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Cryopreservation and cryotherapy of grapevine (*Vitis vinifera* L.)

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Summary

This study aimed at testing the efficiency of a droplet-vitrification cryopreservation protocol in eliminating selected grapevine viruses. The cryopreservation protocol led to approximately 50 % recovery with cultivar 'Portan' and five international cultivars tested, but very low recovery was noted with Croatian cultivars. GFLV and GLRaV-3, two (economically important grapevine) viruses were eliminated in a high percentage (up to 100 %) of plants regenerated from cryopreserved shoot tips. Virus sanitation was observed as well in samples before liquid nitrogen exposure. Genetic stability of plants regenerated after cryopreservation was studied using AFLP markers. Polymorphic fragments were observed in non-cryopreserved and cryopreserved samples treated with PVS2 solution, the number of which increased with increasing durations of exposure to PVS2 solution.

Key words: grapevine; cryopreservation; cryotherapy; genetic stability.

Introduction

Cryopreservation is a method for long-term conservation in which explants are stored at the ultra-low temperature of liquid nitrogen (LN) (Engelmann 2011). In the case of grapevine, cryopreservation protocols have been established for shoot tips sampled from *in vitro* plantlets using encapsulation-dehydration (Plessis *et al.* 1991 and 1993,Wang *et al.* 2000, Zhao *et al.* 2001), vitrification (Matsumoto and Sakai 2003) and, more recently, droplet-vitrification (Marković *et al.* 2013).

Results are quite likely to vary between grapevine cultivars (Matsumoto and Sakai 2003), in as much *Vitis vinifera* numbers around 5000 cultivars (This *et al.* 2006). Indeed, cryopreservation protocols are highly genotype dependent (Ashmore *et al.* 2007) and some cultivars will likely demand a precisely adapted protocol. Cryotherapy is a rather novel application of plant cryopreservation. It eliminates plant pathogens such as viruses, phytoplasmas and bacteria by briefly treating shoot tips with LN using cryopreservation protocols. Healthy plants are regenerated from the surviving pathogen-free meristematic tissue. The first successful cryotherapy experiments were performed in France by De Boucaud *et al.* (1997), who eliminated Plum pox potyvirus from *Prunus* shoot tips. Recently, cryotherapy has been shown to eradicate seven unrelated groups of viruses and/or two types of bacteria-like pathogens from the shoot tips of eleven different crop species (Wang *et al.* 2008). More recently, Gallard *et al.* (2011) showed that *Pelargonium* plants regenerated from shoot tips submitted to cryotherapy were virus-free (according to an ELISA test), although immunolocalization studies still detected some virus signals. This study highlights a strong need for further research on this technique.

Plant cryopreservation is a complex procedure during which explants are exposed to potentially stressing events such as ultra low temperatures and contact with cryoprotectants. Explant manipulations are capable of inducing somaclonal variation in regenerated plants (Harding 2004 and 2009).

The goals of this study were: to test the protocol for cryopreservation on international cultivars, his efficiency in virus eradication and genetic stability of regenerated cryopreserved material.

Material and Methods

Plant materials: In this work, plant materials of different origins were used depending on the experimental purposes. Croatian cultivars, a set of five international cultivars and 'Portan' were used for cryopreservation experiments. International cultivars and their infected counterparts were used in cryotherapy experiments. 'Portan' was used for genetic stability experiments.

Croatian cultivars: Croatian cultivars were taken from *in situ* grapevine collections in Split and Zadar. Selected genotypes had been tested according to the European Union Council Directive 92/34/EEC, for presence of Grapevine leafroll-associated virus 1 (GLRaV-1), Grapevine leafroll-associated virus 3 (GLRaV-3) and for Grapevine virus A (GVA) using enzyme-linked immunosorbent assay (ELISA). ELISA was performed according to manufacturer's instructions (QIAGEN, Germany).

International cultivars and 'Portan': *In vitro* cultures of cultivar 'Portan' were established from

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field-grown plants in the grape germplasm collection of INRA (Institut national de la recherche agronomique) in Vassal (France). Cuttings of 'Chardonnay', 'Cabernet Sauvignon', 'Merlot' and 'Pinot Noir' were taken from IFVV (Institut français de la vigne et du vin) screenhouses (Le Grau du Roi, France). Each cultivar was represented by an infected plant and its derived sanitized clone (sanitation achieved through micro-grafting).

