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Droplet-vitrification of apical shoot tips of *Rubus fruticosus* L. and *Prunus cerasifera* Ehrh.

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ABSTRACT

Apical shoot tips excised from *in vitro* plantlets of blackberry (*Rubus fruticosus* L. 'Čačanska Bestrna') and cherry plum (*Prunus cerasifera* Ehrh.) were tested for recovery after cryopreservation using the droplet-vitrification technique. Following treatment for 30 min with a loading solution comprising 1.9 M glycerol and 0.5 M sucrose, explants were dehydrated with a highly concentrated cryoprotectant solution, so called vitrification solution. Shoot tips were dehydrated for 10, 20 and 30 min at room temperature with a solution derived from the original PVS2 solution (containing 37.5% (w/v) glycerol, 15% (w/v) dimethylsulfoxide, 15% (w/v) ethylene glycol and 22.5% (w/v) sucrose) and for 60, 90 and 120 min using the PVS3 solution (containing 50% (w/v) glycerol and 50% (w/v) sucrose). Explants were cooled by direct immersion in LN in 10 μl droplets of vitrification solution placed on aluminium foil strips. Rewarming was done by direct plunging of foil strips in a preheated (37 °C) unloading solution (0.8 M sucrose) for 30 s, after which an equal volume of unloading solution (at room temperature) was added for further incubation for 30 min. As for regrowth of blackberry, PVS3 proved more effective than the modified PVS2, but the difference was significant ($P < 0.05$) only for the shortest treatment duration. The duration of PVS3 treatment had no significant effect on regrowth of cryopreserved shoot tips (45.8–70%). By contrast, a 30-min treatment with modified PVS2 solution resulted in a significant increase in regeneration percentage (30%), as compared with a 10-min treatment with the same solution (5%). Cherry plum shoot tips were very sensitive to both vitrification solutions and growth recovery of cryopreserved samples was generally lower (5–20%) than that of blackberry explants. No significant influence of PVS treatment (both type of solution and treatment duration) on regrowth of cryopreserved shoot tips was observed with cherry plum shoot tips. Experiments performed in France and in Serbia produced similar results, thereby showing the robustness and reproducibility of the protocols developed.

1. Introduction

The development of *in vitro* techniques for conservation of plant genetic resources as a complementary approach to conventional conservation methods reduces risks linked to management of field germplasm collections and offers additional security for field gene banks. 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). The utilization of new vitrification-based techniques has extended the applicability of cryopreservation to a broad range of tropical and temperate plant species (Engelmann, 2004).

Vitrification procedures involve dehydration of germplasm with highly concentrated cryoprotectant solutions (vitrification solutions, VSs) to prevent lethal ice crystal formation by means of vitrification, *i.e.*, the non-crystalline solidification of remaining water upon direct immersion of plant material in liquid nitrogen (LN) (Sakai, 2000). Although different VSs have been developed for cryopreserving plant tissues, the most commonly used and most efficient are glycerol-based vitrification solutions PVS2 (Sakai et al., 1990) and PVS3 (Nishizawa et al., 1993). However, dehydration procedures need to be carefully controlled in order to prevent biochemical and/or osmotic toxicity of cryoprotectant solutions. Recently, Kim et al. (2009b) developed alternative VSs, modified from the original PVS2 or PVS3 by increasing or decreasing the concentration of the constitutive cryoprotectants, which could be

Abbreviations: BA, N^6-benzyladenine; DMSO, dimethylsulfoxide; EG, ethylene glycol; GA_3, gibberellic acid; IBA, indole-3-butyric acid; LN, liquid nitrogen; MS, Murashige and Skoog (1962); VSs, vitrification solutions.

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successfully employed with plant materials which were very sensitive to these highly toxic VSs.

Until now, vitrification has been successfully applied to very broad range of plant species including representatives of *Prunus* (Niino et al., 1997; Shatnawi et al., 1999; De Carlo et al., 2000) as well as *Rubus* (Gupta and Reed, 2006; Reed et al., 2008; Uchendu et al., 2010) genera, which are two main genera of the *Rosaceae* family that include the majority of temperate fruit crops. Nevertheless, there are only a few reports on the application of the droplet-vitrification cryopreservation technique to fruit species belonging to these genera (De Boucaud et al., 2002; Condello et al., 2010). Droplet-vitrification (Panis et al., 2005) is the combination of droplet-freezing and solution-based vitrification. The main advantage of this technique over the vitrification procedure is the possibility of achieving very high cooling/warming rates due to the very small volume of cryoprotective medium in which the explants are placed (Sakai and Engelmann, 2007). According to Panis et al. (2005), ultra-fast cooling/warming rates significantly increase the probability to obtain a vitrified state during cooling and to avoid devitrification during rewarming. Although droplet-vitrification has so far been applied to a limited number of plant species (reviewed by Sakai and Engelmann, 2007), the promising results achieved encourage a broader application of this technique.

