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APPLICATION OF V AND D CRYO-PLATE METHODS FOR THE CRYOPRESERVATION OF CHERRY ROOTSTOCK GISELA 5

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Abstract

The possibility of improving cryopreservation success using aluminium cryo-plates was investigated in cherry rootstock Gisela 5. The shoot tips were dissected from *in vitro*-grown shoots and precultured for one day at 25 C in the dark, on Murashige and Skoog (MS) medium containing 0.3 M sucrose. The explants were placed on aluminium cryo-plates with 12 wells and embedded in 2% alginate gel. Osmoprotection was performed by immersing the cryo-plates in loading solution (1.9 M glycerol + 0.5 M sucrose) for 30 min at room temperature. In the V cryo-plate protocol, dehydration was performed at room temperature using two types of plant vitrification solutions: modified PVS2 (37.5% glycerol, 15% dimethyl sulfoxide, 15% ethylene glycol and 22.5% sucrose) for 40 min and PVS3 (50% glycerol and 50% sucrose) for 60 min. In the D cryo-plate protocol, explants were desiccated for 2, 2.5 or 3 h in closed glass containers over silica gel. In both protocols, cryo-plates with explants were transferred in uncapped cryotubes and directly plunged into liquid nitrogen. Rewarming was done by direct immersion of cryo-plates in liquid MS medium containing 0.8 M sucrose (30 min at room temperature). In the V cryo-plate procedure regrowth of cryopreserved shoot tips dehydrated with PVS2 was 95.9%, while for those dehydrated with PVS3 was 85%. As for the D cryo-plate procedure, regrowth ranged between 45.8% and 66.7%. After regrowth, shoots were successfully multiplied and rooted. The results obtained clearly indicate that both cryopreservation procedures using aluminium cryo-plates can facilitate efficient cryostorage of Gisela 5 rootstock.

Keywords: *In vitro conservation, Aluminium cryo-plates, Vitrification, Air dehydration.*

Introduction

Tissue culture techniques are not only being commonly applied for collection, rapid multiplication and exchange of plant material, but also for short- and long-term conservation of vegetatively propagated plant species, including different fruit crops. Application of *in vitro* techniques for conservation of plant genetic resources started in the early 1980s with development of slow growth techniques (Engelmann, 2011) mostly intended for medium-term storage due to the likelihood of genetic instability and possible losses caused by contamination in the process of repeated subculturing during long-term period (González-Benito *et al*., 2004).

Cryopreservation is an effective approach for long-term germplasm storage at ultra low temperatures in liquid nitrogen (LN, −196 °C), thus ensuring no metabolic processes to occur (Reed, 2017). In this way, plant material can be conserved in principle indefinitely, with no need for subculturing and with reduced threat of somaclonal variation. In the last three decades a number of different cryopreservation protocols, from the original slow cooling approach to easier and more reproducible vitrification-based cryopreservation techniques such as encapsulation-dehydration, vitrification, encapsulation-vitrification and droplet vitrification have been developed and utilized for germplasm storage (Agrawal *et al*., 2019). Vitrification-based cryopreservation techniques eliminate the need for expensive, programmable freezing equipment (controlled-rate freezers) and permit tissues to be cryopreserved by direct transfer to LN. Implementation of these

techniques has extended the applicability of cryopreservation to a broad range of plant species including different *Prunus* species (Benelli *et al*., 2013). Vitrification and droplet-vitrification techniques were also successfully applied for cryopreservation of cherry rootstock Gisela 5 (Ružić et al., 2013; Ružić *et al*., 2014). However, these methods require skilful manipulation to avoid damage and subsequent loss of shoot tips as well as insufficient or excessive dehydration during procedure (Yamamoto *et al*., 2011).

Recently, two efficient and simple cryopreservation methods using aluminium cryo-plates (V cryo-plate and D cryo-plate) have been developed. V cryo-plate method (Yamamoto *et al*., 2011) is based on dehydration of explants with Plant vitrification solution 2 (PVS2; Sakai *et al*.,1990) while D cryo-plate method (Niino *et al*., 2013) is based on dehydration in the air current of the laminar flow cabinet or over silica gel (desiccation). Both protocols appear promising for cryopreservation of both herbaceous and woody plants (Niino *et al*., 2019) including different fruit tree species after appropriate modifications of the procedures. They were successfully applied to strawberry (Yamamoto *et al*., 2012a), mulberry (Yamamoto *et al*., 2012b), blueberry (Dhungana *et al*., 2017), as well as cherry plum and European plum (Vujović *et al*., 2015).

