Cryopreservation of shallot (*Allium cepa* var. *aggregatum*) shoot tips by droplet-vitrification

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Abstract

Shallot (*Allium cepa* var. *aggregatum*) is an important vegetable crop belonging to the genus *Allium*. The present study attempted to develop an efficient droplet-vitrification cryopreservation method for shallot '10603' shoot tips. In vitro stock shoots were maintained on Murashige and Skoog (1962) medium (MS) supplemented with 30 g L^{-1} sucrose, 0.5 mg L^{-1} BAP, 0.1 mg L^{-1} NAA and 8 g L^{-1} agar (pH=5.8). Shoot tips (2.0-3.0 mm in length) were excised from 4-week-old stock shoots and stepwise precultured with increased sucrose concentrations from 0.3 to 0.5 M, each concentration for 1 day. The precultured shoot tips were then loaded for 20 min with a solution composed of 2 M glycerol and 0.5 M sucrose, before exposure to PVS3 for 3 h at room temperature. Dehydrated shoot tips were transferred onto aluminum foils (2×0.8 cm), prior to direct immersion into liquid nitrogen (LN) for cryostorage. For thawing, frozen aluminum foils were moved from LN and immediately transferred into unloading solution composed of liquid MS containing 1.2 M sucrose. After incubation at room temperature for 20 min, shoot tips were post-cultured on solidified MS medium containing 0.3 M sucrose for 2 days and then transferred onto a recovery medium for shoot regrowth. With this procedure, 94% shoot tips survived, and 58% shoot tips regenerated into shoots following cryopreservation.

Keywords: cryopreservation, shallot, droplet-vitrification, shoot tips

INTRODUCTION

Shallot is an important vegetable belonging to the genus *Allium.* It possesses rich sources of flavonoids and a high level of antioxidant activity (Yang et al., 2004). Flavonoid consumption has been associated with a reduced risk of cancer (Hertog and Hollman, 1996), heart disease (Wattenberg, 1990; Wei et al., 1990) and diabetes (Campos et al., 2003), thus making shallot a healthy dietary option.

As a vegetatively propagated crop, traditional conservation of plant genetic resources ex situ or in vitro requires much more labour input. Field collections may suffer from pathogen infections and in vitro collections may be difficult to continually micropropagate and store (Keller, 2005).

Cryopreservation, i.e., preserving plant tissues at -196°C in liquid nitrogen can potentially obviate these drawbacks and, in the long run, reduce the expense of maintaining germplasm resources (Engelmann, 2004, 2011; Lynch et al., 2016).

The first report of successful cryopreservation of an *Allium* species was for Japanese shallot (*Allium wakegi*) with seven cultivars successfully cryopreserved by vitrification

(Kohmura et al., 1994). This was followed by Niwata (1995) who was the first to succeed in cryopreservation of garlic apices excised from post-dormant cloves. After that, garlic has been the main *Allium* species studied for cryopreservation and various cryogenic protocols have been developed, such as vitrification (Niwata, 1995; Kim et al., 2004, 2007b), droplet-vitrification (Volk et al., 2004; Kim et al., 2006a, 2007a, b) and

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encapsulation-dehydration (Lynch et al., 2012). Among various methods for cryopreservation, droplet-vitrification offers relatively simple handling and generally produces higher survival since ultra-rapid cooling and warming are facilitated within this protocol (Kim et al., 2007a). To date, it has been successfully applied to cryopreservation of various plant species, including chrysanthemum (*Dendranthema grandiflora*) (Halmagyi et al., 2004), potato (*Solanum tuberosum*) (Kim et al., 2006b), sweet potato (*Ipomoea batatas*) (Pennycooke and Towill, 2000) and apple (*Malus domestica*) (Li et al., 2015).

In this paper, we report the successful cryopreservation of *Allium cepa* var. *aggregatum* '10603' using droplet-vitrification.