In vitro culture: *In vitro* plantlets of tested cultivars were cultured on basal medium (BM) composed of half-strength MS (Murashige and Skoog 1962) mineral elements with Morel's vitamins, 3 % sucrose and 0.7 % agar (Sigma) at pH 5.8. They were cultured at 27 ± 2 °C under a 12 h light/12 h dark photoperiod with a light intensity of 40 $\mu E \cdot m^{-2} \cdot s^{-1}$ provided by cool white fluorescent tubes. *In vitro* mother-plants were grown for 2 months to reach a length of approximately 12 cm before use for cryopreservation experiments. They were excised and used as axillary buds in the production of microcuttings as described by Marković *et al*. (2014). *In vitro* plantlets were cut into single- node microcuttings of approx. 1.5 cm in length, which were transferred to 9 cm Petri dishes (20 microcuttings/ dish) and placed on BM medium supplemented with 1 µM benzyladenine (BA). Shoot tips were excised from microcuttings after 2 weeks and used for cryopreservation experiments.

Cryopreservation: Excised buds were cryopreserved using the droplet-vitrification protocol developed by Marković *et al.* (2013). They were precultured on semisolid ½ MS medium with 0.1 M sucrose for 24 h. Precultured buds were then treated with a loading solution (LS) containing 2 M glycerol + 0.4 M sucrose in MS medium (Sakai *et al.* 1991) for 20 min at room temperature. Buds were dehydrated with half-strength PVS2 at room temperature for 30 min, then with full strength PVS2 at 0 °C for 50 min. PVS2 contains 30 % (w/v) glycerol, 15 % (w/v) ethylene glycol (EG), 15 % dimethylsulfoxide (DMSO) and 0.4 M sucrose in MS medium (Matsumoto and Sakai 2003). Buds were then placed in 5 µL PVS2 droplets on sterile aluminium foils which were immersed in LN for at least 1 h. For rewarming, the aluminium foils were immersed for 20 min at room temperature in an unloading solution containing 1.2 M sucrose (Sakai 1997), and buds were then transferred to recovery medium. Explants were post-cultured on medium containing 1µM BAP, maintained in the dark at 26 ± 1 °C for 7 d and then transferred to the conditions described for stock cultures. 20-30 explants per cultivar were inoculated, when possible. In the case of Croatian cultivars, a lower quantity of plant material was used in experiments (15 to 20 explants). Survival and regrowth data are presented in % of inoculated explants.

Cryotherapy: Regeneration of fully developed *in vitro* plantlets was possible only with 'Chardonnay' and 'Cabernet Sauvignon'. These plantlets were subjected to ELISA testing. Regenerated two-months *in vitro* plants from control (with all pretreatment steps performed without exposure to LN) and cryopreserved apices were tested for the presence of Grapevine fanleaf virus (GFLV) (26 plants of 'Chardonnay') and Grapevine leafroll-associated virus 3 (GLRaV-3) (38 plants of 'Cabernet Sauvignon').

Genetic stability: The cryopreservation protocol was performed (with three PVS2 durations tested: 25, 50 and 75 min) to obtain regenerated plants which were subjected to genetic stability testing. After recovery, regenerated plants were maintained in tissue culture conditions for 4 months before samples were taken for DNA isolation. Each condition contained a number of plants that regenerated after LN exposure. Plants cultivated for 2-months *in vitro* were chosen as control samples. AFLP markers were employed to test genetic stability. Eight AFLP primer combinations were used on cryopreserved and non-cryopreserved shoot tips (sampled after sucrose preculture, loading and treatment with half-strength PVS2 solution) for a total of 43 plants tested. The AFLP protocol was designed by Vos *et al.* (1995). The experiments were performed using an ABI3130 Genetic Analyzer (Applied Biosystems).

