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Original scientific article

CRYOPRESERVATION OF SERBIAN AUTOCHTHONOUS PLUM 'CRVENA RANKA' USING ALUMINIUM CRYO-PLATES

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'Crvena Ranka' is one of the oldest autochthonous plum cultivars in Serbia. However, it is endangered due to the long-term pathogen pressure and continuous suppression by commercially important cultivars and therefore faced with genetic erosion. This study was carried out to investigate the suitability of two efficient and simple cryopreservation methods using aluminium cryo-plates for its conservation. Precultured shoot tips of this genotype were embedded in alginate gel into cryo-plates wells. Osmoprotection was performed using loading solution with 1.9 M glycerol and 0.5 M sucrose (30 min at room temperature). In the V cryo-plate protocol, explants were dehydrated at room temperature for 20 or 40 min with PVS A3 (37.5% glycerol, 15% dimethylsulfoxide, 15% ethylene glycol and 22.5% sucrose) or with PVS3 (50% glycerol and 50% sucrose) for 60 min. In the D cryo-plate protocol, desiccation for 2, 2.5 or 3 h was performed over silica gel. Then, the cryo-plates were directly immersed into liquid nitrogen. Unloading was done in MS medium containing 0.8 M sucrose (30 min at room temperature). In the V cryo-plate procedure regrowth of cryopreserved explants dehydrated with PVS A3 was between 50% and 51.9%, while in those dehydrated with PVS3 it was 66.7%. As for the D cryo-plate method, regrowth of cryopreserved explants ranged between 30–40%. After regrowth, shoots were successfully multiplied and rooted. Results prove the feasibility of these new cryogenic methods for a long-term storage of this valuable *Prunus* genotype.

Keywords: *Prunus domestica* L., plant vitrification solution, V cryo-plate, desiccation, D cryo-plate

INTRODUCTION

Growing human population is facing the number of challenges, from predicted climate changes to achieving food security, which implies necessity for sustainable use and conservation of plant genetic resources (PAUTASSO, 2012). The conservation of plant biodiversity is essential

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for classical and modern plant breeding programmes as well. The Republic of Serbia is considered as one of the world's biodiversity centres. Among fruit crops, the European plum (*Prunus domestica* L.) is considered as the leading fruit species in Serbian agriculture. However, it is exposed to climate changes and long-term inoculum pressure of numerous pathogens. In addition, autochthonous plum genotypes of immeasurable genetic and cultural value are endangered and suppressed by newly bred and commercially important cultivars. 'Crvena Ranka' is one of the oldest and the best yielding autochthonous plum cultivars in Serbia. Although highlighted by its suitability as raw material for the production of high-quality brandies (NENADOVIĆ-MRATINIĆ *et al*., 2007), it is also reported that fruits of some local genotypes are suitable for fresh use and drying (MILOŠEVIĆ and MILOŠEVIĆ, 2012). Therefore, this cultivar represents a good genetic basis for clonal selection as well as for further breeding work (GLIŠIĆ and MILOŠEVIĆ, 2015) and should be protected from irreversible disappearance.

Conservation of genetic resources in fruits has a very important place in Serbia with more than 2,370 accessions of *Prunus* genus maintained as *in situ* and *ex situ* field collections (OGNJANOV *et al*., 2009). However, living collections may suffer from poor genetic representation of species and are vulnerable to random genetic drift, artificial selection and mutation accumulation (VOLIS, 2017). Also, these collections are exposed to infestation by numerous pathogens and are labour intensive. The most devastating viral disease in plum is plum pox virus (PPV) that is present in Serbia for more than eight decades infecting about 70% of the plum trees (JEVREMOVIĆ, 2013). PPV represents the main threat to the production and existence of plum genotypes in an open field. Consequently, development of *in vitro* propagation and cryopreservation protocols suitable for storage of selected valuable germplasm, as a gene banking strategy, will contribute to the long-term preservation of autochthonous plum genetic resources which can later be used for breeding or multiplication.

Numerous protocols for cryopreservation have been or are being developed for *Prunus* species including European plum (DE CARLO *et al*., 2000), Japanese plum and different plum rootstocks (DE BOUCAUD *et al*., 2002) and cherry plum (VUJOVIĆ *et al*., 2011). Recently, an effort was also made to cryopreserve different Serbian autochthonous plum genotypes using the droplet-vitrification (VUJOVIĆ *et al*., 2015b) and newly developed V and D cryo-plate techniques (VUJOVIĆ *et al*., 2015a). As cryopreservation success depended markedly on the genotype tested and technique applied, adjustments of these protocols to the genebank level would be necessary to exploit all the advantages of cryopreservation.