MATERIAL AND METHODS

Plant material

In vitro *Allium cepa* var. *aggregatum* '10603' shoot stock cultures were maintained on a basic medium (BM) composed of Murashige and Skoog (1962) medium (MS) supplemented with 30 g L^{-1} sucrose, 0.5 mg L^{-1} 6-benzylaminopurine (BAP), 0.1 mg L^{-1} 1-naphthylacetic acid (NAA) and solidified by 8 g L^{-1} agar. The pH of medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. The cultures were maintained at a temperature of 22±2°C under a 16-h photoperiod with a light intensity of 45 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Subculture was performed every four weeks.

Shoot tips (about 2-3 mm in size; Figure 1b) containing 4-5 primordia were excised from 4-week-old stock cultures (Figure 1a) and were used for cryopreservation by droplet-vitrification.

Droplet-vitrification

Shoot tips were stepwise precultured with sugar enriched MS medium, 0.3 M sucrose for 1 day and followed by 0.5 M sucrose for 1 day, under the same condition as stock cultures. Following the preculture step, shoot tips were loaded for 0 to 40 min at room temperature with a loading solution composed of MS medium containing 2 M glycerol and 0.5 M sucrose. After the loading process, the shoot tips were dehydrated with plant vitrification solution 3 (PVS3) for various periods ranging between 0 h to 4 h at room temperature before transferring on to aluminum foil (2×0.8 cm). PVS3 contains MS supplemented with 50% (w/v) glycerol and 50% (w/v) sucrose (Nishizawa et al*.,* 1993). The shoot tips carried by the aluminum foil were plunged into LN and after a few minutes of immersion were transferred into a 2-mL cryotube, which had been prefilled with LN, for 1 h of cryostorage.

Rewarming and post-culture for recovery

When rewarming, frozen foil strips with shoot tips were quickly transferred into an unloading solution composed of MS and 1.2 M of sucrose at room temperature for 20 min. After unloading, shoot tips were briefly dried by filter paper and post cultured on different medium: 1) directly on BM; 2) MS medium with 0.5 M sucrose for 1 day and 0.3 M sucrose for 1 day before final regeneration on BM; 3) MS with 0.3 M sucrose for 2 days before final regeneration on BM. The regeneration progressed in darkness for the first three days, followed by exposure to weak light condition up to one week. Shoot tips were transferred to normal light condition after 1 week of post-culture.

Experimental design and statistical analysis

In each experiment, 10-12 shoot tips were included in each treatment of three replicates. All experiments were conducted twice. Results are presented as means with their standard error. The data were analyzed using one-directional ANOVA with least significant differences (LSD) were calculated at $P<0.05$.

