DE GRUYTER OPEN

Contemporary Agriculture
Vol. 66, No. 1 - 2, Pp. 44 - 50, 2017.

The Serbian Journal of Agricultural Sciences
ISSN (Online) 2466-4774 UDC: 63(497.1)(051)-"540.2"
www.contagri.info

Short communication

UDC: 582.711.712
DOI: 10.1515/contagri-2017-0008

EFFECT OF THE DURATION OF LIQUID NITROGEN STORAGE ON THE REGROWTH OF BLACKBERRY CRYOPRESERVED BY DROPLET VITRIFICATION*

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Summary: In vitro shoot tips of the blackberry cultivar 'Čačanska Bestrna' were cryopreserved using the droplet vitrification technique. Upon loading (30 min) in a solution of 1.9 M glycerol and 0.5 M sucrose, the explants were dehydrated for 40 min on ice with the PVS A3 vitrification solution (glycerol 37.5%, dimethyl sulfoxide 15%, ethylene glycol 15% and sucrose 22.5%) and for 40 min at room temperature with the PVS3 solution (glycerol 50% and sucrose 50%). They were subsequently frozen in individual microdroplets of vitrification solution, by direct immersion in liquid nitrogen (LN), and kept therein for 2, 4, 8 and 24 h. The explant rewarming was performed in an unloading solution (0.8 M sucrose) for 30 min at room temperature. The duration of LN exposure did not exert significant effects on the survival and regrowth of explants in both types of vitrification solutions. The survival and regrowth of cryopreserved shoot tips dehydrated with PVS3 solution ranged between 90–95% and 80–90%, respectively. However, dehydration with PVS A3 resulted in a lower survival rate (80–90%) and a considerably lower regrowth rate (55–65%) of explants. Monitoring the shoots regenerated in the in vitro culture revealed their normal capacity for multiplication and rooting in comparison with the controls, which fully confirms the purpose of cryopreservation in the long-term preservation of plant material.

Keywords: *blackberry, in vitro cryopreservation, vitrification solutions, liquid nitrogen.*

INTRODUCTION

Advances achieved in plant biotechnology, especially those associated with *in vitro* culture and molecular biology, provide new options for a continuous supply of plant germplasm for replanting, selection of elite individuals, eradication of systemic infections, collection, as well as short- and long-term conservation of plant genetic resources (Cruz-Cruz et al. 2013). Conservation of plant species contributes to a sustainable use of plant diversity and is essential for both conventional and modern plant breeding programmes. Latest biotechnology-based conservation techniques, including different *in vitro* techniques, are complementary to conventional *ex situ* conservation methods and represent a safety precaution against the accidental loss of plant germplasm collections. In recent years, cryopreservation, i.e. the storage of plant material at the ultra-low temperature (-196 ºC) of liquid nitrogen (LN), has become a very important tool for the long-term conservation of plant germplasm. It is the only safe and cost-efficient option for the long-term conservation of various categories of plants, including non-orthodox seed species, vegetatively propagated plants, rare and endangered species, and biotechnology products (Engelmann, 2004). However, a successful large-scale implementation of cryopreservation techniques for long-term plant conservation requires the availability of efficient cryopreservation protocols applicable to diverse germplasm (Panis and Lambardi, 2005). The implementation of new vitrification-based techniques, such as vitrification, encapsulation dehydration, encapsulation vitrification and droplet vitrification, has extended the applicability of cryopreservation to a wide range of plant species (Engelmann, 2004; Sakai and Engelmann, 2007), including numerous fruit tree species (Benelli et al. 2013).

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* This research was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (project grant No.TR-31064)
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Since its establishment, the Tissue Culture Laboratory of the Fruit Research Institute has successfully employed several *in vitro* methods for medium-term conservation of fruit germplasm – the cold storage of *in vitro* shoots and encapsulated shoot tips (synthetic seed technology). In recent years, the application of different *in vitro* cryopreservation techniques (encapsulation dehydration, vitrification, droplet vitrification, V cryo-plate and D cryo-plate methods) for long-term conservation of temperate fruit species has also been studied. Vitrification-based cryopreservation techniques have been successfully used in different representatives of *Rubus* genus. The blackberry cultivar 'Čačanska Bestrna' (*R. fruticosus* L.) was cryopreserved using the encapsulation dehydration technique (Ružić and Vujović, 2012; Ružić et al. 2013). In addition, the procedure of droplet vitrification was also optimized for cryopreserving the raspberry cultivar 'Latham' (*R. idaeus* L.) (Condello et al. 2011) and the blackberry cultivar 'Čačanska Bestrna' (Vujović et al. 2011; 2015). 'Čačanska Bestrna' blackberry indicated a high capacity for regeneration after cryopreservation using the droplet vitrification technique, and the protocol optimisation was performed by evaluating the effect of different vitrification solutions, treatment durations, dehydration temperatures and unloading durations on the recovery of explants after the LN exposure.

