

**IN VITRO CONSERVATION OF TWO PLANT SPECIES
(*Prunus cerasifera* Ehrh. AND *Rubus fruticosus* L.) SHOOT TIPS
BY ENCAPSULATION DEHYDRATION**

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In vitro grown shoot tips of cherry plum (*Prunus cerasifera* Ehrh.) and blackberry 'Čačanska Bestrna' (*Rubus fruticosus* L.) were tested for regrowth after cryopreservation using encapsulation dehydration method. Apical, 2–3 mm long shoot tips, were encapsulated in alginate beads composed of 3, 5 and 10% (w/v) alginic acid sodium salt in calcium-free liquid Murashige and Skoog (MS) medium containing 1.0 mg l⁻¹ benzyladenine, 0.1 mg l⁻¹ indole-3-butyric acid and 0.1 mg l⁻¹ gibberellic acid. Polymerization was done in liquid MS medium with 100 mM CaCl₂ for 30 min at room temperature. Encapsulated shoot tips were pre-treated in liquid MS medium with 0.75 or 1 M sucrose for 24 h in growth room and dehydrated for 4 and 8 h (29% and 20% moisture content respectively) before rapid immersion in liquid nitrogen. Upon thawing which involved placing the cryovials in the air current of the laminar airflow cabinet for 2 min, the beads were directly transferred to regrowth medium. In cherry plum, osmotic dehydration in 0.75 M sucrose followed by 8-hour desiccation gave the highest regrowth (60%) of explants encapsulated in 3% and 5% alginate beads. However, in comparison with cherry plum, blackberry displayed significantly lower capacity for regrowth after cryopreservation under described experimental conditions. In this genotype, osmotic dehydration in 1 M sucrose followed by 8-hour desiccation resulted in the highest regrowth (16.7%) of explants encapsulated in 5% alginate beads. Cryopreserved shoot tips of both

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genotypes multiplied in the three subcultures had normal morphology and similar multiplication capacity in comparison with non-cryopreserved shoots.

Key words: cherry plum, blackberry, cryopreservation, regrowth, multiplication

INTRODUCTION

It was Nikolai Ivanovich Vavilov who founded the first plant gene bank (1920) and initiated collection of genotypes of different plant species in over 50 countries worldwide. To date, *in situ*, *ex situ* and *in vitro* plant gene banks are available in most countries of the world holding altogether 6 million accessions (http://en.wikipedia.org/wiki/Nikolai_Vavilov).

On the COST Action 871 web site, dedicated to long-term preservation of *in vitro* culture, there is information about 100,000 plants in danger of extinction, which accounts for more than 1/3 of the total number of plant species in the world. Biodiversity is seriously affected in Europe, i.e. 64 endemic species were lost over the past decade, 24% species and sub-species are in danger of extinction (<http://www.bjw.kuleuven.be/dtp/tro/cost871/Home.htm>).

Genetic resources, i.e. plant gene banks are maintained mainly *in situ* in the field. These collections are suitable for breeding, but impose high requirements in terms of space (land) and costs. Moreover, collections are exposed to excessive diseases and abiotic external stress factors as well as high pest pressure. Therefore, the establishment of modern germplasm collection necessarily includes the use of *in vitro* techniques for plant/fruit preservation as an important supplement to conventional preservation of germplasm in the field (REED *et al.*, 2008).

Over recent decades, cryopreservation has become a very important tool for long-term conservation of plant germplasm. Nevertheless, a wider application of plant cryopreservation depends on the availability of efficient, reproducible and robust cryopreservation protocols applicable to different plant species (PANIS and LAMBARDI, 2005), i.e. to a broad range of tropical and temperate plant species (ENGELMANN, 2004). Among the currently available cryotechniques, the encapsulation dehydration method has been already applied to different fruit species, such as *Malus* sp. (PAUL *et al.*, 2000) *Pyrus* sp. (REED *et al.*, 1998; CONDELLO *et al.*, 2009), *Prunus avium* (SHATNAWI *et al.*, 2007), *Rubus* sp. (GUPTA and REED, 2006; REED, 2008), *Vitis* L. (WANG *et al.*, 2000), etc.