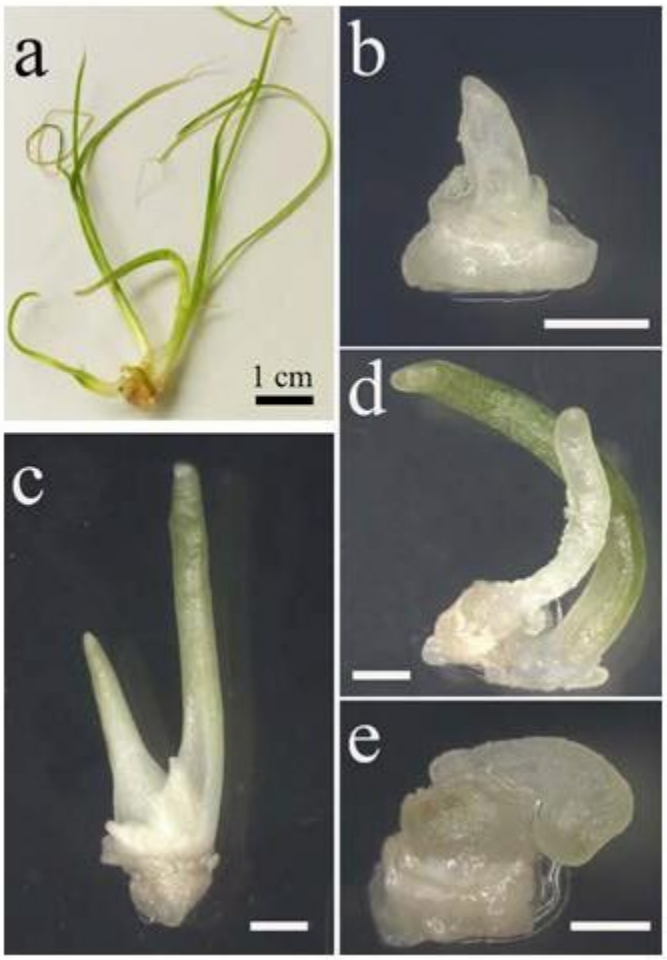

Figure 1. Shoot tips of *Allium cepa* var. *aggregatum* '10603' before and after cryopreservation. a) In vitro shallot '10603' plants after 4 weeks of culture on BM; b) Shallot shoot tip excised for cryopreservation; c) Regenerating shallot shoot tip after 2 weeks of culture post cryopreservation; d) Shallot shoot tips that survived cryopreservation developed hyperhydration after 2 weeks of post culture; e) A dead cryopreserved shallot shoot tip. Bars without numbers = 1 mm.

RESULTS

Overall process of recovery from cryopreserved shoot tips

After 1 week of post culture following cryopreservation, surviving shoot tips with green color were recorded, while dead ones remained white and hyperhydrated (Figure 1e). Some surviving shoot tips also became hyperhydrated within 2 weeks of post culture. New primordia failed to emerge from those shoot tips and the old primordia elongated and retained hyperhydrated (Figure 1d).

After two weeks of post culture, regrowth was evaluated by occurrence of new visible unhyperhydrated leaf primordium (Figure 1c). Regenerated plants were transferred to BM where, after an additional 4 weeks of post culture, grow extended 8 cm with 3-4 newly elongated leaf blades, and roots were initiated.

Loading treatment

Effect of different loading periods on survival and regrowth of cryopreserved shoot tips are shown in Figure 2. Different durations of loading treatment had no significant

influence on survival and regeneration of non-cryopreserved shoot tips or on the survival of cryopreserved shoot tips. Twenty-five percent of regrowth was obtained from the loading free treatment, and the regeneration rate was improved to 40% as shoot tips were pre-dehydrated for 20 min before the subsequent dehydration.