Even though it was possible to cryopreserve the plum cultivar 'Crvena Ranka' by droplet-vitrification technique (VUJOVIĆ *et al*., 2015b) the regrowth rates obtained were considerably lower in comparison with those obtained in other plums (cherry plum and cultivar 'Požegača') cryopreserved using aluminium cryo-plates (VUJOVIĆ *et al*., 2015a). In the present study intended to simplify cryopreservation procedure and improve survival and regrowth, we investigated the suitability of V cryo-plate and D cryo-plate methods for cryopreservation of 'Crvena Ranka' shoot tips.

MATERIAL AND METHODS

Plant material

Healthy plants of autochthonous plum cultivar 'Crvena Ranka' (*Prunus domestica* L.) grown in the containers in the screen-house were used as the source of initial explants for

establishment of *in vitro* culture. Plants were tested on the presence of plum pox virus, prune dwarf virus, prunus necrotic ringspot virus, apple chlorotic leaf spot virus, apple mosaic virus plum bark necrosis stem pitting associated virus, myrobalan latent ringspot virus and '*Candidatus* Phytoplasma prunorum' by ELISA and PCR assays at the Phytopathology Laboratory of the Fruit Research Institute, Čačak. None of the stated pathogens were detected in tested plants.

Aseptic culture was established according to the protocol previously described by RUŽIĆ *et al*. (2012) and shoots were multiplied on MURASHIGE and SKOOG (1962) (MS) medium containing 1 mg l^{-1} N^6-benzyladenine (BA), 0.1 mg l^{-1} indole-3-butyric acid (IBA) and 0.1 mg l^{-1} gibberellic acid (GA_3), 3% sucrose and 0.7% agar. Stock cultures were maintained in a growth chamber at 23 ± 1°C, with 16 h photoperiod under 54 µmol m^{-2} s^{-1} light intensity and subcultured at four-week intervals. Apical shoots (about 5 mm in length) were dissected and cultured on solid multiplication medium for two weeks, under standard growth conditions.

Cryopreservation procedure using aluminium cryo-plates

Excised shoot tips were precultured for 1 day at 23ºC in the dark on solidified MS multiplication medium with 0.3 M sucrose.

Following preculture explants were carefully placed individually in each of the 12 wells of 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). For polymerization, a calcium solution containing 0.1 M calcium chloride in MS basal medium with 0.4 M sucrose was poured on the aluminium plates until shoot tips were fully covered. Calcium solution was removed after 20 min and cryo-plates with adhering explants transferred to loading solution (LS) comprising 1.9 M glycerol and 0.5 M sucrose in liquid MS medium (C4 solution; KIM *et al*., 2009a) for 30 min at room temperature.

The V cryo-plate procedure was performed as described for chrysanthemum by YAMAMOTO *et al*. (2011a) using two types of plant vitrification solutions (PVS): slightly modified PVS2 solution [PVS A3 – 22.5% (w/v) sucrose, 37.5% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethylsulfoxide; KIM *et al*., 2009b] for 20 and 40 min, or PVS3 solution [50% (w/v) glycerol and 50% (w/v) 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 (VUJOVIĆ *et al*., 2015a).

In D cryo-plate procedure (NIINO *et al*., 2013) dehydration step included desiccation of the shoot tips attached to the cryo-plates in closed 100 ml glass 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 adhering shoot tips were transferred to 2 ml uncapped cryotubes held on cryo-canes and directly immersed in liquid nitrogen (LN) where they were kept for at least 1 h. Rewarming of samples was performed by rapid transfer of the aluminium cryo-plates in an unloading solution (0.8 M sucrose) for 30 min incubation at room temperature (KIM *et al*., 2009b). Thereafter, explants were transferred onto the regrowth medium (corresponds to the previously described multiplication medium), cultivated in the dark for seven days, and then under standard conditions.

Each critical step in the cryopreservation procedures had the appropriate control: pregrowth control – immediately after pregrowth explants were transferred onto the regrowth

medium; loading control – shoot tips 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.

Assessment of survival and regrowth and statistical analysis

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.

