Cryopreservation of cherry plum and blackberry shoot tips by droplet-vitrification

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1. Introduction

During a STSM performed in IRD Montpellier in the framework of COST Action 871, the droplet-vitrification technique was applied to *in vitro* grown cherry plum and blackberry cultivar 'Čačanska Bestrna'. The main advantage of this technique compared with vitrification is the possibility of achieving very high cooling/warming rates due to the very small volume of cryoprotectant medium in which the explants are placed and the direct contact with liquid nitrogen (Sakai and Engelmann 2007). This paper presents a report on preliminary results with cryopreservation of cherry plum and blackberry cultivar 'Čačanska Bestrna' *in vitro* grown shoot tips using droplet-vitrification. The establishment of the droplet-vitrification protocol was performed by evaluating the effect of different vitrification solutions (VSs) and treatment durations on recovery after liquid nitrogen (LN) exposure.

2. Materials and Methods

2.1. Plant material

Cryopreservation experiments were performed with *in vitro* shoot tips of cherry plum (*Prunus cerasifera* Ehrh.) and blackberry (*Rubus fruticosus* L. cv. 'Čačanska Bestrna'). Aseptic cultures of these species had been previously established at the Tissue Culture Laboratory of Fruit Research Institute, Čačak, Serbia. Shoot tips (1–2 mm in length) were sampled from nodal segments maintained for 3 weeks on MS medium (Murashige and Skoog 1962) containing 0.1 mg l^{-1} N^6-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. Cultures were maintained in 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}.

2.2. Cryopreservation technique employed

Isolated shoot tips were precultured in the dark at 23°C, in liquid MS medium with progressively increasing sucrose concentration (0.3 M for 15 h, then 0.7 M for 5 h). Loading involved 30 min incubation of explants in a solution comprising 1.9 M glycerol and 0.5 M sucrose (Kim *et al*. 2009a). Explants were dehydrated at room temperature using a modified PVS2 solution (solution A3, Kim *et al*. 2009b) for 10, 20 and 30 min and the PVS3 solution (Nishizawa *et al.* 1993) for 60, 90 and 120 min. Explants were cooled by direct immersion in LN in 10 µl droplets of vitrification solution placed on aluminium foil strips. Foil strips were retrieved from LN and plunged in a preheated (37°C) unloading solution (0.8 M sucrose) for 30 s, then an equal volume of unloading solution at room temperature was added for a further 30 min incubation (Kim *et al*. 2009b). Dehydration controls refer to replicates carried out under the same conditions as cryopreservation experiments but without immersion in LN. Shoot tips were transferred on regrowth medium, cultivated in the dark for 7 days, and then transferred to standard conditions. Survival was evaluated 3 weeks after cryopreservation by

counting the number of shoots that showed any kind of growth, while regrowth was defined as further development of apices into shoots with developed leaves 4–8 weeks after rewarming.

2.3. Statistical analysis

The experiments were replicated twice in two different laboratories (IRD Montpellier, France and Fruit Research Institute, Čačak, Serbia) and 10–15 shoot tips were used per experimental condition. Data presented in the form of percentages were subjected to arcsin transformation and subsequently analysed by ANOVA, followed by Duncan's Multiple Range Test for mean separation.

3. Results

3.1. Cryopreservation of Rubus fruticosus shoot tips

There was no significant effect of PVS treatment (both nature of solution and treatment duration) on regrowth of non-cryopreserved shoot tips of *R. fruticosus* (Fig. 1a). Regrowth of control shoot tips dehydrated with PVS3 ranged between 40% and 77.5%, and 45% and 60% for those dehydrated with modified PVS2 (A3). As for regrowth of cryopreserved shoot tips, PVS3 was better than the modified PVS2, but the difference was significant ($P< 0.05$) only for the shortest treatment duration. Also, PVS3 treatments provided fast-growing and more vigorous plantlets after cryopreservation (Fig. 2) in comparison with PVS2 (A3) treatments. The duration of PVS3 treatment had no significant effect on regrowth of cryopreserved shoot tips (45.8–70%), while the longest (30 min) exposure of explants to A3 solution resulted in a significant increase in regeneration percentage (30%) in comparison with a 10-min treatment with the same solution (5%).

3.2 Cryopreservation of Prunus cerasifera shoot tips

No significant influence of PVS treatment on regrowth of both dehydrated controls and cryopreserved shoot tips was observed in *P. cerasifera*. Regrowth of control explants ranged between 15% and 45.6%, and between 5% and 20% for cryopreserved samples (Fig. 1b). In addition, control shoot tips (both non-dehydrated and non-cryopreserved) loaded with C4 solution showed poor regrowth (37.3%). 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) (Fig. 3). Very often, regenerating explants formed clusters of small bud rudiments. The same signs were also visible on loading controls.