Although previous research on cryopreservation of cherry rootstock Gisela 5 showed that vitrification and droplet-vitrification techniques can be successfully applied for long-term storage of *in vitro* shoot tips of this genotype (Ružić *et al*., 2013; Ružić *et al*., 2014), the present study was intended to simplify cryopreservation procedure and to improve survival and regrowth success using V cryo-plate and D cryo-plate methods.

Material and Methods

Tissue-cultured shoots of cherry rootstock Gisela 5 (*Prunus cerasus* × *Prunus canescens*) were used for the experiments. Aseptic culture of this genotype was established according to the protocol previously described by Ružić *et al*. (2015) and shoots were multiplied on Murashige and Skoog (1962) medium (MS) containing 3.37 mg l^{-1} N6-benzyladenine (BA). Cultures were maintained in a growth chamber at 23 ± 1°C (16 h photoperiod, light intensity 54 µmol m^{-2} s^{-1}) and subcultured at 3-week intervals to obtain a sufficient number of plantlets for cryopreservation experiments. Excised axillary buds were precultured for 1 day at 23 ºC in the dark on solidified MS medium with 0.3 M sucrose.

Precultured explants were placed in wells on aluminium cryo-plates previously filled with 2% (w/v) sodium alginate in calcium-free MS basal medium with 0.4 M sucrose (about 4 µl). MS basal medium containing 0.1 M calcium chloride and 0.4 M sucrose was poured on the aluminium plates over shoot tips and left for 20 min to achieve complete polymerization of sodium alginate. Cryo-plates with explants were transferred to loading solution (LS) with 1.9 M glycerol and 0.5 M sucrose in liquid MS medium for 30 min at room temperature.

The V cryo-plate procedure was performed using PVS A3 (22.5% sucrose, 37.5% glycerol, 15% ethylene glycol and 15% dimethyl sulfoxide) (Kim *et al*. 2009) for 40 min, or PVS3 (50% glycerol and 50% sucrose) (Nishizawa *et al*. 1993) for 60 min. Dehydration with both types of VSs was done at room temperature while durations of treatments were selected as the most efficient ones in the previous droplet-vitrification experiments (Ružić *et al*., 2013).

In D cryo-plate procedure dehydration included desiccation of the explants attached to the cryo-plates in closed containers over 40 g of silica gel at 23°C for 2, 2.5 and 3 h.

After dehydration, in both protocols cryo-plates with explants were transferred to 2 ml uncapped cryotubes, directly immersed in LN and kept for 1 h. For rewarming, aluminium cryo-plates were immersed in an unloading solution (0.8 M sucrose) for 30 min at room temperature. Then, explants were transferred onto the regrowth medium (MS medium with 3.37 mg l^{-1} BA), cultivated in the dark for seven days, and then under standard conditions.

Each critical step in both procedures had proper control: pregrowth control – after pregrowth explants were directly transferred onto the regrowth medium; loading control – explants

exposed to loading solution but neither dehydrated nor cryopreserved; dehydration controls – following loading explants were dehydrated with VS or desiccated and directly unloaded without immersion in LN.

Survival was evaluated two weeks after samples retrieval from LN by counting the number of explants showing any signs of regeneration. Regrowth was defined as further development of apices into viable shoots up to the sixth week. Each experimental treatment was replicated three times with each replication containing 10–12 shoot tips. Statistical analysis was performed using one-way ANOVA and then Duncan's Multiple Range Test ($P < 0.05$) to compare the means. Data presented in the form of percentage were subjected to arcsine transformation before analysis of variance.

Shoots originating from different treatments were separately transferred onto the MS multiplication medium. Multiplication index and length of axial and lateral shoots were monitored in the third subculture after regrowth. In the following subculture the shoots were rooted on the medium with ½ MS mineral salts, organic complex unchanged, 1 mg l^{-1} 1-naphthaleneacetic acid and 0.1 mg l^{-1} gibberellic acid. The following parameters were monitored: percentage of rooting, number and length of roots, and length of rooted shoots.

Results and Discussion

In the present study, V cryo-plate and D-cryo-plate methods were successfully applied to *in vitro* shoot tips of Gisela 5 cherry rootstock. During cryopreservation process we monitored both survival (presence of green tissue) and regrowth of explants (shoot growth and development) after every critical step in both protocols (Table 1). Namely, pregrowth, osmoprotection, exposure to highly concentrated VSs (V cryo-plate) or air dehydration (D cryo-plate) are of vital importance for successful cryopreservation, but each of these steps also affects viability of non-cryopreserved explants.

