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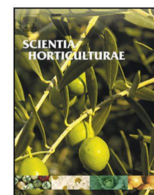

Cryopreservation of *Prunus* spp. using aluminium cryo-plates

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ABSTRACT

Cryopreservation using aluminium cryo-plates was successfully applied to *in vitro*-grown shoot tips of two *Prunus* genotypes, cherry plum (*Prunus cerasifera* Ehrh.) and the plum cultivar 'Požegača' (*Prunus domestica* L.). Shoot tips were dissected from the shoots and precultured for 1 day at 23 °C in the dark on Murashige and Skoog medium containing 0.3 M sucrose. The precultured shoot tips were placed on aluminium cryo-plates containing 10 or 12 wells and embedded in alginate gel. Osmoprotection was performed by immersing the cryo-plates in two types of loading solution (LS1 – 2 M glycerol + 0.4 M sucrose or C4 – 1.9 M glycerol + 0.5 M sucrose) for 30 min at room temperature. In the V cryo-plate protocol, dehydration was performed for 30 min at room temperature in a plant vitrification solution containing 37.5% (w/v) glycerol, 15% (w/v) dimethylsulfoxide, 15% (w/v) ethylene glycol and 22.5% (w/v) sucrose. In the D cryo-plate protocol, dehydration was performed by placing the cryo-plates for 2, 2.5 or 3 h under the air current of the laminar flow cabinet or in closed glass containers over silica gel. In both protocols, cooling was performed by placing the cryo-plates in uncapped 2 mL cryotubes, which were immersed in liquid nitrogen. Rewarming was done by direct plunging of cryo-plates in liquid MS medium containing 0.8 M sucrose at room temperature for 30 min (plum cultivar) or 60 min (cherry plum). All experiments were conducted twice in two different laboratories (IRD Montpellier, France and FRI Čačak, Serbia). In the V cryo-plate procedure regrowth (calculated as average values for two laboratories) of cryopreserved shoot tips loaded with C4 solution was 41.7% (cherry plum) and 34.2% (plum cultivar 'Požegača'), while in those loaded with LS1 solution regrowth was 56.1% and 44.6%, respectively. As for the D cryo-plate procedure, the average regrowth of cryopreserved explants ranged between 57.7% and 77.5% in cherry plum and between 28.5% and 47.5% in plum cultivar 'Požegača'. Although during the first subculture after regrowth, the multiplication capacity of cryopreserved explants was lower compared with those originating from dissection controls, by the third subculture they regained and even exceeded the multiplication capacity of shoots regenerated from explants placed on regrowth directly after dissection. The results obtained clearly indicate that both cryopreservation procedures using aluminium cryo-plates can facilitate efficient cryostorage of *Prunus* germplasm.

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1. Introduction

The significant progress achieved in *in vitro* culture and cryopreservation technologies has helped improving conservation of genetic resources, especially for vegetatively propagated plant species. Most efforts have focused on the development of procedures that eliminate the need for controlled cooling and enable direct transfer of plant material into liquid nitrogen (LN), which involves application of different vitrification-based techniques (Sakai and Engelmann, 2007). However, successful large-scale implementation of cryopreservation techniques for long-term conservation of vegetatively propagated crops depends on the availability of efficient and reproducible cryopreservation protocols applicable to diverse germplasm (Panis and Lambardi, 2005) and validated in different laboratories (Reed et al., 2004). Many cryopreservation protocols have been developed using at best few genotypes only and usually require some modification/changes in order to achieve optimal post-storage recovery of plants belonging to other species.

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

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Prunus is an economically very important genus in the Rosaceae family that includes over 200 species cultivated worldwide (Bortiri et al., 2001), mainly for their fruits and as ornamentals. Furthermore, in many species, numerous cultivars have been developed over time, leading to an extremely rich array of genotypes. It is therefore very important to maintain the diversity of this genus, as this conservation is essential for classical and modern plant breeding programs. To date, vitrification-based techniques including vitrification, encapsulation-dehydration, encapsulation-vitrification and droplet-vitrification have been successfully applied to a broad range of plant species (Engelmann, 2004; Sakai and Engelmann, 2007), including different fruit species belonging to the *Prunus* genus (Benelli et al., 2013). However, the routine implementation of cryopreservation technologies in plant genetic resources programs worldwide is hampered by such drawbacks as insufficient or excessive dehydration, damage and subsequent loss of material, and manipulation problems (Yamamoto et al., 2011a). Recently, cryopreservation methods using aluminium cryo-plates were developed at the National Institute of Agrobiological Sciences (NIAS) Genebank (Tsukuba, Japan). Briefly, explants are made to adhere to the aluminium plates (cryo-plates) in sodium alginate droplets before sample dehydration is achieved using various vitrification solutions (V cryo-plate method) or air-dehydration (D cryo-plate method). High regrowth was reported for different plant species. Indeed, the V cryo-plate method has been successfully applied to Dalmatian chrysanthemum (Yamamoto et al., 2011a), strawberry (Yamamoto et al., 2011b), carnation (Sekizawa et al., 2011), potato (Yamamoto et al., 2012a), mint (Yamamoto et al., 2012b), mulberry (Yamamoto et al., 2012c) and mat rush (Niino et al., 2013). The D cryo-plate method has been adapted to mat rush (Niino et al., 2014), chrysanthemum (Tanaka et al., 2014) and potato (Yamamoto et al., 2015). Both cryopreservation methods are very practical, efficient and also appear to be promising for other plant species with only minor adaptations in the procedures.