This paper investigates the influence of the duration of LN storage on the survival and regrowth of blackberry shoot tips dehydrated with two vitrification solutions and cryopreserved by droplet vitrification technique, using the protocol previously described by Vujović et al. (2015). In addition, the multiplication and rooting ability of regenerated shoots were monitored and compared.

MATERIALS AND METHODS

Plant material. 'Čačanska Bestrna' (*Rubus fruticosus* L.) is a blackberry cultivar developed at the Fruit Research Institute Čačak as a cross of 'Dirksen Thornless' and 'Black Satin'. The cultivar displays excellent performance with regard to cropping and resistance to diseases and low winter temperatures. It is suitable for fresh consumption, freezing and various forms of processing. The aseptic culture of this genotype was previously established using the procedure described by Ružić and Lazić (2006). The *in vitro* shoots were multiplied on the Murashige and Skoog (1962) medium (MS medium) containing 1 mg l^{-1} N6-benzyladenine (BA), 0.1 mg l^{-1} indole-3-butyric acid (IBA), 0.1 mg l^{-1} gibberellic acid (GA_3), 20 g l^{-1}sucrose and 7 g l^{-1}agar. The cultures were maintained in a growth chamber at 23 ± 1 °C, under a 16 h light/8 h dark photoperiod and a light intensity of 54 µmol m^{-2} s^{-1}.

Cryopreservation protocol. The cryopreservation experiments were performed using 1–2 mm long apical shoot tips excised from *in vitro* shoots, which were maintained on the MS multiplication medium for 3 weeks. The explants taken were cryopreserved using the optimized droplet vitrification protocol (Vujović et al. 2015): (1) pre-treatment of explants in the liquid MS medium with BA 1, IBA 0,1 and GA_3 0,1 mg l^{-1} and a progressively increasing sucrose concentration (0.3 M for 15 h and 0.7 M for 5 h), in the dark at 23 °C; (2) loading treatment with the hormone-free liquid MS medium comprising 1.9 M glycerol and 0.5 M sucrose (C4 solution; Kim et al. 2009a), 30 min at room temperature; (3) dehydration with PVS3 [50% glycerol (w/v) and 50% (w/v) sucrose; Nishizawa et al. 1993] 40 min at room temperature and the modified PVS2 solution [PVS A3 – 37.5% (w/v) glycerol, 15% (w/v) dimethylsulfoxide, 15% (w/v) ethylene glycol and 22.5% (w/v) sucrose; Kim et al. 2009b] 40 min on ice; (4) transfer of explants in 10 µ droplets of vitrification solution attached to aluminium foil strips; (5) direct plunging of aluminium foil strips in LN where they were kept for 2, 4, 8 and 24 h; (6) rapid warming by direct plunging of aluminium foils in a preheated (37°C) unloading solution (0.8 M sucrose) for 30 s, and further 30 min-incubation in the equal volume of unloading solution at room temperature. The following adequate controls were included for each step of the procedure: (1) pre-growth control refers to shoot tips just pre-treated in the liquid MS medium and directly placed on the growth medium; (2) loading controls were loaded but both non-dehydrated and non-cryopreserved; (3) dehydration controls were treated in the same way as the cryopreserved explants but without the LN immersion.

Monitoring of survival and regrowth. For recovery, shoot tips were transferred to the solid MS medium for regeneration (corresponding to the previously described multiplication medium), cultivated in the dark for 7 days, and then transferred to the standard growth conditions (16 h light/8 h dark). Survival was recorded as a percentage of the total number of shoot tips showing any signs of growth 3 weeks after rewarming and culture, whereas regrowth was defined as a further development of apices into shoots with developed leaves 7 weeks after rewarming.

Two replicates of 10 shoot tips were used in each treatment. Statistical significance between the treatments was tested using the analysis of variance (ANOVA) and Duncan's Multiple Range Test for mean separation (at $P < 0.05$). Prior to the analysis, the data presented in the form of a percentage were subjected to arcsine transformation.

