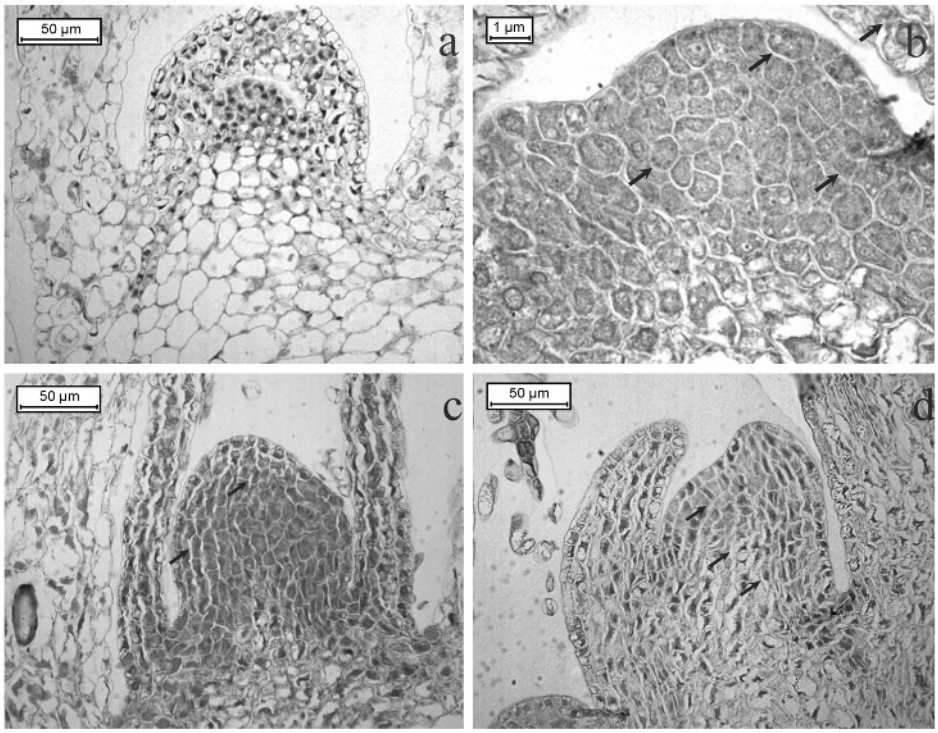

Fig. 1. Anatomical modifications in Kober 5BB buds during the cryopreservation procedure. The sections were stained with PAS-Amido black: *a* - bud longitudinal section after 0.4 M sucrose pre-culture (2 d) and 30 min CP treatment; parenchyma cells display evident symptoms of dehydration; *b* - bud longitudinal section after 0.3 M sucrose pre-culture (2 d), 30 min CP treatment and 30 min PVS2 treatment; starch granules are visible (as dark spots) in meristem cells (*arrows*); *c* - evident plasmolysis (*arrows*) in 60 min PVS2 treated buds; *d* - marked plasmolysis (90 min exposure to PVS2), *arrows* indicate location of visible plasmolysis in apex cells.

of cells of the meristematic dome.

Explant analysis after PVS2 treatments showed a marked dehydration of subapical cells and, in some instances, the protocol led to a lysis of differentiated cells and to marked dehydration of the meristematic cells (such as in shoot tips which underwent the sequence 0.4 M sucrose + CP treatment + 90 min loading in PVS2) (Table 2).

The cause of cell damage could lie in the protoplasm insufficient ability to shrink and concentrate cell sap due to water content reduction. The more vacuolated cells showed the higher plasmolysis level when treated with sucrose, followed by cryoprotective and vitrification treatments. The water quantity lost from the protoplasm of these cells appeared to be lethal since these cells do not generally survive the imposed osmotic stress (Volk and Caspersen 2007). According to Withers (1980), cell suspensions with less vacuoles are preserved better than those with more or larger vacuoles. Strongly vacuolated cells showed two different types of shrinkage, *i.e.* concave and convex plasmolysis (Attree and Sheffield 1985, Oparka 1994). Plasmolysis type may also vary in cold-hardened and non cold-hardened plant cells. In cabbage seedlings, plasmolysis was more convex in the hardened state and more concave in the unhardened state (Levitt 1983). In our study, the plasmolysis was dependent on PVS2 exposure time, and independent on sucrose-concentration in pre-culture medium. Indeed, following a 30 min PVS2 treatment, plasmolysis was minimal (Fig. 1*b*), but it increased with longer exposure times (Fig. 1*c*,*d*). While in the shortest PVS2 treatments (30 min) plasmolysis was either absent or confined to very few cells (Fig. 1*b*), with the 90-min treatment the phenomenon concerned the whole shoot-tip (Fig. 1*d*); the 60-min treatment resulted in an intermediate situation (Fig. 1*c*). According to Drake *et al.* (1978) and Ruan *et al.* (2004), the lack of rehydration after sucrose, CP and PVS2 treatments could be ascribed to plasmodesm obstruction (callose deposition) due to the severe plasmolysis of cells.

It is worthy of note that, in this study, starch was always detectable following sucrose-treatment, and persisted after the following CP and PVS2 treatments, but was absent after 5 weeks of culture.

In conclusion, our results confirm that cell dehydration is a fundamental step to protect them from the ultra-low temperature of LN. In this study, dehydration greatly increased after CP treatment, while cell osmotic potential decreased gradually only when PVS2 exposure time was of 30 min. Prolongation of the exposure time to PVS2 produced toxic effects on Kober 5BB shoot tips. On the other hand, the 30 min exposure in PVS2 was not sufficient to induce optimal shoot-tip dehydration and protection from damages due to ultra-rapid freezing in

LN, as no regeneration was observed in post-cryopreservation.

Unlike *Vitis vinifera*, the grape rootstock Kober 5BB (an hybrid of *V. Berlandieri* and *V. riparia*) shows to be recalcitrant to cryopreservation even if its meristematic tissue still looks vital after recovering from storage in LN. The lack of regrowth of shoot tips, which appear to be green and alive after cryopreservation, is not an uncommon event. It was observed in olive (Lambardi *et al.* 2002, Lynch *et al.* 2007) and underlines once more the necessity to get a deeper knowledge of the physiological and biochemical events at the basis of the acquisition of tolerance of plant cells and organs, such as shoot tips, to ultra-rapid freezing in LN. Hence, the present investigation with Kober 5BB will continue along this way.

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