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Three vitrification-based cryopreservation procedures cause different cryo-injuries to potato shoot tips while all maintain genetic integrity in regenerants

Biao Wang[a,1], Jing-Wei Li[a,1], Zhi-Bo Zhang[a,b], Ren-Rui Wang[a], Yan-Li Ma[a], Dag-Ragnar Blystad[b], E.R. Joachim Keller[c], Qiao-Chun Wang[a,*]

[a] State Key Laboratory of Crop Stress Biology in Arid Areas, Key Laboratory of Genetic Improvement of Horticultural Crops of Northwest China of Ministry of Agriculture of China, College of Horticulture, Northwest A&F University, Yangling 712100 Shaanxi, PR China
[b] Plant Health and Plant Protection Division, Bioforsk, N-4213 Ås, Norway
[c] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), 06466 Seeland OT Gatersleben, Germany

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ABSTRACT

We previously reported successful cryopreservation of shoot tips of potato 'Zihuabai' by three vitrification-based protocols. In the present study, cryo-injury to shoot tips and genetic stability in regenerants recovered from cryopreserved shoot tips by the three vitrification-based protocols were further investigated. The results showed that sucrose preculture caused no obviously different injuries, while dehydration with plant vitrification solution 2 (PVS2) was the step causing major damage to cells of shoot tips, regardless of the cryogenic procedures. Compared with droplet-vitrification and encapsulation–vitrification, vitrification caused the most severe injury to cells of the shoot tips, thus resulting in much longer time duration for shoot recovery and much lower shoot regrowth rate. Cells in apical dome and the youngest leaf primordia were able to survive and subsequently some of them regrew into shoots following all three vitrification-based cryopreservation procedures. Analyses using inter-simple sequence repeat (ISSR) and amplified fragment length polymorphism (AFLP) markers in shoots regrown from all three vitrification-based protocols did not find any polymorphic bands. The results reported here suggest that vitrification-based cryo-procedures can be considered promising methods for long-term preservation of potato genetic resources.

1. Introduction

Cryopreservation is an ideal means for long-term storage of plant germplasm. Over the last two decades, considerable progresses have been made in plant cryobiology (Benelli et al., 2013; Benson, 2008; Reed, 2008). To date, various cryopreservation procedures have been successfully established for almost all of the important agricultural and horticultural crops (Benelli et al., 2013; Feng et al., 2011; Reed, 2008; Wang et al., 2008). In addition, cryo-techniques have great potential applications to plant pathogen eradication (Wang and Valkonen, 2009) and genetic transformation (Wang et al., 2012).

Yet, genotype specificity is a major factor limiting much wider application of cryopreservation (Benson, 2008; Feng et al., 2011; Reed, 2008; Wang et al., 2009, 2012; Wang and Valkonen, 2009). In order to break down this limitation, great efforts have been made to either develop various protocols for the same genotype (Agrawal et al., 2004; Gogoi et al., 2012; Halmagyi et al., 2004; Wang et al., 2005, 2013) or seek for a widely applicable cryopreservation protocol for different genotypes within the same species (Feng et al., 2013; Panis et al., 2005). The former is of great significance, because if one protocol fails to some cultivars within the species, another protocol may work (Halmagyi et al., 2004; Wang et al., 2013). In addition, a number of comparative studies on cryo-injury, genetic stability and pathogen eradication efficiency can be performed upon availability of various cryopreservation protocols for the same genotype (Halmagyi et al., 2004; Wang et al., 2013).

Potato (*Solanum tuberosum*) is one of the most studied crops with regard to cryopreservation (Kaczmarczyk et al., 2011; Wang et al., 2008), and so far various cryogenic procedures have been developed, including dimethyl sulfoxide (DMSO) droplet

* Corresponding author. Tel.: +86 29 87081660; fax: +86 29 87081660.
E-mail addresses: qiaochun.wang@hotmail.com, qiaochunwang@nwsuaf.edu.cn (Q.-C. Wang).
[1] Those two authors contributed equally to the present study.

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freezing (Keller and Dreiling, 2002; Kryszczuk et al., 2006; Schäfer-Menuhr et al., 1996), droplet-vitrification (Halmagyi et al., 2005; Hirai, 2011; Kim et al., 2006; Panta et al., 2006; Wang et al., 2013), encapsulation–dehydration (Bouafia et al., 1996; Fabre and Dereuddre, 1990; Hirai and Sakai, 1999), encapsulation–vitrification (Hirai and Sakai, 1999; Wang et al., 2013), pregrowth-desiccation (Faltus et al., 2006) and vitrification (Kryszczuk et al., 2006; Panta et al., 2006; Wang et al., 2013). Several cryo-banks have been set up and used for the conservation of potato genetic resources. Cryoprocedures upon which potato cryo-banking was established differed from laboratory to laboratory. For example, DMSO droplet freezing was applied in Germany (Keller and Dreiling, 2002), vitrification and droplet-vitrification at International Potato Center (CIP) in Peru (Panta et al., 2006), pregrowth-desiccation in Czech Republic (Faltus et al., 2006) and droplet-vitrification in Korea (Kim et al., 2006). The same is true for other plant species, *e.g.*, droplet-vitrification for banana (Panis, 2009), two-step freezing for apple (Towill et al., 2004), two-step freezing and vitrification for pear (Reed et al., 2000).