All experimental treatments and corresponding controls were performed in three independent replicates, with 10–12 explants per each replicate. Statistical analysis was performed by one-way Analysis of variance (ANOVA) and Duncan's Multiple Range Test ($P < 0.05$) for mean separation. Before analysis of variance, data presented in the form of percentage were subjected to arcsine transformation.

Shoot multiplication and rooting after regrowth

Following regrowth, 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 second subculture after regrowth (four-week culture interval). In the following subculture the shoots were rooted on the MS medium with mineral salts reduced to ½-strength, organic complex unchanged, 1 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 0.1 mg l^{-1} GA_3 (RUŽIĆ *et al*., 2012). The monitoring of rooting capacity included the following parameters: percentage of rooted plantlets, number and length of roots, and length of rooted shoots. All data were analysed by ANOVA, followed by the Duncan's Multiple Range Test, at $P < 0.05$.

RESULTS

Cryopreservation of 'Crvena Ranka' shoot tips using aluminium cryo-plates

The experiments evaluated both survival and regrowth capacity of control, non-frozen and cryopreserved shoot tips. In all treatments survival rates of explants (Figure 1a) after two weeks of culturing were higher than corresponding regrowth rates (Figure 1b) recorded at the end of the sixth week.

Pregrowth, loading and most of the PVS treatments had no significant effect on survival (100%) of non-cryopreserved shoot tips (Figure 1a). However, desiccation significantly decreased survival of control explants which ranged between 60% and 80%, the lowest rate obtained for the longest treatment duration. Cryopreservation additionally decreased survival of shoot tips both in V cryo-plate protocol (60–75%) and in D cryo-plate protocol (45–64.2%).

Compared with pregrowth control (100% of regrowth), loading treatment in our experiments decreased regrowth capacity of shoot tips to 90% (Figure 1b). Regrowth rates of dehydration controls in V cryo-plate protocol (80–90%) were similar to that obtained for loading control. However, the significant decrease in regrowth capacity was noticed in cryopreserved explants for all PVS treatments (50–66.7%). The highest regrowth rate was recorded with PVS3 dehydration. Similar to survival, regrowth of both control and cryopreserved explants in D cryo-plate protocol was considerably more affected by desiccation, varying between 30% and 50%

and between 30% and 40%, respectively. The highest regrowth of both control and frozen shoot tips was obtained after 2 h of desiccation.