Recently, the tissue culture group of the Fruit Research Institute Čačak has initiated the application of cryopreservation techniques for the long-term conservation of temperate fruit species as a valuable supplement to traditional germplasm preservation to prevent the extinction of extremely valuable germplasm (RUŽIĆ *et al.*, 2008; 2010; 2011; CONDELLO *et al.*, 2010; VUJOVIĆ *et al.*, 2011).

This paper presents the results on the application of the encapsulation dehydration technique for cryopreservation of *in vitro* grown shoot tips of cherry plum (*Prunus cerasifera* Ehrh.) and blackberry 'Čačanska Bestrna' (*Rubus fruticosus* L.). The aim of the study was to compare regrowth capacity of two diverse genotypes after cryopreservation using the same protocol. The establishment and development of the encapsulation dehydration protocol was performed by evaluating the effect of alginate concentration used for encapsulation of shoot tips, as well as the effect of sucrose concentration during osmotic dehydration and duration of air desiccation of encapsulated explants on recovery after liquid nitrogen (LN) exposure.

MATERIALS AND METHODS

Plant material and experimental design

In vitro grown shoot tips of cherry plum (*Prunus cerasifera* Ehrh.) and blackberry ‘Čačanska Bestrna’ (*Rubus fruticosus* L.) were tested for regrowth after cryopreservation using encapsulation dehydration method described by DEREUDDRE *et al.* (1990).

Prunus cerasifera Ehrh. is a plum species known by the common names ‘cherry plum’ or ‘Myrobalan plum’. In Serbia it is widely used as a main rootstock for plum. ‘Čačanska Bestrna’ is blackberry cultivar developed at Fruit Research Institute Čačak as the result of the breeding program based on application of planned hybridization. As this cultivar displays excellent performance in respect of cropping, fruit quality and resistance to diseases, it is very valuable for further breeding work.

In the experiment, 12 combinations/treatments were monitored and 9–10 apices (explants) x 2 replications and were used per each experimental condition for both genotypes (Tab. 1).

Table 1. Experimental design

| Treatments | Alginate (%) | Concentration of sucrose (M) | Desiccation in GR (hour) | Combination mark |
|------------|--------------|------------------------------|--------------------------|------------------|
| 1 | 3 | 0.75 | 4 | *0.75–3–4 |
| 2 | 3 | 1 | 4 | 1–3–4 |
| 3 | 5 | 0.75 | 4 | 0.75–5–4 |
| 4 | 5 | 1 | 4 | 1–5–4 |
| 5 | 10 | 0.75 | 4 | 0.75–10–4 |
| 6 | 10 | 1 | 4 | 1–10–4 |
| 7 | 3 | 0.75 | 8 | 0.75–3–8 |
| 8 | 3 | 1 | 8 | 1–3–8 |
| 9 | 5 | 0.75 | 8 | 0.75–5–8 |
| 10 | 5 | 1 | 8 | 1–5–8 |
| 11 | 10 | 0.75 | 8 | 0.75–10–8 |
| 12 | 10 | 1 | 8 | 1–10–8 |

*1st number = sucrose concentration (mM); 2nd number = alginate concentration (%); 3rd number = desiccation time (hours)

Cryopreservation method employed

For cryopreservation, the encapsulation dehydration method (E-D) was used. Excised, 2–3 mm long shoot tips (leaves excluded), were encapsulated in alginate beads composed of 3%, 5% and 10% (w/v) low viscosity alginic acid – sodium salt (ACRÖS Organics, Belgium) in liquid MURASHIGE and SKOOG (MS) medium (1962) without CaCl₂ supplemented with 1 mg l⁻¹ benzyladenine (BA), 0.1 mg l⁻¹ indole-3-butyric acid (IBA) and 0.1 mg l⁻¹ gibberellic acid (GA₃), pH 5.7, and were allowed to polymerize for 30 min at room temperature in MS medium supplemented with 100 mM CaCl₂ and 0.06 M sucrose (Fig. 1a). Encapsulated shoot tips were pre-treated in liquid MS medium with 0.75 or 1 M sucrose for 24 h in growth room.