Results and Discussion

The basal cryopreservation protocol, which was established on cultivar 'Portan' (Marković *et al.* 2014) was tested as to its applicability to healthy international cultivars ('Pinot Noir', 'Chardonnay', 'Cabernet Sauvignon' and 'Merlot') and their infected counterparts. The same protocol was applied to Croatian cultivars ('Plavac mali', 'Maraština', 'Pošip' and 'Škrlet') and to two infected genotypes ('Plavac mali' and 'Maraština'). The infected cultivars were included in order to assess the effect of sanitary status on recovery after cryopreservation.

Survival of non-cryopreserved and cryopreserved shoot tips of the four international cultivars tested was generally high (Tab. 1). However, no regrowth was achieved with either control or cryopreserved shoot tips of cultivar 'Pinot Noir'. With the other three cultivars tested, intermediate (30 %) ('Chardonnay') to high (70 %) ('Merlot') regrowth was achieved before and after cryopreservation, which was comparable to the results obtained by Matsumoto and Sakai (2003) on 10 *Vitis* cultivars, which produced an average recovery of 64 %. Similar results were obtained with healthy and virus infected cultivars.

The same protocol was performed on Croatian cultivars. Low survival and recovery were obtained with only one ('Maraština') of the four cultivars tested. This low success might be due to the fact that cryopreservation experiments were performed using cultures which had been introduced *in vitro* very recently and which were thus not adapted to *in vitro* culture conditions. Another possibility could be that the *in vitro* culture conditions employed were not adapted to the multiplication of these Croatian cultivars (Marković *et al.* in press). Plant tissue culture techniques are genotypic, cultivar and also clone specific (George 1993).

Our results illustrate the fact that the response to cryopreservation can be genotype-specific, and that different conditions for the same protocol or different protocols may have to be used to achieve cryopreservation of different genotypes (Reed 2008).

Cryotherapy: The results of the cryotherapy experiments revealed that a high proportion of 'Chardonnay'

Table 1

Survival and regrowth of non-cryopreserved (-LN) and cryopreserved (+LN) shoot tips of healthy and virus infected genotypes

Cultivar and sanitary status	Survival (%)		Regrowth (%)	
	-LN	+LN	-LN	+LN
Portan	50.0	50.0	40.0	50.0
Pinot Noir, healthy	76.6	47.7	0.0	0.0
Pinot Noir (GFLV)	90.0	37.9	0.0	0.0
Chardonnay, healthy	77.5	61.4	25.0	30.0
Chardonnay (GFLV)	69.4	50.9	55.6	30.7
Cabernet Sauvignon, healthy	75.0	57.7	35.0	46.6
Cabernet Sauvignon (GLRaV-3)	86.9	61.8	62.3	41.6
Merlot, healthy	90.0	75.0	90.0	70.0
Merlot (GLRaV-3)	85.5	67.9	79.2	61.0
Plavac mali, healthy	0.0	0.0	0.0	0.0
Plavac mali (GLRaV-1)	0.0	0.0	0.0	0.0
Maraština, healthy	44.0	22.0	33.0	11.0
Maraština (GLRaV-3)	60.0	25.0	0.0	0.0
Pošip, healthy	0.0	0.0	0.0	0.0
Škrlet, healthy	22.0	15.0	22.0	0.0

and 'Cabernet Sauvignon' plants were virus-free after being subjected to cryopreservation. GLRaV-3 was completely eliminated, while GLRV was eliminated in about 78 % of the regenerated plants (Tab. 2). Virus testing was also performed on control plants treated with PVS2 (-LN) and the proportion of healthy plants was almost the same as with LN exposed ones for both viruses.

This differential response may be connected with different capacities of parenchymatic (GFLV) and phloematic (GLRaV-3) viruses to invade and spread through the tissues of the host plant. Closterolike-virus particles of GLRaV-3 are known to be restricted to vascular tissues (phloem) (Namba *et al.* 1979) while GFLV particles are present even in very young meristematic tissues (i.e. meristematic dome of shoot apices) of infected grapevine.