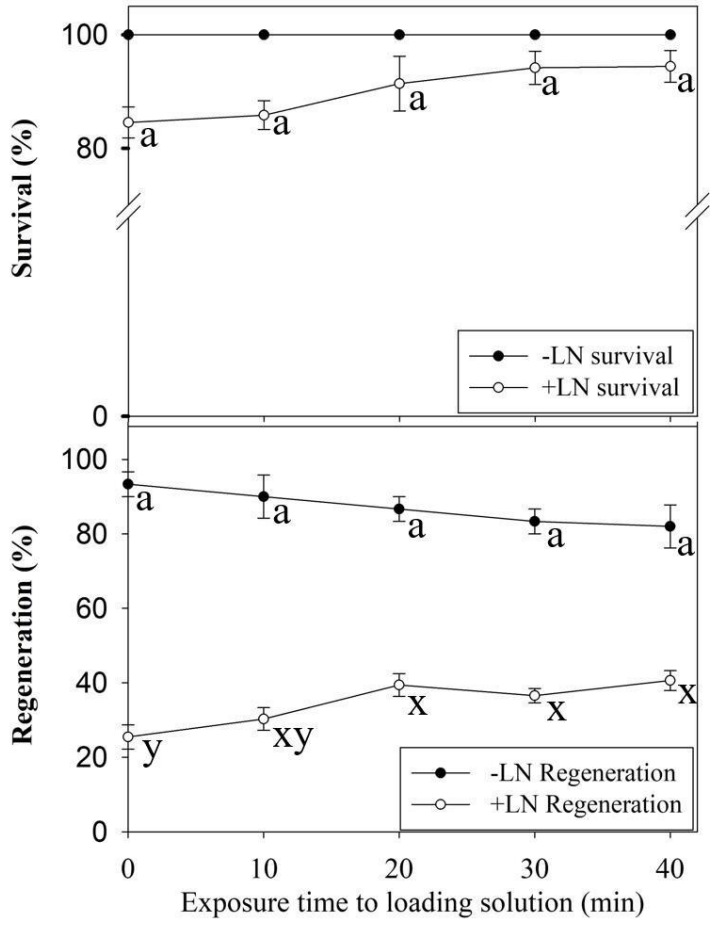

Figure 2. Effects of different durations of loading treatment on survival and shoot regrowth of cryopreserved shoot tips of *Allium cepa* var. *aggregatum* '10603' by droplet-vitrification. Shoot tips were precultured for 2 days on 0.3 M sucrose-enriched MS medium before different periods of loading. Afterwards, shoot tips were dehydrated with PVS3 for 2 h before cryopreservation. Shoot tips were maintained 2 days at 0.3 M sucrose-enriched medium before final regeneration on BM. Data are presented as means ± SE analyzed using one-directional ANOVA. Means with different letters in the same parameter indicate least significant differences (LSD) at $P<0.05$.

PVS3 dehydration

Both survival and regeneration rate after cryopreservation were improved as the duration of dehydration prolonged (Figure 3). More than 90% of shoot tips survived after 2 h of dehydration; the highest regrowth (58%) was obtained after 3 h of PVS3 treatment. The regrowth rate was slightly decreased as the PVS3 treatment extended to 4 h.

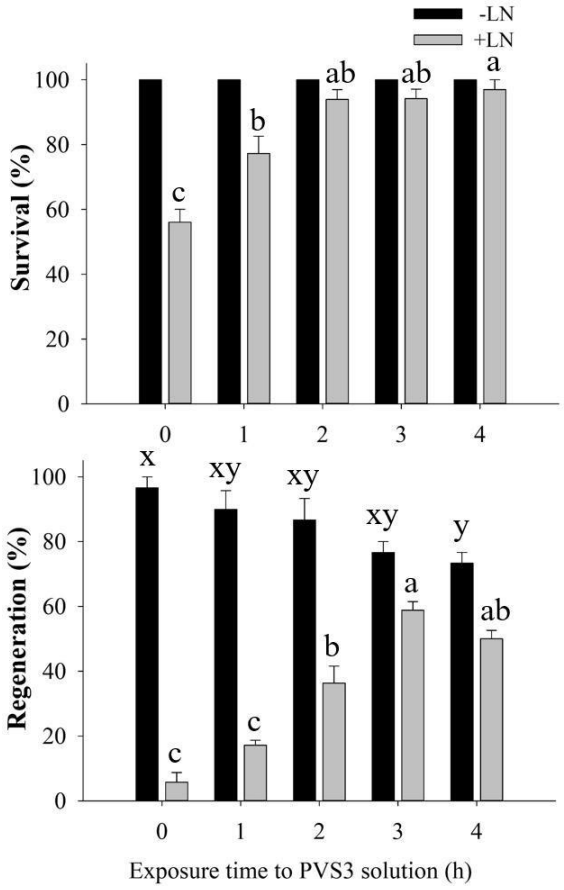

Figure 3. Effects of different durations of PVS3 treatment on survival and shoot regrowth of cryopreserved shoot tips of *Allium cepa* var. *aggregatum* '10603' by droplet-vitrification. Shoot tips were precultured 2 days on 0.3 M sucrose-enriched MS medium followed by 20 min of loading treatment. Then, shoot tips were dehydrated by PVS3 for different periods (h) before cryopreservation. After cryopreservation, shoot tips were maintained 2 days at
0.3 M sucrose-enriched medium before final regeneration on BM. Data are presented as means ± SE analyzed using one-directional ANOVA. Means with different letters in the same parameter indicate least significant differences (LSD) at $P<0.05$.

Post-culture after cryopreservation

Cryopreserved shoot tips of shallot '10603' that were post-cultured on MS containing different sucrose concentration regenerated differently. When only post cultured on BM for regeneration, the regrowth rate was 36, and 45% of regenerated shoot tips were hyperhydrated. When cryopreserved shoot tips were transferred to sucrose-enriched medium either at 0.5 M followed by 0.3 or 0.3 M for two days, more than 50% of regrowth rate could be obtained and hyperhydration rates decreased significantly compared with direct post culture on BM (Table 1).