4. Discussion

One of the most critical steps in developing vitrification-based cryopreservation protocols is dehydration with highly concentrated cryoprotectant solutions. Application of VSs is usually highly species-specific and the duration of dehydration has to be determined very precisely in order to find a correct balance between toxicity and an adequate dehydration to reduce possibility of ice crystal formation in cryopreserved tissues (Panis *et al*. 2005). Our preliminary experiments, conducted in two different laboratories, demonstrated that it is possible to cryopreserve both *Rubus* and *Prunus* genotypes using droplet-vitrification including treatment with both PVS3 and PVS2 (A3) solutions. However, the regrowth percentages achieved and the qualitative characteristics of regrowing shoots were lower in *P. cerasifera*. Although the utilization of PVS3 is limited due to the high osmotic stress it may induce (Kim *et al*. 2009b), in our experiments *R. fruticosus* explants withstood a broad range of treatments with PVS3 without the occurrence of significant decrease in regrowth

percentage. As for the qualitative aspects of different PVS3 treatment durations, a 60 min treatment produced the highest quality plantlets after cryopreservation. This indicates that further optimisation of the protocol for this genotype may be achieved by decreasing the duration of PVS3 treatment. As for droplet-vitrification experiments performed with PVS2, a slight increase duration of PVS2 (A3) treatment and/or the use of other PVS2-based solutions with lower concentrations of dimethylsulfoxide (DMSO) and ethylene glycol (EG) may improve regrowth of cryopreserved shoot tips. The low regrowth of non-frozen controls and the low quality of regenerated shoots of *P. cerasifera* indicate the necessity of identifying the step(s) of the protocol which is/are harmful to shoot tips. Further research should focus on optimizing the preconditioning step (suppression of sucrose pretreatment and/or addition of cold acclimation) and comparing the effect of other loading and vitrification solutions and of shorter treatment durations.

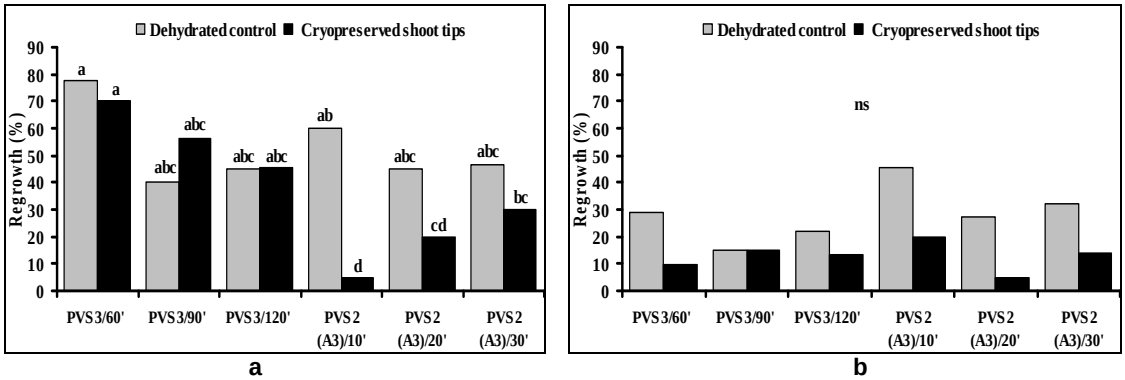

Figure 1. Regrowth of dehydrated controls and cryopreserved shoot tips of *R. fruticosus* (a) and *P. cerasifera* (b) dehydrated with PVS3 and modified PVS2 solution (A3). Mean values followed by the same letter are not significantly different ($P < 0.05$); ns – non significant.

Figure 2. Regrowth of cryopreserved shoot tips of *R. fruticosus* dehydrated for 60 min with PVS3.

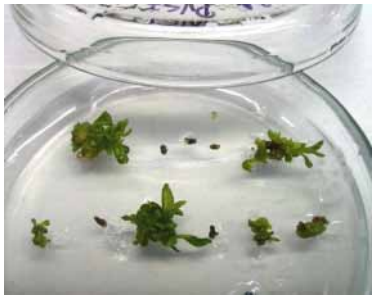

Figure 3. Regrowth of cryopreserved shoot tips of *P. cerasifera* dehydrated for 120 min with PVS3.

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