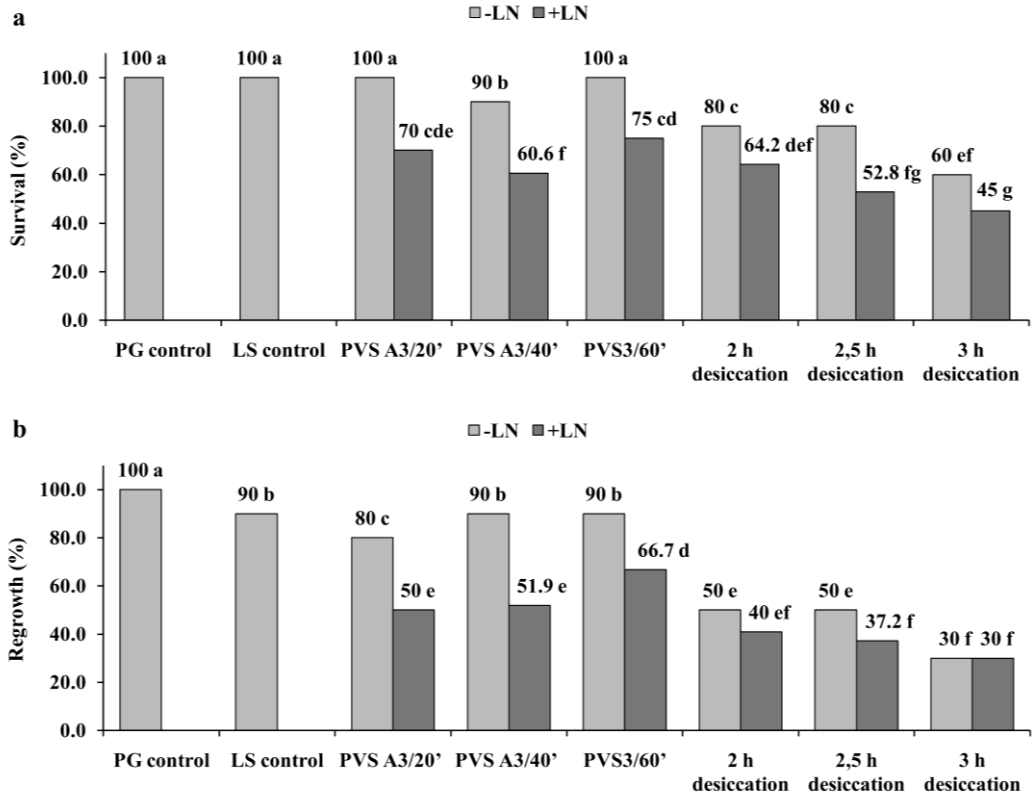

Figure 1. Survival and regrowth of pregrowth (PG), loading (LS) and dehydration (-LN) controls and cryopreserved (+LN) shoot tips of autochthonous plum 'Crvena Ranka' using the V cryo-plate and D cryo-plate methods. Mean values for both survival and regrowth followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$)

Plantlets regenerated after LN exposure mostly displayed normal morphology (Fig. 2a–f). Nevertheless, growth and vigour of shoots regenerated from cryopreserved explants was affected by cryopreservation method applied as well as by duration of dehydration/desiccation treatment. The most vigorous growth was noticed after 40-min dehydration with PVS A3 (Figure 2b) and after 4 h of desiccation, although the latter displayed pronounced signs of hyperhydricity (Figure 2f).

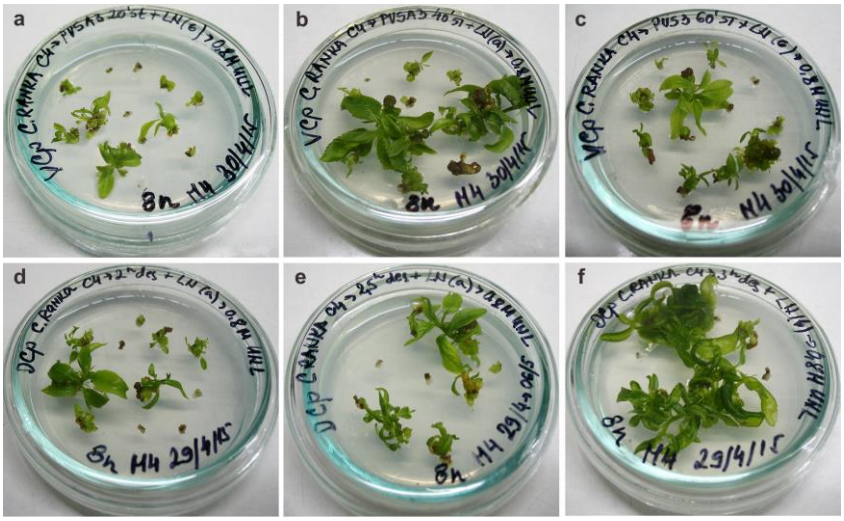

Figure 2. Regrowth of cryopreserved shoot tips of autochthonous plum 'Crvena Ranka'. Explants dehydrated with PVS A3 solution for 20 min (a) and 40 min (b) and with PVS3 solution for 60 min (c) in V cryo-plate protocol. Explants desiccated for 2 h (d), 2.5 h (e) and 3 h (f) in D cryo-plate protocol

Shoot multiplication and rooting after regrowth

In the present study we monitored individual effect of each step of both cryopreservation protocols on multiplication and rooting capacity of shoots after regrowth (Tables 1 and 2).

Table 1. Multiplication of plum shoots of different origin in the second subculture after regrowth

Shoot origin	Multiplication index	Length of axial shoot (mm)	Length of lateral shoots (mm)
Pregrowth control	3.2 a	11.1 efg	6.5 f
Loading control	3.1 ab	14.4 a	6.8 ef
PVS A3 20' – LN	2.1 f	11.0 fg	7.0 ef
PVS A3 20' + LN	2.0 f	9.7 h	6.7 f
PVS A3 40' – LN	2.2 ef	10.8 g	6.4 f
PVS A3 40' + LN	2.4 e	12.3 cd	8.4 a
PVS3 60' – LN	2.8 cd	11.7 defg	8.3 ab
PVS3 60' + LN	2.2 ef	11.6 defg	7.1 def
2 h desiccation – LN	2.7 d	13.9 ab	7.5 cde
2 h desiccation + LN	2.7 d	12.6 cd	7.8 abcd
2.5 h desiccation – LN	2.9 bc	12.2 cde	7.7 bcd
2.5 h desiccation + LN	2.6 d	12.2 cde	7.7 bcd
3 h desiccation – LN	2.0 f	13.2 bc	8.2 abc
3 h desiccation + LN	1.7 g	11.7 defg	6.7 f

