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Shoot recovery and genetic integrity of *Chrysanthemum morifolium* shoot tips following cryopreservation by droplet-vitrification

Ren-Rui Wang[a,1], Xiao-Xia Gao[a,1], Long Chen[a], Liu-Qing Huo[a], Ming-Fu Li[b], Qiao-Chun Wang[a,*]

[a] State Key Laboratory of Crop Stress Biology for Arid Areas, Key Laboratory of Genetic Improvement of Horticultural Plants of Northwest China, Ministry of Agriculture of China, College of Horticulture, Northwest A&F University, Yangling 712100, Shaanxi, People's Republic of China
[b] Chinese Academy of Inspection and Quarantine (CAIQ), Chaoyang DistrictBeijing 100029, People's Republic of China

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ABSTRACT

We reported here an efficient, widely applicable droplet-vitrification cryopreservation for shoot tips of *Chrysanthemum morifolium*. Nodal segments, each being 0.5 cm in length and containing one bud positioned on nodes 3–7, were taken from 6 weeks old stock shoots and cultured on a shoot maintenance medium (SMM) for 12 days to promote bud elongation. Shoot tips (2.0 mm in size) containing 5–6 leaf primordia were excised from elongated buds and precultured on Murashige and Skoog medium (MS) containing 0.5 M sucrose for 1 day. Precultured shoot tips were loaded, dehydrated with PVS2 for 30 min at 0 °C and then transferred onto droplets containing 2.5 μl PVS2 on aluminum foils (2 cm × 0.8 cm), prior to a direct immersion in liquid nitrogen (LN) for 1 h. Thawed shoot tips were post-cultured on shoot recovery medium containing MS supplemented with 0.05 mg L^{-1} GA_3 in the dark for 3 days and then transferred on the same medium under standard culture conditions for shoot recovery. The droplet-vitrification procedure resulted in the highest (83%) and lowest (43%) shoot regrowth rates for *C. morifolium* 'Japanese Red' and 'Xizi Qiuzhuang', with an average rate of 68% in six *C. morifolium* genotypes tested. Histological observations showed that the pattern and percentage of surviving cells were similar in cryopreserved shoot tips of these two genotypes. No polymorphic bands were detected by simple sequence repeats (SSR) and ploidy levels analyzed by flow cytometry (FCM) were maintained in plantlets regenerated from cryopreserved shoot tips of the two genotypes.

1. Introduction

Chrysanthemum (*Chrysanthemum morifolium* Ramat.) is globally the second most important floricultural crop following rose (Teixeira da Silva, 2004). In Asian countries like China, Japan and Korean, *Chrysanthemum* has also long been used as medicines, mainly due to its pharmacological functions (China Pharmacopoeia Committee, 2005).

China is an original center for many *Chrysanthemum* species (Chen, 2012). Conservation of genetic resources is a prerequisite for breeding of novel cultivars by both classic and genetic engineering programs, and further exploitations of medicine-valued species. However, threats imposed by industrialization and urbanization that have been occurring since the last years in China are making plant genetic resources including *Chrysanthemum* face dangers of extinction. Cryopreservation, *i.e.* storage of living samples at ultra-low temperatures, usually in that of liquid nitrogen (LN, −196 °C), has long been recognized as an ideal means for the long-term conservation of plant germplasm including ornamental species (Wang and Perl, 2006; Kulus and Zalewska, 2014).

Fukai (1990) and Fukai et al. (1991) were the first to successfully cryopreserve *Chrysanthemum* shoot tips using two-step cooling. Since then, various cryopreservation protocols have been described, such as preculture-desiccation (Hitmi et al., 1999, 2000), encapsulation–dehydration (Sakai et al., 2000; Halmagyi et al., 2004; Martín and González-Benito, 2005; Martín et al., 2011), vitrification (Martín and González-Benito, 2005), DMSO droplet (Halmagyi et al., 2004) and droplet-vitrification (Halmagyi et al., 2004; Lee et al., 2011). Two-step cooling had to use dimethylsulfoxide (DMSO), which resulted in abnormal plants from cryopreserved shoot tips (Fukai, 1990; Fukai et al., 1991). In vitrification and encapsulation–dehydration, the stock cultures had to be cold-hardened for 3 weeks (Sakai et al., 2000; Martín and

* Corresponding author. Tel.: +86 29 87081660; fax: +86 29 87081660.
E-mail address: qiaochunwang@nwsuaf.edu.cn (Q.-C. Wang).
[1] These authors contributed equally to the present study.

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González-Benito, 2005; Martín et al., 2011), which requires expensive growth chamber and is time-consuming. In addition, genotype-dependent response is still very common, and efficient and widely applicable cryopreservation protocol is still lacking for *Chrysanthemum* (Martín and González-Benito, 2009).

Droplet-vitrification has been proved to be a suitable protocol for cryopreservation of a wide range of genotypes within the same species such as *Musa* (Panis et al., 2005), *Solanum* (Kim et al., 2006), *Malus* (Halmagyi et al., 2010) and *Allium* (Kim et al., 2012). Highly effective and user-friendlier characteristics of droplet-vitrification have been demonstrated in *Chrysanthemum* (Lee et al., 2011). However, a droplet-vitrification cryopreservation for diverse *Chrysanthemum* genotypes has not yet been reported, thus limiting routine applications of cryopreservation to establishment of cryo-banking of *Chrysanthemum* germplasm (Martín and González-Benito, 2009).