Various reports on different viruses and species have shown that explant size is crucial for the success of virus eradication (Brison *et al.* 1997, Faccioli and Marani 1998, Ding *et al.* 2008). One of the advantages of cryotherapy, compared with meristem culture, is the possibility of using large explants. These explants contain large cells, which are more likely to be virus- infected than smaller meristematic ones. However, cryopreservation kills large cells, which cannot survive because of their higher water content, and thus increases the proportion of healthy cells in large shoot tips. Based on our results on cryopreservation protocol optimization (Marković *et al.* 2014), 1 mm long explants were used in these experiments. Such small explants may already be free of infected cells, which could be one of the reasons for the high percentage of healthy plants obtained even without LN exposure. This is in accordance with the study of Youssef *et al.* (2009) who reported that meristem tips 1 mm in length were found to be optimal for elimination of GFLV and GLRaV-1 from infected grapevines using meristem tip culture.

Another explanation for the high percentage of virus-free plants obtained from control and cryopreserved shoot tips might be the exposure of explants to the PVS2 cryoprotectant solution which penetrates deeply in shoot tips and may display some sort of "chemotherapeutic" effect. Yet another explanation for this result might be that the osmotic shock caused by exposure of shoot tips to the highly concentrated PVS2 solution kills differentiated and virus-infected cells, without the need for LN exposure. This was already reported by Helliot *et al*. (2002) who obtained 2 % virus-free plants for cucumber mosaic virus (CMV) and 95 % virus-free plants for banana streak virus (BSV) in *Musa spp.* after 1-h cryoprotection with PVS2 solution. By contrast, Wang *et al.* (2003) did not report the occurrence of any virus-free plants without LN exposure. Additional experiments are thus needed to confirm our preliminary observations of virus elimination without LN exposure.

Table 2

Results of ELISA tests for two grapevine viruses (GFLV and GFLaV-3) performed on plantlets regenerated from control (-LN) and cryopreserved (+LN) buds of cultivars 'Chardonnay' and 'Cabernet Sauvignon'

Cultivar	Virus	Number of samples for ELISA virus testing		% of negative (healthy) samples	
		-LN	+LN	-LN	+LN
Chardonnay (GFLV)	GFLV	17	9	82.4	77.8
Cabernet Sauvignon (GLRaV-3)	GLRaV-3	15	16	100	100

Genetic stability: AFLP markers were employed to test the genetic stability of grapevine *in vitro* 'Portan' plantlets regenerated following a cryopreservation experiment. With the eight AFLP primer combinations employed on the 43 plants tested, cryopreserved and non-cryopreserved shoot tips (sampled after sucrose preculture, loading and treatment with half-strength PVS2 solution) were included. No significant differences in AFLP profiles were observed after sucrose preculture, treatment with the loading solution and with half-strength PVS2. However, an increase in polymorphic fragments was observed in non-cryopreserved and cryopreserved samples treated with PVS2 solution, the number of polymorphic fragments increasing with longer durations of exposure to PVS2 solution.

All plants regenerated were compared after the successive steps of the cryopreservation protocol (Figure). It was noted that differences between samples increased with the number of treatments to which they were exposed. Looking at the last two clusters, it was visible that increasing the duration of exposure to PVS2 influenced the number of polymorphic fragments, even without LN exposure. Exceptions were samples CON 50_5 and CON 50_6, which were very close to control samples.

Genetic stability of cryopreserved grapevine material was tested by ZHAI *et al*. (2003) using RAPD markers and by CASTILLO *et al.* (2010) using SSR and AFLP analysis on cryopreserved grapevine shoot tips. ZHAI *et al*. (2003) observed no difference between DNA patterns of regenerated plantlets from control and cryopreserved *Rubus* plantlets. CASTILLO *et al*. (2010) detected AFLP polymorphisms in three of the four *Rubus* genotypes after they had been subcultured for 7 months, but they were no longer detected when plants were grown *ex vitro*. This study showed that polymorphism can vary depending on the environmental conditions of the plantlets. It is also obvious that different techniques used for polymorphism detection may yield different results. Our study, which revealed differences between non-cryopreserved and cryopreserved material, is in accordance with the results of CASTILLO *et al*. (2010) who found polymorphism in plantlets derived from cryopreserved shoot tips subcultured for 7 months but contradicts the results of ZHAI *et al*. (2003) who did not observe any difference between control and cryopreserved samples.