Desiccation included placing beads in air-tight containers (3.5 x 4.5 cm; 5 beads per container) with 8 g silica gel for 4 and 8 h (moisture content ~29% and 20%, respectively). Desiccation curves were previously formed to calculate the residue of water after desiccation

from 0 to 24 h. Dried beads were placed in 2-ml polypropylene cryovials (5 beads/cryovial) and plunged directly into liquid nitrogen (LN) for at least 1 h.

Thawing involved placing the cryotubes in the air current of the laminar flow cabinet for 2 min. White ice bearing beads were not well dehydrated and were therefore rejected (Fig. 1b).

The beads were then transferred to Petri dishes containing standard medium (MS medium supplemented with BA 1 mg l⁻¹, IBA 0.1 mg l⁻¹ and GA₃ 0.1 mg l⁻¹, pH 5.7, 0.06 M sucrose and 7.2 g l⁻¹ agar), kept in growth room in darkness for 7 days, and then transferred to standard growth conditions.

Explants that resumed normal development (production of new leaves and /or expansion of small shootlets) 28 days after bead burst were considered as regrowing.

Culture conditions and shoot multiplication capacity after regrowth

Before and after cryopreservation cultures were maintained in a growth chamber at 23 ± 1°C, under a 16 h light/8 h dark photoperiod and light intensity of 54 μmol m⁻² s⁻¹. Multiplication parameters (index of multiplication, length of axial and lateral shoots) of cryopreserved shoots in 3 successive subcultures after regrowth were compared with control shoots (non cryopreserved).

Statistical analysis

Data collected from cryopreservation experiments as well as those from micropropagation experiments after regrowth were analyzed by ANOVA, followed by the Duncan's Multiple Range Test for mean separation. Data presented in the form of percentage were subjected to arcsine transformation.

RESULTS

After 1-hour immersion into LN some beads were frozen, particularly after 4-hour desiccation period (Tab. 2, Fig. 1b). In cherry plum, the highest incidence of frozen beads, i.e. 40% (with 1M sucrose pre-treatment) and 100% (with 0.75 M sucrose pre-treatment), was observed in the treatments with 10% alginate (Tab. 2). Ice bearing beads were not observed for any of 8-hour desiccation treatments in cherry plum. As for blackberry 'Čačanska Bestrna', the rate of frozen beads was very high (70–100%) in all 4-hour desiccation treatments, regardless of alginate concentration (Tab. 2). However, the incidence of frozen beads was very low for 8-hour desiccation treatments, i.e. 5.6% and 11.1%, for explants encapsulated in 5% and 10% alginate.

It was only 8 days after placing cryopreserved explants of cherry plum in the light that the first alginate bead was observed to burst (so called 'survival'), whereas in blackberry, the first alginate bead burst occurred 10 days later (Fig. 1c).

In regard to 4 h of desiccation, regrowth was obtained only in cherry plum explants encapsulated in 3% alginate reaching the same rate (40%) in both sucrose pre-treatments. However, the highest regrowth rate in this genotype, amounting up to 60%, was obtained with 0.75 M sucrose pre-treatment and 8-hour desiccation, in explants encapsulated in 3% and 5% alginate (Tab. 2, Fig. 1d). In blackberry, osmotic dehydration in 1 M sucrose followed by 8-hour desiccation gave the highest regrowth (16.7%) of explants encapsulated in 5% alginate beads (Fig. 1e).

Table 2. Regrowth of cryopreserved shoot tips of cherry plum and blackberry 28-days after bead burst