Mean values of multiplication parameters within each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$); –LN: dehydrated controls; +LN: cryopreserved shoot tips

As regards index of multiplication, all PVS treatments as well as all desiccation treatments significantly decreased capacity for multiplication of regenerated shoots in comparison with those originated from pregrowth and loading controls (Table 1). On the other side, cryopreservation either did not or slightly decreased multiplication index of regenerated plantlets compared with corresponding controls. As regards length of axial shoots, the highest values were recorded for shoots originated from loading control. Shoots originated from most of the dehydration and correspondent cryopreservation treatments were of the similar or even greater length comparing to pregrowth controls, displayed normal morphology and were well developed (Figure 3a–c). Similar tendency was observed for the length of lateral shoots, especially for those regenerated from explants dehydrated with PVS3 or desiccated over silica gel in D cryo-plate protocol (regardless the treatment duration).

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 greater in shoots originating from all types of control and cryopreserved explants in comparison with those originating from pregrowth and loading controls (Figure 3d–f). Among shoots regenerated from cryopreserved shoot tips the highest rooting rate (76.67%) was noticed for those desiccated for 2 h in D cryo-plate protocol.

Table 2. Rooting of plum shoots of different origin in the third subculture after regrowth

Shoot origin	Rooting rate (%)	Number of roots	Root length (mm)	Rooted shoot length (mm)
Pregrowth control	56.67 cde	2.3 cd	16.5 gh	12.2 c
Loading control	56.67 cde	2.3 cd	17.8 fg	11.9 c
PVS A3 20' – LN	70.00 ab	2.6 c	21.7 d	12.6 c
PVS A3 20' + LN	66.67 bc	2.0 de	15.6 ghi	12.8 c
PVS A3 40' – LN	63.33 bcde	2.3 cd	20.1 de	12.1 c
PVS A3 40' + LN	66.67 bc	2.5 c	15.3 hi	12.5 c
PVS3 60' – LN	46.67 f	1.7 e	13.6 ij	12.6 c
PVS3 60' + LN	56.67 cde	3.1 b	12.1 j	10.0 d
2 h desiccation – LN	70.00 ab	2.4 c	24.7 c	20.2 a
2 h desiccation + LN	76.67 a	3.8 a	27.0 b	19.0 a
2.5 h desiccation – LN	76.67 a	3.1 b	30.1 a	18.5 a
2.5 h desiccation + LN	50.00 ef	4.1 a	19.1 ef	15.9 b
3 h desiccation – LN	53.33 def	3.1 b	15.4 hi	11.1 cd
3 h desiccation + LN	56.67 cde	2.3 cd	13.4 ij	12.3 c

Mean values of rooting parameters within each column followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P < 0.05$); –LN: dehydrated controls; +LN: cryopreserved shoot tips