In our experiments, we used the PVS2 solution, which is known for its efficiency but also for its toxicity (SAKAI and ENGELMANN 2007). One hypothesis may be that, even though, to the best of our knowledge, no such changes have been reported in the literature, exposure of grape shoot tips to PVS2 for 50 min induced these polymorphisms. LN exposure increased the level of polymorphism observed. Nevertheless, *in vitro* culture can also cause some changes in the structure of plants, without any involvement of toxic chemicals. GRIBAUDO *et al*. (2000) observed that after some years in the vineyard, plants produced by micropropagation reached a higher yield and had a different leaf shape compared to plants multiplied in the nursery.

Conclusion

From the results presented, we can conclude that a comprehensive study should be performed with selected grapevine cultivars for the development of an efficient cryopreservation protocol. Cryotherapy as a method can be efficient for virus eradication even in the preconditioning steps of a cryopreservation protocol, even though each of these steps should be thoroughly tested for confirmation of these results. A genetic stability study should be included in post-cryopreservation experiments, because our results

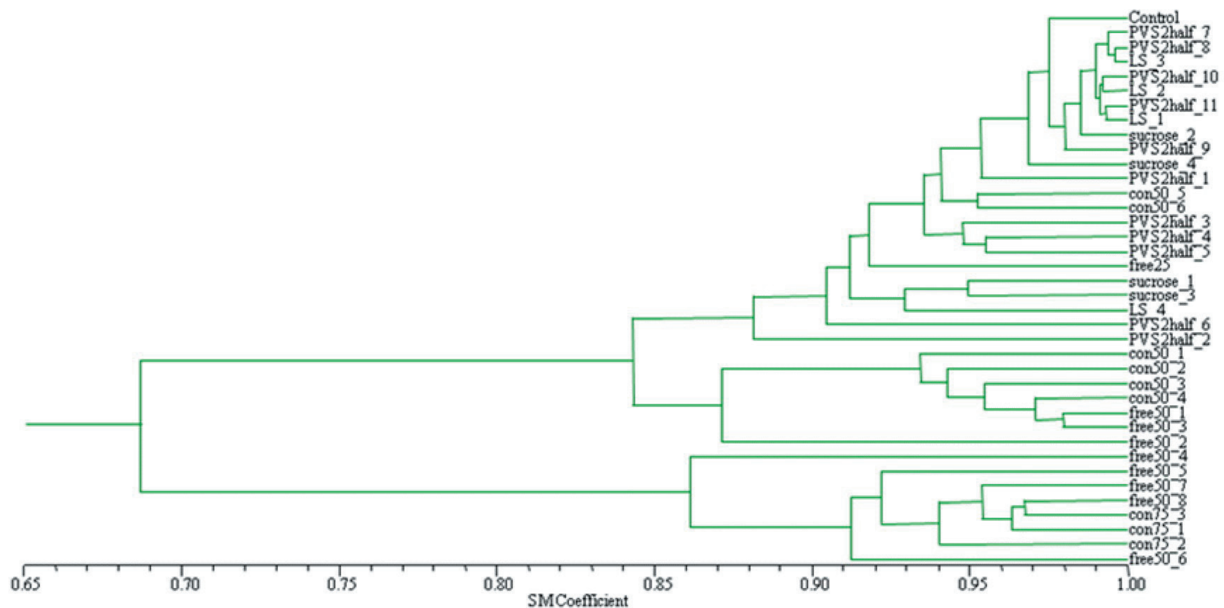

Figure: Comparison between all steps of cryopreservation protocol with control (*in vitro* plants)) LS: treatment with loading solution for 20 min; Sucrose: treatment with 0.3 M sucrose for 24 h; PVS2half: treatment with half-strength PVS2 solution for 30 min; FRE25: treatment with PVS2 for 25 min followed by LN exposure; CON50: treatment with PVS2 for 50 min; FRE50: treatment with PVS2 for 50 min followed by LN exposure; CON75: treatment with PVS2 for 75 min.

suggest that DNA polymorphism may increase after some steps of the cryopreservation protocol and that caution is necessary; moreover, using eight AFLP primer combinations and amplifying 736 fragments, only a small portion of the genome was covered. The experiments presented should be confirmed on a larger scale.

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