As regards survival, no significant differences were observed among pregrowth, loading, dehydration controls and cryopreserved explants (Table 1). Survival rates were very high and ranged between 95.9% and 100%. However, loading treatment significantly reduced regrowth capacity of explants (90%) in comparison with pregrowth control (100%; Table 1). In the V cryo-plate protocol dehydration treatments (PVS A3 and PVS3) did not further affect regrowth of control explants (90%). After cryostorage, significantly higher regrowth rate was obtained in explants dehydrated with PVS A3 solution (95.9%) in comparison with those dehydrated with PVS3 (85%). Plantlets, regenerated after LN exposure, were vigorous and displayed normal morphology (Figure 1a–b). Regrowth rates of dehydration controls (both PVS A3 and PVS3) and cryopreserved shoot tips achieved in our experiment are more than twice higher than those obtained by Ružić *et al.* (2013) in droplet-vitrification experiments conducted under the same dehydration conditions. Similarly, Vujović *et al.* (2015) reported significant improvement in the regrowth of cryopreserved explants of cherry plum using V cryo-plate method. Yamamoto *et al.* (2011) assumed that calcium alginate gel used to attach explants to aluminium plates mitigates the cytotoxicity of PVSs. In addition, V cryo-plate method enables more precise time control of dehydration and reduces possibility of injuring shoot tips during manipulations.

In contast to results obtained with V cryo-plate method, considerable lower regrowth rates in control (50–83.3%) and frozen explants (45.8–66.7%) were achieved using D cryo-plate method (Table 1). The highest regeneration ability of both types of explants was obtained after 2.5 h of desiccation. In all experimental treatments shoots regenerated from cryopreserved explants were vigorous, but exhibited symptoms of hyperhydricity (Figure 1 c–e). According to Niino *et al.* (2014) the D cryo-plate method may be used with larger explants, that enables more uniform physical dehydration. Further, optimisation of hormonal composition of recovery

medium and adding antioxidants to the cryoprotectant and/or to recovery medium may improve regrowth after LN exposure (Uchendu *et al.*, 2010).

Table 1. Survival and regrowth of pregrowth, loading and dehydration controls (-LN) and cryopreserved explants (+LN) of Gisela 5 rootstock using V and D cryoplate methods

Treatment/duration	Survival (%)		Regrowth (%)	
	-LN	+LN	-LN	+LN
Pregrowth control	100.0*	-	100.0 a	-
Loading control	100.0	-	90.0 bc	-
PVS A3/40 min room t°	100.0	100.0	90.0 bc	95.9 ab
PVS3/60 min room t°	100.0	100.0	90.0 bc	85.0 c
2 h desiccation	100.0	95.9	58.3 ef	45.8 f
2.5 h desiccation	100.0	95.9	83.3 c	66.7 de
3 h desiccation	100.0	100.0	50.0 ef	66.7 de
Significance	ns		P < 0.05	

Mean values for regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test; ns – non significant.

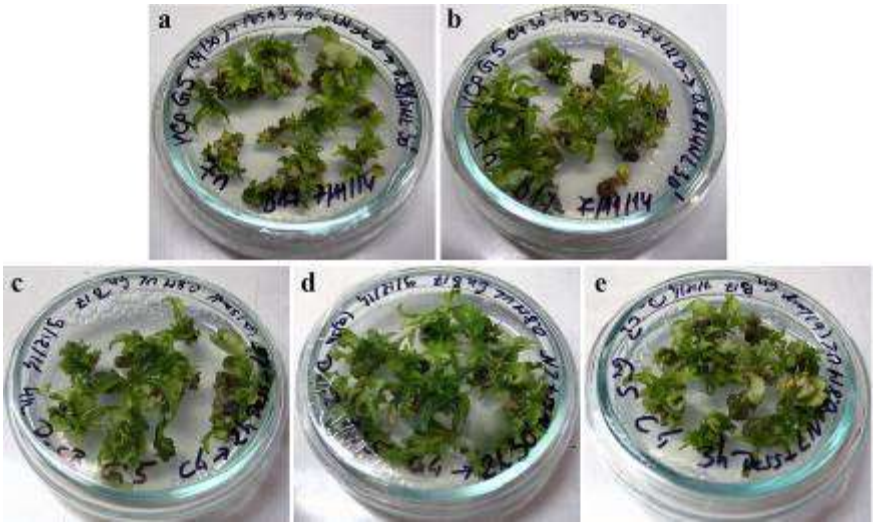

Figure 1. Regrowth of cryopreserved shoot tips of Gisela 5 rootstock: explants dehydrated with PVS A3 for 40 min (a) and with PVS3 for 60 min (b); explants desiccated for 2 h (c), 2,5 h (d) and 3 h (e)