Multiplication and rooting after regrowth. The shoots originating from different treatments were isolated separately, labelled according to their origin and transferred onto the MS multiplication medium of the same hormonal composition as the regrowth medium. The multiplication capacity was monitored in the second subculture after regrowth (a 28 day-culture interval), and the multiplication index was observed alongside the length of axial and lateral shoots. The shoots were rooted on the MS medium with mineral salts reduced to ½-strength, organic complex unchanged, 1.0 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3

and 1 g l^{-1} of active charcoal. The rooting capacity of the shoots originating from the control and cryopreserved shoot tips was monitored after 28 days of culture, and the following parameters were included: percentage of rooted plantlets, number and length of roots, and length of rooted shoots. All the data were analysed by ANOVA, followed by the Duncan's Multiple Range Test, at $P < 0.05$.

RESULTS

Preculture and loading had no significant effect on the survival (100%) and regrowth (100%) of non-cryopreserved blackberry shoot tips (Table 1). The duration of LN exposure did not significantly affect the survival and regrowth of cryopreserved explants dehydrated with the same vitrification solution. However, the type of vitrification solution had significant influence on the regrowth of explants. The survival and regrowth of cryopreserved explants dehydrated for 40 min at room temperature with the PVS3 solution ranged between 90–95%, and 80–90%, respectively. Both parameters were not significantly different in comparison with the corresponding control parameters, i.e. the non-cryopreserved explants dehydrated with the same vitrification solution (90% for both parameters). Dehydration with the PVS A3 solution for 40 min on ice resulted in a considerably lower survival (75–85%) and regrowth rates (55–65%) of cryopreserved explants compared to the PVS3 treatments. In addition, the survival and regrowth of control shoot tips dehydrated with the PVS A3 solution were significantly higher (100% for both parameters) in comparison with those obtained for the cryopreserved explants after different periods of storage in LN.

Table 1. Survival and regrowth of the control explants and cryopreserved shoot tips of 'Čačanska Bestrna' blackberry – the effect of the liquid nitrogen (LN) exposure duration

Treatment/duration	Survival (%)	Regrowth (%)	No. of regenerants per explant
Pregrowth control	100,0 a*	100,0 a	3,3 a
Loading control	100,0 a	100,0 a	2,0 e
PVS3 - LN	90,0 bc	90,0 b	2,9 ab
PVS3/2h LN	90,0 bc	85,0 b	3,0 ab
PVS3/4h LN	95,0 ab	80,0 b	2,2 de
PVS3/8h LN	95,0 ab	85,0 b	2,4 cd
PVS3/24h LN	90,0 bc	90,0 b	2,2 de
PVS A3 - LN	100,0 a	100,0 a	3,1 a
PVS A3/ 2h LN	80,0 c	65,0 c	3,1 a
PVS A3/ 4h LN	85,0 bc	60,0 c	2,7 bc
PVS A3/ 8h LN	75,0 c	55,0 c	3,3 a
PVS A3/ 24h LN	80,0 c	65,0 c	3,0 ab

*The mean values within each column followed by the same letter are not significantly different according to the Duncan's Multiple Range Test ($P < 0.05$); LN – dehydrated controls

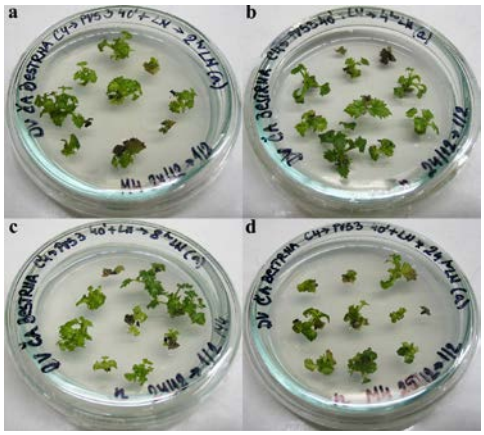

Figure 1. Regrowth of cryopreserved shoot tips of the blackberry dehydrated for 40 min with PVS3 at room temperature and stored in LN for 2 h (a), 4 h (b), 8 h (c) and 24 h (d) (6 weeks after thawing)