Table 1. Effect of different post-culture media on regeneration of cryopreserved shoot tips of *Allium cepa* var. *aggregatum* '10603' by droplet-vitrification.

Post culture procedure	Sucrose concentration and postculture duration	Regeneration after cryopreservation (%)[1]	
		Non-hyperhydration	Hyperhydration
Direct	0.09 M (BM)	36±3b	45±4e
Stepwise	0.3 M for 2 d and BM	55±5a	35±7f
Stepwise	0.5 M for 1 d, 0.3 M for 1 d and BM	53±3a	32±3f

[1]Data are presented as means ± SE analyzed using one-directional ANOVA. Means with different letters in the same parameter indicate least significant differences (LSD) at P<0.05.

DISCUSSION

In the present study, a cryopreservation protocol has been established using shallot in vitro cultures. In previous studies, newly harvested and sterilized propagules have mostly been used for cryopreservation of *Allium* species (Kim et al., 2006a).

In vitro tissue culture is the fundamental technique for plant virus elimination, the viability of cryopreservation of in vitro propagating materials can facilitate the cryopreservation of virus-free *Allium* materials.

In cryopreservation, preculturing the explant is essential before freezing (Engelmann, 1997). In this study, excised shoot tips have been maintained on MS medium with 0.3 and 0.5 M sucrose medium 2 days. As for the vitrification-based cryopreservation, loading of samples is used to alleviate possible damage to shoot tips under the high osmotic dehydration of PVS (Sakai et al., 2008). Two molar glycerol with 0.5 and 0.6 M sucrose has been used as loading treatments for the successful cryopreservation of garlic shoot tips (Volk et al., 2004; Kim et al., 2006a, 2007b). Similarly, in this study, treatments with a loading solution containing 2.0 M glycerol and 0.5 M sucrose for 20-40 min significantly enhanced shallot shoot tips regrowth after cryopreservation.

Plant vitrification solution (PVS) can protect cells from lethal intracellular freezing that occurs during cooling and thawing (Sakai et al., 1990). Among different vitrification solutions, PVS2 has been shown to be effective at improving tolerance of cryopreservation in many plant species (Halmagyi et al., 2004; Leunufna and Keller, 2003; Panis et al., 2005; Pennycooke and Towill, 2000; Li et al., 2015; Wang et al., 2017). Among *Allium* species, garlic has been successfully cryopreserved using PVS2 vitrification procedure (Volk et al., 2004); however, PVS3-based procedure produced better regrowth (Makowska et al., 1999; Kim et al., 2006a, 2012). PVS2 contains DMSO which is potentially toxic to plant cells, therefore the duration of exposure time should be carefully controlled. In sufficient cryoprotection and inadequate dehydration can result in more crystallization injury. In our study, the optimal regrowth rate was obtained after 3 h of PVS3 dehydration. When exposure time was prolonged to 4 h, survival rate showed no difference, while the regrowth level decreased.

When cryopreserved shoot tips were directly transferred to BM for regeneration, more shoots became hyperhydric within 2 weeks of post culture. Regrowth rate was significantly improved when sucrose enriched medium was used for 2 days before regeneration on BM medium. After cryopreservation, rehydration occurred as soon as the highly osmotically protected shoot tips were transferred to the unloading solution (1.2 M sucrose) and continued during the regeneration at lower osmotic potential of BM (0.09 M sucrose). This was revealed using differential scanning calorimetry, as higher water contents were measured when cryoexposed garlic shoot tips were cultured on solid regenerated medium (Volk and Walters, 2006). In the present study, treatment of 0.3 M sucrose after the unloading may lead to decreased rehydration potential which contributed to the reduction of hyperhydration.

In the present study, a cryopreservation protocol for shallot (*Allium cepa* var. *aggregatum*) was firstly established using in vitro shoot tips. We will apply this protocol on cryopreservation of more shallot cultivars and assess shallot virus elimination.

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