For long-term storage of plant germplasm, a major concern is the genetic integrity of plants regrown from cryopreservation (Benson, 2008). Previous studies have shown that survival pattern in cryopreserved shoot tips varied with cryogenic protocols in plant species such as *Solanum tuberosum* (Wang et al., 2013), *Eutrema japonicum* (Matsumoto et al., 1995) and *Rubus idaeus* (Wang et al., 2005). As a result, differences in cryo-injury may alter genetic stability of plants recovered from shoot tips cryopreserved by different protocols (Harding, 2004; Martín and González-Benito, 2005). Vitrification-based cryopreservation procedures such as vitrification, droplet-vitrification and encapsulation–vitrification have successfully been applied to more than 100 plant species including agricultural and horticultural crops (Benelli et al., 2013; Reed, 2008) and are, at present, most frequently employed for long-term preservation of plant genetic resources.

We previously reported successful cryopreservation of potato 'Zihuabai' shoot tips using three vitrification-based procedures (Wang et al., 2013). The objective of the present study was, therefore, to investigate cryo-injury to potato shoot tips and genetic alteration in regenerants recovered from droplet-vitrification, encapsulation–vitrification and vitrification cryopreservation.

2. Materials and methods

2.1. Plant material

Potato (*Solanum tuberosum*) 'Zihuabai' a major cultivar widely grown in China, was employed in the present study. *In vitro* shoot stock cultures were maintained on a basal medium (BM) composed of Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) containing 30 g/l sucrose and 7 g/l agar (A1296, Sigma Chemical Co., USA). The pH of the medium was adjusted to 5.8 prior to autoclaving at 121 °C for 20 min. The stock shoots were grown at a temperature of 22 ± 2 °C under a 16-h photoperiod with a light intensity of 45 μmol s^{-1} m^{-2} provided by cool-white fluorescent tubes. *In vitro* stock cultures were micropropagated using single nodal segments. Subculture was performed once every three weeks. Nodal segments (1 cm in length), each containing an axillary bud, were removed from three-week-old stock cultures and cultured on BM under the same conditions as described for the *in vitro* stock cultures. After seven days of culture, shoots (1–1.5 cm in length) developing from axillary buds were moved into a growth chamber and were cold-hardened in the dark at 5 °C for three weeks. Shoot tips (2 mm in length) containing 5–6 leaf primordia were excised from the cold-hardened shoots and used for all three vitrification-based cryopreservation procedures.

2.2. Cryopreservation

2.2.1. Droplet-vitrification

Droplet-vitrification was conducted as described by Wang et al. (2013). Shoot tips were precultured on BM containing 0.3 M sucrose in the dark at 5 °C for 3 days. Precultured shoot tips were loaded for 30 min with a loading solution containing 2 M glycerol and 0.4 M sucrose in MS and then dehydrated with PVS2 (Sakai et al., 1990) at 0 °C for 40 min. PVS2 consisted of 30% (w/v) glycerol, 15% (w/v) dimethyl sulfoxide (DMSO), 15% (w/v) ethylene glycol and 0.4 M sucrose in MS medium. Dehydrated shoot tips were transferred onto aluminum foils followed by a direct immersion in liquid nitrogen (LN) for one hour. Cooled foil strips with shoot tips were rapidly plunged into an unloading solution composed of MS supplemented with 1.2 M sucrose at 25 °C for 20 min.

2.2.2. Encapsulation–vitrification

Encapsulation–vitrification was performed according to Wang et al. (2013). Shoot tips were precultured on BM containing 0.3 M sucrose for 1 day and then suspended in MS solution medium supplemented 2.5% (w/v) Na-alginate and 0.4 M sucrose, alginate beads were gelled by dropping into 0.1 M $CaCl_2$ solution containing 0.4 M sucrose. The encapsulated beads were immersed in the loading solution containing 2 M glycerol and 0.6 M sucrose in MS for 90 min and then dehydrated with PVS2 at 0 °C for 4 h. Ten dehydrated beads were transferred into cryovials, followed by a direct immersion into LN for 1 h. Rewarming was performed in a water bath at 38 °C for 2 min and then unloaded with 1.2 M sucrose at 25 °C for 20 min.

2.2.3. Vitrification

Vitrification was carried out as reported by Wang et al. (2013). Shoot tips were precultured on BM containing 0.45 M sucrose for 1 day and then stepwise dehydrated with 60% and 80% PVS2, with duration of each step being 30 min and finally with 100% PVS2 for 40 min. All dehydration steps were carried out at 0 °C. Dehydrated shoot tips were transferred into cryovials, followed by a direct immersion of the cryovials into LN for one hour. Rewarming and unloading were performed as described in the encapsulation–vitrification procedure described above.

2.2.4. Post-culture for shoot recovery

Shoot recovery was performed according to Wang et al. (2013), with some modifications described as following. Rewarmed, unloaded shoot tips from three cryopreservation methods were post-cultured on a shoot recovery medium 1 (SRM1) composed of BM supplemented with 0.5 mg/l indoleacetic acid (IAA), 0.5 mg/l zeatin riboside (ZR) and 0.2 mg/l gibberellic acid (GA_3) (Towill, 1983) and kept in the dark at 22 ± 2 °C for 3 days. For encapsulation–vitrification, shoot tips were extracted from the beads before post-culture for recovery. Subsequently, shoot tips cryopreserved by encapsulation–vitrification were transferred onto a shoot recovery medium 2 (SRM2) composed of BM containing 0.8 mg/l ZR and 2 mg/l GA_3 (Beaujean et al., 1998), while those by vitrification and droplet-vitrification onto a shoot recovery medium 3 (SRM3) consisting of BM supplemented with 0.05 mg/l GA_3, for shoot regrowth. Use of these two shoot recovery media was because SRM2 was found optimal for shoot recovery in encapsulation–vitrification, while SRM3 for those in vitrification and droplet-vitrification (Wang et al., 2013). Survival was recorded seven days after post-culture and expressed as percentage of the total number of shoot tips showing any new tissue growth, while shoot regrowth was defined as percentage of the total number of shoot tips regenerating into normal shoots (≥5 mm) 4 weeks after after post-culture.