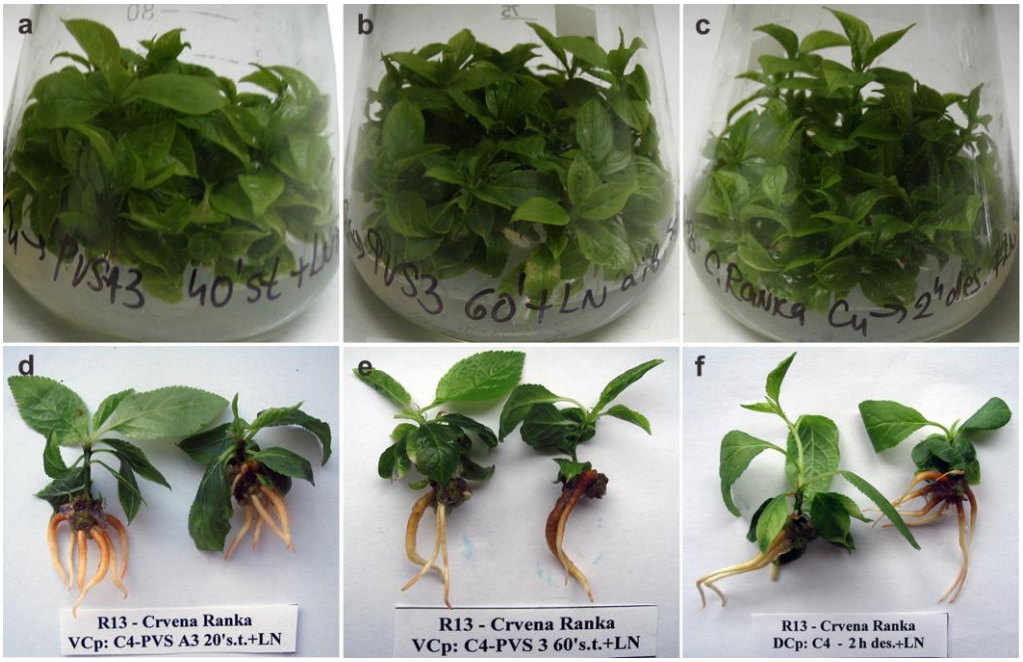

Figure 3. Shoots of autochthonous plum 'Crvena Ranka' regenerated from cryopreserved explants in multiplication stage: dehydrated with PVS A3 solution for 40 min (a), dehydrated with PVS3 solution for 60 min (b), desiccated for 2 h (c); and in rooting stage: dehydrated with PVS A3 solution for 20 min (d), dehydrated with PVS3 solution for 60 min (e), desiccated for 2 h (f)

DISCUSSION

One of the main goals of the cryopreservation is to simplify the procedure and to minimize the use of expensive equipment and tools to make this technique available to the laboratories in developing countries (DE BAUCAUD *et al*., 2002). Vitrification based cryopreservation techniques eliminated the need for expensive control rate freezers, still most of these methods require skilful manipulation and very precise control of critical steps such as osmoprotection and dehydration and excessive manipulation of explants during cryogenic procedure especially in droplet-vitrification protocol. Vitrification protocol using cryo-plates (YAMAMOTO *et al*., 2011b) and dehydration protocol using cryo-plates (NIINO *et al*., 2013) were developed with the aim to simplify the procedure and to avoid damage and wastage of explants during manipulations by adhering them on the cryo-plates. In addition, V cryo-plate enables precise control of dehydration with PVSs and very high cooling and warming rates of samples while D cryo-plate allows overcoming problems associated with sensitivity to PVSs (NIINO and YAMAMOTO, 2017).

Autochthonous plum 'Crvena Ranka' displayed sensitivity to biochemical toxicity of PVS A3 and notably to osmotic toxicity of PVS3 solution over a wide range of treatment durations in our previous droplet-vitrification experiments (VUJOVIĆ *et al*., 2015b). As for PVS A3, 20 min and 40 min dehydration steps combined with 30 min and 60 min unloading respectively gave the best regrowth (around 35%), while for PVS3 regeneration of cryopreserved explants was achieved only with 60 min dehydration (30%). Utilizing V cryo-plate method in the present study, under the same dehydration conditions, we achieved 60% higher regrowth of cryopreserved explants using PVS A3 and more than twice as high regeneration in those dehydrated with PVS3. Similar observations were reported for cherry plum too (VUJOVIĆ *et al*., 2015a). According to YAMAMOTO *et al*. (2011b) V Cryo-plate method enables not only the more precise time control of dehydration but also reduction of chemical toxicity of cryoprotectant solutions by the alginate gel used to fix explants on aluminium plates. A further increase of tolerance to vitrification solutions can be achieved by increasing the sucrose concentration in the loading solution containing 2.0 M glycerol (YAMAMOTO *et al*., 2011a).

Compared to V cryo-plate method lower rates of survival and later regrowth of both control and frozen explants were observed in plum 'Crvena Ranka' using D cryo-plate method that is contrary to results obtained in our previous experiments with cherry plum and plum cultivar 'Požegača' (VUJOVIĆ *et al*., 2015a). The duration of physical dehydration is critical for achieving high recovery after LN exposure so that GONZALEZ-ARNAO and ENGELMANN (2006) suggested desiccation in air-tight containers with silica gel to standardize this step in D cryo-plate protocol. In our experiments low recovery of both control and cryopreserved specimens as well as gradual decrease in regrowth with prolonged desiccation indicate that additional optimisation of this step is required. Further, larger explants should be used in the D cryo-plate method in order to achieve more uniform dehydration (NIINO *et al*., 2013). Considerable differences noticed between survival and regrowth rates in all D cryo-plate treatments also indicate that the recovery medium should be optimised, because suboptimal conditions or regrowth media may limit recovery even though the cryopreservation was done correctly (REED, 2008).