This paper presents the results on the application of the droplet-vitrification technique for cryopreservation of *in vitro* grown shoot tips of blackberry cultivar 'Čačanska Bestrna' (*Rubus fruticosus* L.) and cherry plum (*Prunus cerasifera* Ehrh.). The establishment of the droplet-vitrification protocol was performed by evaluating the effect of two vitrification solutions and different treatment durations on recovery of explants after LN exposure, along with the assessment of reproducibility and comparison of results obtained in two different laboratories – UMR DIADE, Centre IRD de Montpellier and Fruit Research Institute, Čačak.

2. Materials and methods

2.1. Plant material

In vitro grown shoot tips of blackberry (*R. fruticosus* L. 'Čačanska Bestrna') and cherry plum (*P. cerasifera* Ehrh.) were the starting material for cryopreservation experiments. Aseptic cultures of these species had previously been established at the Tissue Culture Laboratory of Fruit Research Institute, Čačak, Serbia. Shoot tips were sampled from nodal segments maintained for 3 weeks on MS medium (Murashige and Skoog, 1962) containing 1 mg l^{-1} BA, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3, 20 g l^{-1} sucrose and 7 g l^{-1} agar. 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}. Individual shoot tips (1–2 mm in length) were dissected under a binocular microscope, transferred on solidified MS medium supplemented with 0.3 M sucrose and kept in the dark (Petri dishes wrapped with aluminium foil) until all shoots were excised.

2.2. Cryopreservation

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) and continuous shaking (90 rpm). Immediately after preculture, pregrowth control explants were transferred directly onto regrowth medium (MS medium supplemented with 1 mg l^{-1} BA, 0.1 mg l^{-1} IBA, 0.1 mg l^{-1} GA_3, 20 g l^{-1} sucrose and 7 g l^{-1} agar). Loading involved 30 min incubation of explants in a solution comprising 17.5% (w/v) glycerol and 17.1% (w/v) sucrose (C4 solution, Kim et al., 2009a). Loading controls refers to non-dehydrated and non-cryopreserved shoot tips loaded with C4 solution. Loading controls were directly unloaded in 27.4% (w/v) sucrose solution for 30 min and subsequently transferred onto medium for regrowth.

Shoot tips were dehydrated at room temperature using a modified PVS2 and PVS3 solutions. The former contained 37.5% (w/v) glycerol, 15% (w/v) dimethylsulfoxide (DMSO), 15% (w/v) ethylene glycol (EG) and 22.5% (w/v) sucrose (solution A3, Kim et al., 2009b) and was applied for 10, 20 and 30 min, whereas the latter comprised 50% (w/v) glycerol and 50% (w/v) sucrose (Nishizawa et al., 1993) and was applied for 60, 90 and 120 min. Just a few minutes before the end of dehydration, one explant was placed in each of the five droplets of vitrification solution (10 μl) placed on aluminium foil strips. Explants were cooled by direct immersion of foil strips in LN. For rewarming, foil strips were retrieved from LN and plunged in a preheated (37 °C) unloading solution (27.4% [w/v] sucrose) for 30 s, after which an equal volume of unloading solution (at room temperature) was added for further 30 min incubation (Kim et al., 2009b). Dehydration controls (−LN) refer to replicates carried out under the same conditions as cryopreservation experiments but without immersion in LN.

2.3. Assessment of survival and regrowth and statistical analysis

Upon unloading, shoot tips were transferred onto 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. Both survival and regrowth percentages were expressed relative to the total number of shoot tips treated. Mean number of regenerating shoots per explant was obtained by dividing the total number of regenerated shoots with the number of explants showing regrowth in each treatment.

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. Results for survival and regrowth, presented as mean percentages with standard error of the mean (SD) were subjected to arcsine transformation. Statistical differences between mean values of all parameters were assessed by analysis of variance (ANOVA) and Duncan's Multiple Range Test for mean separation.

2.4. Shoot multiplication capacity after regrowth

Shoots originating from different treatments were isolated separately, labeled according to their origin and transferred onto MS multiplication medium of the same hormonal composition as the regrowth medium. Multiplication capacity was monitored after a 28 day-culture interval and included the following parameters: multiplication index and length of axial and lateral shoots. Multiplication index was defined as the number of newly formed shoots (>0.5 cm) per initial shoot recorded after the stated subculture interval. Data were also analyzed by ANOVA, followed by Duncan's Multiple Range Test for mean separation.

3. Results

3.1. Cryopreservation of blackberry shoot tips

Preculture, loading and PVS treatments (both type of solution and treatment duration) had no significant effect on survival and regrowth of non-cryopreserved shoot tips of blackberry (Table 1). In all other experimental treatments, regrowth percentages were lower in comparison with survival percentages, without and with