With consideration given to factors of high survival, high regrowth, and simplicity of the protocols, both methods have been demonstrated as a highly effective for cryogenic storage of Gisela 5 rootstock. However, successful utilisation of any cryopreservation technique dependends not only on efficient regrowth after thawing, but also on efficient propagation and subsequent rooting of true-to-type regenerants. In our experiments, loading as well as all PVS and desiccation treatments significantly increased capacity for multiplication of regenerated shoots in comparison with those originated from pregrowth controls (Table 2). Cryopreservation slightly decreased multiplication index of regenerated plantlets compared with corresponding controls. The only exception was PVS A3 treatment where shoots originated from cryopreserved explants displayed the highest multiplication capacity (index of multiplication being 4). As regards length of axial and lateral shoots, significantly higher values were recorded for shoots originated from the dehydration and correspondent cryopreservation treatments in V cryo-plate than in D cryo-plate protocol. Monitoring of rooting parameters also revealed significant variations among the shoots originating from different treatments (Table 2). In general, with few exceptions, in the third subculture after regrowth, rooting ability, particularly the rooting rate, was similar or even greater in shoots originating from all types of

control and cryopreserved explants in comparison with those originating from pregrowth and loading controls. Among shoots regenerated from cryopreserved shoot tips the highest rooting rate (100%) was noticed for those dehydrated with PVS A3 in V cryo-plate protocol. Contrary to these results, Pawłowska and Szewczyk-Taranek (2015) did not find significant differences in multiplication and rooting ability between plants regenerated from control and cryopreseved explants of rosa species.

Table 2. Multiplication (third subculture) and rooting (fourth subculture) of Gisela 5 shoots of different origin after regrowth

Shoot origin	Multiplication parameters			Rooting parameters			
	MI	Length of axial shoot (mm)	Length of lateral shoots (mm)	Rooting rate (%)	No. of roots	Root length (mm)	Shoot length (mm)
Pregrowth control	2.1 f	14.1 bc	5.7 f	75.6 b	5.3 cd	29.0 f	24.2 a
Loading control	2.9 d	14.4 b	7.8 a	68.9 bc	5.9 b	36.0 ab	15.5 cde
PVS A3 40 min-LN	3.7 b	14.2 bc	7.1 bc	75.6 b	4.9 de	33.8 abcd	23.9 a
PVS A3 40 min+LN	4.0 a	15.8 a	7.6 a	100.0 a	4.6 ef	31.3 cdef	18.1 c
PVS3 60 min-LN	3.3 c	13.5 bcd	7.4 ab	73.3 bc	4.6 ef	33.4 bcde	15.3 de
PVS3 60 min+LN	2.9 d	16.3 a	7.7 a	62.2 c	4.8 de	28.0 fg	16.8 cde
2 h desiccation-LN	3.0 d	12.8 cde	5.7 f	63.5 c	4.4 ef	34.7 abc	15.0 de
2 h desiccation+LN	2.5 e	11.9 e	6.0 ef	76.7 b	7.0 a	34.6 abc	17.5 cd
2.5 h desiccation-LN	2.5 e	11.7 e	6.8 cd	80.0 b	3.9 g	37.4 a	14.6 e
2.5 h desiccation+LN	2.6 e	13.6 bcd	6.5 de	80.0 b	4.5 ef	29.7 def	17.3 cd
3 h desiccation-LN	2.9 d	12.2 de	6.3 ef	76.7 b	4.2 fg	25.0 g	20.7 b
3 h desiccation+LN	2.5 e	12.3 de	6.2 ef	76.7 b	5.6 bc	30.0 def	17.6 cd

Mean values of all analyzed parameters within each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$); MI – multiplication index.

Conclusions

In conclusion, we showed that cryopreservation methods using aluminium cryo-plates, especially V cryo-plate method, markedly improve cryostorage success of *in vitro* shoot tips of Gisela 5 rootstock. After marginal modifications of the procedure, D cryo-plate method could efficiently complement the V cryo-plate method. Also, these procedures are simple and user-friendly and can be performed by semi-skilled operators with limited experience in cryopreservation. However, before their large scale application can be foreseen, the optimized protocols should be validated through their application to a range of *Prunus* genotypes.

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