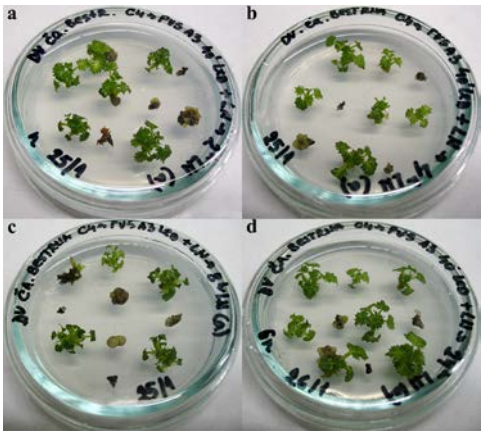

Figure 2. Regrowth of cryopreserved shoot tips of the blackberry dehydrated for 40 min with PVS A3 on ice and stored in LN for 2 h (a), 4 h (b), 8 h (c) and 24 h (d) (6 weeks after thawing)

The mean number of regenerated shoots per explant varied significantly among the explants kept in LN for different periods, that was particularly prominent in those dehydrated with the PVS3 solution (2.2–3.0), with the highest value observed after 2 hours of storage in LN. The range of variation was narrow (2.7–3.3) in the explants dehydrated with the PVS A3 solution, and the lowest value was observed after 4 hours of storage in LN. However, the shoots regenerated from the explants originating from different treatments had similar morphology, and they developed into normal plantlets with green leaves and without any signs of hyperhydricity (Figures 1 and 2).

The multiplication capacity in the second subculture after regrowth varied significantly among the shoots originating from different treatments (Table 2). The highest values of the multiplication index were recorded in the dehydration controls (4.89 for PVS3 and 4.19, for PVS A3) and in the shoots originating from the explants stored for 2 h in LN (5.17 and 4.22, resp.). The length of axial shoots was greater in those originating from apices dehydrated with the PVS A3 and ranged between 0.96 cm and 1.07 cm, whereas the value of this parameter was between 0.83 cm and 0.99 cm in the shoots regenerated from PVS3-treated explants. However, regardless of the type of vitrification solution and the duration of LN exposure, the shoots regenerated from cryopreserved explants were well-developed, vigorous and displayed normal morphology typical of the genotype (Figures 3a and b).

Table 2. *Multiplication (the second subculture after regrowth) and rooting parameters (the third subculture after regrowth) of the 'Čačanska Bestrna' blackberry shoots of different origin*

	Multiplication parameters			**Rooting parameters**			
Treatment	**Multiplication index**	**Length of axial shoot (cm)**	**Length of lateral shoots (cm)**	**Rooting rate (%)**	**Number of roots**	**Root length (cm)**	**Rooted shoot length (cm)**
Pregrowth control	4,25 bc*	0,95 bc	0,66 def	68,8 e	2,0 d	2,8 bc	1,5 c
Loading control	4,25 bc	1,07 a	0,69 cde	72,2 de	2,6 bcd	2,4 cd	1,6 c
PVS3 - LN	4,89 a	0,84 d	0,62 ef	83,3 cde	2,6 bcd	2,4 cd	2,0 a
PVS3/2h LN	5,17 a	0,84 d	0,65 def	75,0 de	2,4 cd	2,6 cd	1,9 ab
PVS3/4h LN	4,94 a	0,99 ab	0,69 cde	77,3 de	2,6 cd	2,4 cd	2,1 a
PVS3/8h LN	4,17 bc	0,87 cd	0,61 f	93,8 bc	2,9 bc	2,9 bc	2,0 a
PVS3/24h LN	4,39 b	0,82 d	0,61 f	95,8 ab	3,2 b	2,1 def	1,9 ab
PVS A3 - LN	4,19 bc	1,05 ab	0,68 def	87,5 cd	2,4 cd	3,1 b	2,0 a
PVS A3/2h LN	4,22 bc	1,05 ab	0,78 a	100,0 a	4,7 a	1,8 ef	1,8 abc
PVS A3/4h LN	3,78 c	0,96 bc	0,70 bcd	100,0 a	4,3 a	3,7 a	2,0 a
PVS A3/8h LN	4,00 bc	1,05 ab	0,77 ab	78,1 de	4,3 a	1,6 f	1,7 bc
PVS A3/24h LN	4,22 bc	1,07 a	0,76 abc	82,1 cde	4,3 a	2,2 de	1,5 c

*The mean values within each column followed by the same letter are not significantly different according to the Duncan's Multiple Range Test ($P < 0.05$); LN – dehydrated controls