| Combinati on mark | % of frozen beads after 1h in LN | | Non-regenerated explants (%) | | Regrowth (%) | | Mean No. of regenerated shoots | |
|----------------------|-------------------------------------|---------------------|---------------------------------|---------------------|-----------------|---------------------|-----------------------------------|---------------------|
| | Cherry plum | Čačanska Bestrna | Cherry plum | Čačanska Bestrna | Cherry plum | Čačanska Bestrna | Cherry plum | Čačanska Bestrna |
| | 0.75–3–4 | 40.0 d* | 90.0 ab | 20.0 ef | 10.0 fg | 40.0 b | 0.0 e | 2.5 cd |
| 1–3/4 | 0.0 f | 80.0 bc | 60.0 cd | 20.0 ef | 40.0 b | 0.0 e | 2.5 cd | - |
| 0.75–5–4 | 0.0 f | 100.0 a | 100.0 a | 0.0 g | 0.0 e | 0.0 e | - | - |
| 1–5–4 | 0.0 f | 100.0 a | 100.0 a | 0.0 g | 0.0 e | 0.0 e | - | - |
| 0.75–10–4 | 100.0 a | 90.0 ab | 0.0 g | 10.0 fg | 0.0 e | 0.0 e | - | - |
| 1–10–4 | 40.0 d | 70.0 c | 60.0 cd | 30.0 e | 0.0 e | 0.0 e | - | - |
| 0.75–3–8 | 0.0 f | 0.0 f | 40.0 de | 88.9 b | 60.0 a | 11.1 c | 2.3 cd | 3.5 b |
| 1–3–8 | 0.0 f | 0.0 f | 100.0 a | 94.4 ab | 0.0 e | 5.6 d | - | 7.0 a |
| 0.75–5–8 | 0.0 f | 0.0 f | 40.0 de | 100.0 a | 60.0 a | 0.0 e | 1.0 d | - |
| 1–5–8 | 0.0 f | 5.6 ef | 100.0 a | 77.8 bc | 0.0 e | 16.7 c | - | 5.0 b |
| 0.75–10–8 | 0.0 f | 11.1 e | 100.0 a | 77.8 bc | 0.0 e | 11.1 c | - | 6.0 ab |
| 1–10–8 | 0.0 f | 5.6 ef | 80.0 bc | 94.4 ab | 20.0 c | 0.0 e | 1.0 d | - |
| | P ≤ 0.05 | | P ≤ 0.05 | | P ≤ 0.05 | | P ≤ 0.05 | |

*Mean values for each parameter followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).

In the first subculture after regrowth, shoots of cherry plum had short stems with tiny, emerging buds. However, cryopreserved shoot tips multiplied in three successive subcultures regained normal morphology with similar multiplication capacity in comparison with non-cryopreserved shoots (Tab. 3; Fig. 1f). Although multiplication index and length of axial shoots of blackberry were significantly lower in first subculture after regrowth, regenerants displayed normal morphology and were well developed, with wide green leaves (Fig. 1g). They retrieved multiplication capacity by the third subculture.

Table 3. Shoot multiplication of cherry plum and blackberry in three successive subcultures after regrowth compared with the control – non-cryopreserved shoots

| No of subculture | Multiplication index | | Length of axial shoot Average (cm) | | Length of lateral shoots Average (cm) | |
|---------------------|----------------------|-----------------------|---------------------------------------|-----------------------|--|-----------------------|
| | Cherry plum | ‘Čačanska Bestrna’ | Cherry plum | ‘Čačanska Bestrna’ | Cherry plum | ‘Čačanska Bestrna’ |
| | 1 st | 1.73 d* | 2.19 bc | 0.48 e | 0.66 d | 0.38 d |
| 2 nd | 2.08 c | 2.16 c | 0.82 c | 0.81 c | 0.52 c | 0.57 c |
| 3 rd | 1.91 cd | 2.55 a | 1.01 b | 0.85 c | 0.73 a | 0.60 bc |
| Control | 1.94 cd | 2.46 ab | 1.59 a | 0.77 cd | 0.70 ab | 0.56 c |
| | P ≤ 0.05 | | P ≤ 0.05 | | P ≤ 0.05 | |

*Mean values for each parameter followed by the same letter are not significantly different according to Duncan's Multiple Range Test ($P \leq 0.05$).



Figure 1. Encapsulated shoot tips (a); Frozen beads after 1 h in LN (b); Bead burst – survival (c); Regrowth of cherry plum: 5% alginate – 0.75 M sucrose – 8-hour desiccation (d); Regrowth of blackberry: 5% alginate – 1 M sucrose – 8-hour desiccation (e); Multiplication of cherry plum – 2nd subculture after E-D (e); Multiplication of blackberry – 1st subculture after E-D (g)

DISCUSSION

Encapsulation dehydration is a widely used cryotechnique which has been until now experimented with over 70 different plant species (ENGELMANN, 2011). According to the author, from the practical point of view, it has several advantages over other preservation techniques. It is a simple-to-implement, user-friendly technique, as encapsulated explants are easy to manipulate in comparison to the non-encapsulated ones. Also this technique allowed use of larger explants that can survive LN exposure, as well as the use of sucrose as the only cryoprotectant, thereby avoiding the toxic effect of other cryoprotectants (GONZALES-ARNAO and ENGELMANN, 2006).