Sensitivity of plant germplasm to the stresses of the cryopreservation method applied can affect not only cryopreserved cultures but also regenerated plantlets (REED *et al*., 2005). Therefore, successful implementation of any cryopreservation protocol is largely dependent on clonal fidelity, vigorous growth, rapid multiplication and efficient rooting of shoots regenerated after cryopreservation. In the present study, plum shoots originating from cryopreserved explants recovered their multiplication capacity up to the second subculture, although index of multiplication was lower compared to pregrowth control explants. VUJOVIĆ *et al*. (2015a) reported similar or even higher multiplication capacity of *Prunus* shoots regenerated from cryopreserved explants compared to those recovered from untreated specimens. By contrast shoots of cherry plum were unable to multiply over two successive subcultures after regrowth (VUJOVIĆ *et al*., 2011). In the literature, little attention was paid to rooting of the plantlets after cryopreservation. An increased rooting efficiency was observed in 'Crvena Ranka' shoots produced after cryopreservation compared to the pregrowth control. These results are in compliance with findings of AI *et al*. (2012), while HAO *et al*. (2002) did not observe any

influence of storage in LN on citrus rhizogenesis. On the contrary, KULUS *et al*. (2017) reported that cryopreservation inhibited spontaneous rooting of chrysanthemum.

CONCLUSIONS

In this study, we investigated the suitability of two efficient and simple cryopreservation methods using aluminium cryo-plates for conservation of autochthonous plum cultivar 'Cvena Ranka'. Successful recovery of cryopreserved explants, especially in V cryo-plate protocol, followed by vigorous growth, efficient multiplication and rooting of regenerated plantlets proved the feasibility of these new cryogenic methods for a long-term storage of this valuable *Prunus* genotype. Additional modification of critical steps in both protocols (osmoprotection, dehydration and recovery conditions) is needed for further improvement of regrowth capacity after cryogenic storage.

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KRIOPREZERVACIJA SRPSKE AUTOHTONE SORTE ŠLJIVE 'CRVENA RANKA' PRIMENOM ALUMINIJUMSKIH KRIO-PLOČICA

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Izvod

'Crvena Ranka', jedna od najstarijih autohtonih sorti šljive u Srbiji, je zbog dugogodišnjeg patogenog pritiska i kontinualnog potiskivanja od strane komercijalnih sorti izložena genetičkoj eroziji. Ova istraživanja su sprovedena sa ciljem da se ispita pogodnost dve novorazvijene metode krioprezervacije („V" i „D cryo-plate" metode) za njenu dugotrajnu konzervaciju. *In vitro* vrhovi izdanaka ovog genotipa su posle pretretmana pričvršćeni na aluminijumske krio-pločice pomoću 2% (w/v) rastvora natrijum alginata u tečnom MS medijumu. Osmotska priprema eksplantata je izvršena u rastvoru 1.9 M glicerola i 0.5 M saharoze (30 min, sobna temperatura). U „V cryo-plate" protokolu eksplantati su dehidratisani na sobnoj temperaturi 20 ili 40 min sa vitrifikacionim rastvorom PVS A3 (37.5% glicerol, 15% dimetilsulfoksid, 15% etilen glikol i 22.5% saharoza) i 60 min sa PVS3 rastvorom (50% glicerol i 50% saharoza). U „D cryo-plate" protokolu, vršena je desikacija na silika gelu u trajanju 2, 2,5 ili 3 h. Krio-pločice sa eksplantatima su direktno uranjane u tečni azot (LN) gde su držane 1 h. Po vađenju iz LN osmotska rehidracija je vršena u MS medijumu sa 0.8 M saharozom (30 min, sobna temperatura). Procenat regeneracije izdanaka iz krioprezerviranih eksplantata u "V cryo-plate" protokolu iznosio je 50% i 51.9% kod eksplantata dehidratisanih PVS A3 rastvorom, odnosno 66.7% kod onih dehidratisanih sa PVS3 rastvorom. Primenom „D cryo-plate" metode, postignuta je regeneracija u rasponu 30–40%. Posle regeneracije, izdanci su uspešno multiplicirani i ožiljeni *in vitro*. Dobijeni rezultati opravdavaju primenu "V" i "D cryo-plate" metoda u cilju dugotrajnog čuvanja ove vredne autohtone sorte.

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