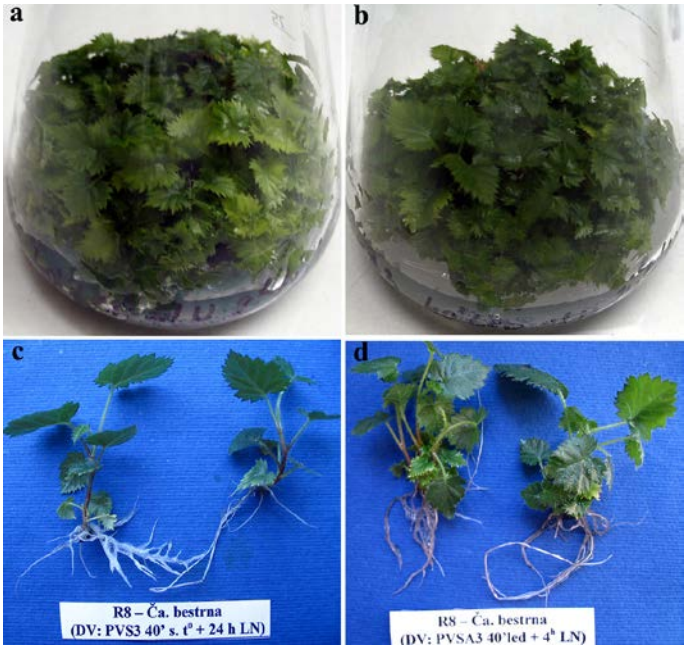

Figure 3. Shoots regenerated from cryopreserved explants in the multiplication stage – a) dehydrated with PVS3 and stored for 2 h in LN; b) dehydrated with PVS A3 and stored for 24 h in LN, and in the rooting stage – c) dehydrated with PVS3 and stored for 24 h in LN; b) dehydrated with PVS A3 and stored for 4 h in LN

The monitoring of rooting parameters also revealed significant variations among the shoots originating from the explants stored for different periods in LN (Table 2). The rooting percentage of the shoots originating from the cryopreserved explants was between 75.0–95.8% for those dehydrated with PVS3, and between 78.1–100% for the explants dehydrated with PVS A3 (Figures 3c and d). A significantly greater root number (4.3–4.7) was observed in the shoots originated from the PVS A3-treated explants in comparison with those treated with PVS3 (2.4–3.2). Variations among the treatments were notably less pronounced with regard to other rooting parameters.

DISCUSSION

The droplet vitrification technique, which combines the application of highly concentrated vitrification solutions with ultra-high cooling/warming rates, has been proven very effective in cryopreservation of broad range of plant species and tissues (Panis et al. 2011). According to the authors, the optimized protocol can be developed within the period ranging from 4 months to 2 years, depending on plant species and previous research related to *in vitro* culture and cryopreservation of selected species/genotype. The previous studies on the optimization of the protocol for the cryopreservation of 'Čačanska Bestrna' blackberry revealed that the droplet vitrification technique is favourable for cryopreservation of *in vitro* shoot tips of this genotype, particularly if samples to be frozen are dehydrated with the PVS3 solution (Vujović et al. 2015). The research results revealed that the genotype is highly tolerant to osmotic stress induced by vitrification solutions and can withstand a broad range of treatment durations (40–120 min). The highest regrowth rate of the cryopreserved explants (90%) was achieved after 40 min-dehydration at room temperature. The present study also shows that the type of vitrification solution has significantly affected both the survival and regrowth of cryopreserved explants. Namely, the regrowth rates after the PVS3-based dehydration was similar to those previously obtained by Vujović et al. (2015). A modified PVS2 vitrification solution (PVS A3) was less effective for cryopreservation of this genotype, probably due to its high chemical toxicity. The regrowth rates of cryopreserved shoot tips achieved after the PVS A3 dehydration for 40 min on ice did not notably differ from the previous results (61.8%). Although the most commonly employed vitrification solution for cryopreservation of plant tissues is PVS2, it is very toxic for most plant species, thus the dehydration duration has to be determined very precisely (Kim et al. 2009b). Sample dehydration at 0 °C mitigates the cytotoxicity of this vitrification solution. The positive effect of PVS2 treatment on ice in comparison with room temperature was also reported in other plant species, such as *Musa* sp. (Panis et al. 2005), *Citrus* sp. (Cho et al. 2002), *Colocasia* sp. (Sant et al. 2008). In addition, Cho et al. (2002) highlighted the operational flexibility of the technique employing dehydration treatment at 0 °C, since optimal recovery can be achieved over much wider range of treatment durations.