One of the most concerned issues is genetic stability in regenerants recovered form cryopreservation. Studies have been conducted on assessments of genetic stability in regenerants of *Chrysanthemum* following cryopreservation by encapsulation–dehydration and vitrification (Martín and González-Benito, 2005; Martín et al., 2011). Cryo-injury varied with cryo-procedures (Wang et al., 2005, 2013, 2014), which may result in differences in genetic stability in regenerants recovered from different cryopreservation protocols (Martín and González-Benito, 2005). However, data have been quite limited on assessments of genetic stability in regenerants from droplet-vitrification cryopreservation (Lee et al., 2011). Therefore, it is necessary to assess genetic stability in regenerants following droplet-vitrification, in order for this cryo-procedure to be applied for establishment of cryo-banking of *Chrysanthemum* germplasm.

The objective of the present study was, therefore, to develop an efficient droplet-vitrification cryopreservation for shoot tips of diverse *Chrysanthemum* genotypes. Histological observations on cell survival pattern, and assessments of genetic stability by single sequence repeats (SSR) and by flow cytometry (FCM) in the regenerants following cryopreservation were also conducted.

2. Materials and methods

2.1. Plant materials

C. morifolium 'Japanese Red' was used for optimizing the parameters in droplet-vitrification procedure. Five additional genotypes were subsequently used for testing the developed droplet-vitrification procedure. Of the six genotypes, 'Fall Color', 'Japanese Red' and 'Xizi Qiuzhuang' are used as pot flowers, and 'Roma Red' and 'Jinba' as cut flowers. 'Hangju' is of pharmacological functions and mainly used as medicine in China (China Pharmacopoeia Committee, 2005). *In vitro* stock shoots were maintained on a shoot maintenance medium (SMM) composed of Murashige and Skoog (1962) medium (MS) supplemented with 30 g L^{-1} sucrose and 7 g L^{-1} agar (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 cultures were maintained at 22 ± 2 °C under a 16-h photoperiod with a light intensity of 50 μmol s^{-1} m^{-2} provided by cool-white fluorescent tubes (standard culture conditions). Subculture was done once every 4 weeks.

2.2. Cryopreservation and shoot recovery

Three sizes of shoot tips: 0.5, 1.0 and 2.0 mm (Fig. 1a) in length containing 1–2, 3–4, 5–6 leaf primordia (LPs), respectively, were excised from terminal buds of 4 weeks old stock shoots. Based on our preliminary studies, shoot tips were directly precultured on MS enriched with 0.25, 0.5, 0.75 M sucrose for 1 day, to select an optimal sucrose concentration for cryopreservation. Precultured shoot tips were treated for 20 min at room temperature with a loading solution composed of MS containing 0.4 M sucrose and 2 M glycerol contained in sterile plastic Petri dishes (9 cm in diameter). Loaded shoot tips were exposed to plant vitrification solution 2 (PVS2) (Sakai et al., 1990) at 0 °C for 10–40 min. PVS2 is composed of MS supplemented with 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO and 0.4 M sucrose (pH 5.8). Each shoot tip was transferred onto a droplet containing 2.5 μl PVS2 carried on an aluminum foil (2 cm × 0.8 cm) (Fig. 1b), followed by a direct immersion in LN. After staying in LN for a few minutes, the foils with shoot tips were transferred into a 2 ml cryotube filled with LN for cryostorage for 1 h. Frozen foils with shoot tips were moved out from LN and immediately placed into an unloading solution containing liquid MS containing 1.2 M sucrose at room temperature for 20 min. Based on our preliminary studies, cryopreserved shoot tips were post-cultured for shoot recovery on three shoot recovery medium (SRM): (1) on SRM1 containing SMM supplemented with 1.0 mg L^{-1} 6-benzylaminopurine (BA) and 2.0 mg L^{-1} α-naphthalene acetic acid (NAA) under standard culture conditions, as described by Hitmi et al. (1999, 2000); (2) on SRM2 containing SMM supplemented with 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA under standard culture conditions, as described by Halmagyi et al. (2004); (3) on SRM3 containing SMM supplemented with 0.05 mg L^{-1} gibberellic acid-3 (GA_3) in the dark for 3 days and then transferred on the same medium under standard culture conditions. Filter-sterilized GA_3 was added to the medium after autoclaving, while BA and NAA to the medium before autoclaving. Survival was expressed as the percentage of shoot tips showing any green tissues of the total samples used for cryopreservation after 7 days of post-culture (Fig. 1c), while shoot regrowth was defined as percentage of shoot tips regenerating into shoots ≥0.5 mm of the total samples after 4 weeks of post-culture. Shoots (≥0.5 cm in length) were transferred on SMM for further shoot growth and root development. Shoots with roots developed after 8 weeks on SMM were acclimated and transferred to soil under greenhouse conditions, according to a method described by Wang et al. (2005).

2.3. Improvement of shoot recovery

When we applied the droplet-vitrification procedure established above to other five *Chrysanthemum* genotypes, shoot regrowth rates were low and some genotypes even failed to shoot regrowth. In addition, shoot tips from terminal buds were used, meaning that only one shoot tip can be taken from each of *in vitro* stock shoots. Therefore, an experiment was further designed in order to improve cryopreservation efficiency in terms of shoot recovery rate and shoot tip production. Nodal segments, each being about 0.4 cm in length and containing one bud, were taken from nodes positioned from 1 (youngest, apical bud) to 8 (oldest, axillary bud) of 4 and 6 weeks old stock shoots and numbered as 1–8 (Fig. 1d). The segments were cultured on SMM to promote bud elongation (Fig. 1e). After 12 days of culture, shoot tips (2.0 mm in size) containing 5–6 LPs were excised from the elongated buds (Fig. 1e1) and used for cryopreservation using the optimized parameters as described above.

2.4. Histological observation

Histological observations were conducted to compare pattern and number of surviving cells in cryopreserved shoot tips of two genotypes: 'Japanese Red' and 'Xizi Qiuzhuang' that produced the highest (83%) and lowest (43%) shoot regrowth rate,