According to results obtained in the present paper, shoot tips of cherry plum could be successfully cryopreserved by encapsulation dehydration method achieving 60% regrowth. It is also demonstrated that shoot tips of blackberry could be cryopreserved by this technique, but the genotype displayed significantly lower capacity for regrowth after cryopreservation under described experimental conditions. REED *et al.* (2008) cryopreserved shoot tips of 25 genotypes in 9 *Rubus* species using the same cryotechnique and obtained better regrowth (60–100%), but cold acclimation was used as a cold pre-treatment. The higher regrowth obtained in their experiments may also be due to better optimization of cell moisture content in *Rubus* tissues.

As recovery following encapsulation-dehydration mainly depends on the amount of freezable water in cells, the duration of the dehydration treatment is very important factor for apex survival after cryopreservation (GUPTA and REED, 2006). After 1-hour immersion in LN some beads were frozen, particularly those desiccated for 4 hours, which was also confirmed by CLAVERO-RAMIREZ *et al.* (2005) in several strawberry (*Fragaria × ananassa* Duch ex Rozier) cultivars.

Freezable water in plant tissues encapsulated in calcium-alginate matrix was removed by both osmotic dehydration and air desiccation. The highest regrowth of cryopreserved shoot tips in both genotypes (60% in cherry plum and 16.7% in blackberry) was achieved with 20% water content, which is the same as water content recommended for ensuring high survival rates and rapid growth recovery of cryopreserved tissues (ENGELMANN, 2004).

SHATNAWI *et al.* (2007) obtained slightly better regrowth (77%) of *Prunus avium* shoots with 6 hrs dehydration, 29% bead moisture content and 0.75 M sucrose (pre-culture medium – 1 day) than that obtained in cherry plum in our experiment. On the other hand, compared to results obtained in blackberry 'Čačanska Bestrna', GUPTA and reed (2006) achieved much higher regrowth in 17 blackberry cultivars and selections, although encapsulated shoot tips were desiccated to the same moisture content (~20%) as in our experiment. However, it seems that using the same protocol, success is not only species-specific, but probably genotype-dependent response, mainly due to their differences in dehydration tolerance (WANG *et al.*, 2000).

Further, the concentration of sucrose used for osmotic dehydration is important as well. The sucrose concentration most commonly employed for cryoprotection is 0.75 M (ENGELMANN, 2011). Although in cherry plum regrowth was achieved both from explants dehydrated with 0.75 M and 1 M sucrose, the best results were achieved in treatments where 3% and 5% alginate beads were pre-treated in 0.75 M sucrose followed by 8-hour desiccation. In blackberry, the effect of sucrose pre-treatment on regrowth depended on alginate concentration. Explants encapsulated in 5% alginate beads gave regrowth only if the beads were pretreated in 1M sucrose. In contrast, regrowth of explants encapsulated in 10% alginate was achieved only after osmotic dehydration in 0.75 M sucrose.

It seems that E-D cryotechnique is suitable for cherry plum preservation, because droplet vitrification technique applied on the same species gave poorer results, regrowth rate varying between 5% and 20%, depending on plant vitrification treatment (VUJOVIĆ *et al.*, 2011). Additionally, all explants (both dehydrated controls and cryopreserved shoot tips) grew slowly and showed pronounced signs of hyperhydricity (their stems and leaves were thick, rigid and fragile) which was not observed with E-D method. As for blackberry 'Čačanska Bestrna', higher regrowth rate, reaching 70%, was obtained by droplet vitrification (VUJOVIĆ *et al.*, 2011) in comparison with E-D technique (16.7%) used in this experiment. According to ENGELMANN (2011) it can be expected that different cryotechniques will have to be applied to different genotypic groups for successful implementation of cryopreservation in large germplasm collection.