This research was conducted with the aim to determine whether the storage time in liquid nitrogen significantly affects the survival and regrowth of cryopreserved explants. During cryopreservation, plant material is stored at the ultra-low temperature (-196 ^{o}C), at which all cellular divisions and metabolic processes are stopped. Therefore, plant material is expected to be stored without alteration or modification for a theoretically unlimited period of time (Engelmann, 2004). The results obtained revealed that the duration of LN storage in the tested time intervals (2 h, 4 h, 8 h and 24 h) did not significantly affect the survival and regrowth of the explants dehydrated with the same vitrification solution. Soliman (2013) also reported that the survival and regrowth percentage of the apricot shoot tips did not differ with storage duration.

However, the development of an *in vitro* system, which provides the efficient and vigorous regrowth after retrieving explants from LN and rapid multiplication of regenerated shoots, is very important in the cryopreservation process. Cryopreservation imposes a series of stresses to plant material, which can affect cryopreserved cultures, as well as regenerated plantlets. The research on the multiplication and rooting capacity of shoots after the LN exposure revealed that the multiplication and rooting capacity varied among both the shoots regenerated from apices dehydrated with different vitrification solutions and the shoots kept in LN for different period of time. Higher values of the multiplication index in the shoots regenerated from the PVS3-dehydrated explants were also reported in our previous study (Vujović et al. 2015). Various effects of the duration of LN storage were observed in the multiplication and rooting ability among the shoots originating from the cryopreserved explants. However, the shoots regenerated from the cryopreserved explants mostly did not indicate significantly lower values of these parameters in comparison with the corresponding controls.

CONCLUSION

The results obtained confirmed that the PVS3-based droplet vitrification technique is highly effective for the cryopreservation of the blackberry cultivar 'Čačanska Bestrna'. Satisfactory percentages of recovery were also obtained with the explants dehydrated in the modified PVS2 solution. Examining the influence of the duration of liquid nitrogen storage indicated that there were no significant variations in the regeneration capacity of the cryopreserved explants within the tested time intervals (2 h, 4 h, 8 h and 24 h). The further monitoring of the regenerated shoots in the *in vitro* culture revealed that they have a normal capacity for multiplication and rooting compared to the controls, which fully confirms the purpose of cryopreservation in the long-term preservation of plant material.

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UTICAJ DUŽINE ČUVANJA U TEČNOM AZOTU NA REGENERACIJU KUPINE KRIOPREZERVIRANE TEHNIKOM „DROPLET" VITRIFIKACIJE

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Izvod: Vrhovi *in vitro* izdanaka sorte kupine Čačanska bestrna su krioprezervirani primenom tehnike „droplet" vitrifikacija. Po završenom osmotskom tretmanu (30 min) u rastvoru sa 1,9 M glycerolom i 0,5 M saharozom, eksplantati su dehidrirani 40 min na ledu vitrifikacionim rastvorom PVS A3 (glicerol 37,5%, dimetil sulfoksid 15%, etilen glikol 15% i saharoza 22,5%) ili 40 min na sobnoj temperaturi PVS3 rastvorom (glicerol 50% i saharoza 50%). Eksplantati postavljeni u kapljice vitrifikacionog rastvora na aluminijumskoj foliji su direktno uranjani u tečni azot gde su držani 2, 4, 8 i 24 h. Osmotska rehidracija je vršena 30 min u rastvoru sa 0,8 M saharozom, na sobnoj temperaturi. Dužina čuvanja eksplantata u tečnom azotu nije imala značajan uticaj na procenat preživljavanja i regeneracije eksplantata dehidriranih sa oba vitrifikaciona rastvora. Preživljavanje i regeneracija eksplantata koji su dehidrirani sa PVS3 su iznosili 90–95% i 80–90%, resp. Međutim, dehidracija sa PVS A3 je rezultirala nižim procentima preživljavanja (80–90%) i regeneracije (55–65%) eksplantata. Praćenjem regenerisanih izdanaka u *in vitro* kulturi utvrđeno je da oni imaju normalan kapacitet za multiplikaciju i ožiljavanje u poređenju sa kontrolnim izdancima čime je u potpunosti potvrđena svrha krioprezervacije za dugotrajno čuvanje biljnog materijala.

Ključne reči: kupina, *in vitro* krioprezervacija, vitrifikacioni rastvori, tečni azot.

Received / Primljen: 20.03.2017.
Accepted / Prihvaćen: 10.07.2017.