Although both cryo-plate methods are expected to facilitate the use of cryopreservation for efficient and cost-effective long-term storage of vegetatively propagated plants, it is necessary to expand and assess the applicability of these methods to additional crop species, especially those demonstrating poor regrowth capacities after cryo-storage using other techniques. This paper investigates the possibility to cryopreserve *in vitro*-grown shoot tips of two *Prunus* genotypes, *i.e.*, cherry plum (*P. cerasifera* Ehrh.) and the plum cultivar 'Požegača' (*P. domestica* L.) using both the V cryo-plate and D cryo-plate methods. To assess the reproducibility of the protocols used, we performed all experiments in two different laboratories (IRD, UMR DIADE, Centre de Montpellier, France and the Fruit Research Institute—FRI, Čačak, Serbia) and compared the results obtained. Another important aspect of this study was the monitoring of multiplication capacity of shoots regenerated from shoot tips after LN exposure.

2. Materials and methods

2.1. Plant material and tissue culture protocol

Tissue-cultured shoots of cherry plum (*Prunus cerasifera* Ehrh.) and 'Požegača' plum cultivar (*Prunus domestica* L.) were used for the experiments. Cherry plum or myrobalan (*Prunus cerasifera* Ehrh.) is a plum species native to south-eastern Europe or south-western Asia. Cherry plum seedlings are mostly used as rootstock for plum, but also for apricot, almond and peach. Being an autochthonous species, cherry plum is widely used in Serbian and other countries plum breeding programs (Nikolić and Rakonjac, 2007). 'Požegača' is today the most widespread and most important prune variety in Europe known by different names such as, 'Quetsche Commune' in France, 'Hauszwetsche' in Germany, 'Common Plum', *etc.* (Hartmann and Neumuller, 2009).

Aseptic cultures of both genotypes were established at the Tissue Culture Laboratory of the FRI, Čačak, using actively growing leaf buds selected from screenhouse-grown plants. The surface sterilization procedure involved washing explants under running water for 2 h, sterilization in 70% ethanol (1 min), and 10 min-soaking in 10% (v/v) commercial bleach solution (0.4%, w/v, final concentration of sodium hypochlorite), followed by triple rinsing with sterile water. Buds (0.3–0.8 cm long) were isolated under a stereomicroscope and placed onto MS nutritive medium (Murashige and Skoog, 1962) containing 2 mg L^{-1} *N*6-benzyladenine (BA), 0.5 mg L^{-1} indole-3-butyric acid (IBA) and 0.1 mg L^{-1} gibberellic acid (GA_3). Upon rosette initiation, shoots were multiplied on MS shoot proliferation medium containing 1 mg L^{-1} BA, 0.1 mg L^{-1} IBA and 0.1 mg L^{-1} GA_3 (cherry plum), or 2.25 mg L^{-1} BA and 0.1 mg L^{-1} IBA (plum cultivar 'Požegača') and subcultured at 3-week intervals to obtain a sufficient number of mother stock axillary shoots for cryopreservation experiments. *In vitro* shoots were at the forth and sixth subculture for cryopreservation experiments conducted in IRD Montpellier, France and FRI Čačak, Serbia, respectively. Cultures were maintained in a growth chamber at 23 ± 1 °C, with 16 h photoperiod under 54 μmol m^{-2} s^{-1} light intensity, provided by cool white fluorescent tubes.

2.2. Vitrification using aluminium cryo-plate (V cryo-plate procedure)

In order to obtain uniform material, shoots about 5 mm in length were cut and plated onto 30 mL solid MS multiplication medium (hormonal composition for each genotype as mentioned above) in Petri dishes (90 mm × 20 mm), and cultured for 2 weeks at 23 °C under standard growth conditions. Shoots for cryopreservation experiments were brought to IRD Montpellier, France at this stage. Then, shoot tips (approx. 1.5 mm long) were dissected from the shoots and precultured for 1 day at 23 °C in the dark on MS multiplication medium with 0.3 M sucrose.

The V cryo-plate procedure was performed as described for chrysanthemum by Yamamoto et al. (2011a). The cryo-plates were custom-made by Taiyo Nippon Sanso Corp., Tokyo, Japan (courtesy of Dr. Shin-ichi Yamamoto, Genebank, National Institute of Agrobiological Sciences, Tsukuba, Japan). The size of the aluminium cryo-plates (fitting in 2 mL cryotubes) was 7 mm × 37 mm × 0.5 mm with 10 or 12 oval shaped wells (length 2.5 mm, width 1.5 mm, depth 0.75 mm). Precultured shoot tips were carefully fitted individually in each well 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 dropwise on the aluminium plates with the shoot tips until they were fully covered. After 20 min, the calcium solution was removed by gently tapping the cryo-plate onto filter paper and cryo-plates with adhering explants were transferred to a loading solution (LS) for 30 min at room temperature. Two types of LSs were used for osmoprotection of explants in both genotypes: LS1 solution—2.0 M glycerol and 0.4 M sucrose in liquid MS medium (Nishizawa et al., 1992) and C4 solution—1.9 M glycerol and 0.5 M sucrose in liquid MS medium (Kim et al., 2009a). Following the loading treatment, explants were dehydrated for 30 min at room temperature with a vitrification solution comprising 37.5% (w/v) glycerol, 15% (w/v) dimethylsulfoxide (DMSO), 15% (w/v) ethylene glycol (EG) and 22.5% (w/v) sucrose (PVS A3, Kim et al., 2009b). Cryo-plates with adhering shoot tips were then transferred to 2 mL uncapped cryotubes held on cryo-canes and directly immersed in LN where they were kept for at least 30 min. For rewarming, cryotubes were retrieved from LN and cryo-plates were plunged into an unloading solution (0.8 M

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