All the factors above are important for better survival and regrowth of explants after cryopreservation, which can be achieved with modifications of current technique in order to overcome genotypic variation. Thus, further research should focus on optimization of these important factors of successful cryopreservation, as well as on evaluation of genetic stability of cryopreserved shoots/plants.

CONCLUSION

The results obtained indicate that successful application of cryopreservation using the same protocol is highly genotype dependent and requires some modifications in order to achieve optimal recovery of plants belonging to diverse species. Encapsulation dehydration cryotechnique under described experimental conditions is favorable for cherry plum conservation, while further optimization of the protocol is needed for successful application of this technique in blackberry 'Čačanska Bestrna'.

Explants showing normal development 28 days after bead burst were considered as regrowing. The osmotic dehydration in 0.75 M sucrose followed by 8-hour desiccation gave the highest regrowth of cherry plum (60%) after freezing in LN of explants encapsulated in 3 and 5% alginate beads. The osmotic dehydration in 1 M sucrose followed by 8-hour desiccation gave the highest regrowth of blackberry 'Čačanska Bestrna' (16.7%) after freezing in LN of shoots encapsulated in 5% alginate beads.

Cryopreserved shoot tips of both genotypes multiplied in the three subcultures after regrowth had normal morphology and similar multiplication capacity in comparison with non-cryopreserved shoots.

Given our efforts to help establishing a national *in vitro* fruit gene bank, these results will contribute to the development of standard protocols for maintenance of *in vitro* fruit germplasm.

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IN VITRO KONZERVACIJA VRHOVA IZDANAKA DVE BILJNE VRSTE (*Prunus cerasifera* EHRH. I *Rubus fruticosus* L.) PRIMENOM INKAPSULACIJE DEHIDRACIJE

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Izvod

In vitro vrhovi izdanaka generativne podloge džanarika (*Prunus cerasifera* Ehrh.) i kupine 'Čačanska Bestrna' (*Rubus fruticosus* L.) su testirani na mogućnost ponovnog rastjenja (konverzije) posle krioprezervacije primenom metode inkapsulacija dehidracija. Vrhovi izdanaka veličine 2–3 mm su inkapsulirani u 3, 5 i 10% natrijum-alginatnom rastvoru u tečnom Murashige i Skoog (MS) medijumu bez CaCl₂, koji je sadržavao 1,0 mg l⁻¹ benziladenina, 0,1 mg l⁻¹ indol-3-buterne kiseline i 0,1 mg l⁻¹ gibernelne kiseline. Polimerizacija je vršena u tečnom MS medijumu koji je sadržavao 100 mM CaCl₂, u trajanju 30 min na sobnoj temperaturi. Inkapsulirani vrhovi izdanaka su izloženi u pretretmanu u tečnom MS medijumu sa 0,75 i 1 M saharozom, 24 h u klima komori za gajenje biljaka i dehidrirani 4 h i 8 h (29% i 20% sadržaj vlage, respektivno), pre brzog uranjanja u tečni azot. Posle vađenja iz tečnog azota krioposude sa inkapsuliranim eksplantatima su ostavljane 2 min u struji sterilnog vazduha u Laminaru, a zatim su otvarane i perlice su direktno stavljane na medijum za multiplikaciju. Kod podloge džanarika, osmotska dehidracija u 0,75 M saharozu praćena desikacijom u trajanju od 8 h je dala najveći procenat konverzije (60%) vrhova izdanaka inkapsuliranih u 3% i 5% alginatnim perlicama. Međutim, u poređenju sa džanarikom, vrhovi izdanaka kupine krioprezervirani u istim eksperimentalnim uslovima su pokazali znatno manji kapacitet za ponovno rastjenje. Kod ovog genotipa, osmotska dehidracija u 1 M saharozu praćena desikacijom u trajanju od 8 h je dala najveći procenat konverzije (16.7%) vrhova izdanaka inkapsuliranih u 5% alginatnim perlicama. Krioprezervirani vrhovi izdanaka oba genotipa, multiplicirani u tri sukcesivne supkulture, su imali normalnu morfologiju i sličan kapacitet za multiplikaciju u poređenju sa kontrolnim izdancima koji nisu krioprezervirani.

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