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CRYOPRESERVATION OF ADVENTITIOUS SHOOT TIPS OF *PARAISOMETRUM MILEENSE* BY DROPLET VITRIFICATION

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Abstract

BACKGROUND: Gesneriaceae family contains numerous species endemic to China, and many of them are listed as endangered species. There is a need for a simple and efficient method for long term conservation of these species. **OBJECTIVE:** The study aimed to establish an efficient procedure for cryopreserving *Paraisometrum mileense*, a critically endangered species endemic to Yunnan, China. **METHODS:** Effects of sucrose concentration of preculture solution, duration of sucrose preculture, duration of plant vitrification solution 2 (PVS2) treatment, and cold acclimation on regeneration of cryopreserved adventitious shoot tips (ASTs) were assessed. **RESULTS AND CONCLUSION:** Among different sucrose preculture regimes tested, preculture with 0.3M sucrose for 24h resulted in best regeneration of cryopreserved ASTs. PVS2 treatment also affected regeneration considerably with the maximum survival of ASTs after incubation in PVS2 for 90 min at 0°C. With the optimised parameters, the level of shoot regeneration from cryopreserved ASTs reached 86%. No morphological abnormalities were observed during one year's growth of the plantlets developing from cryopreserved ASTs. Procedure established in this research is a promising technique for the cryopreservation of ASTs of this species.

Keywords: Gesneriaceae, endangered species, sucrose preculture, cold acclimation

INTRODUCTION

Paraisometrum mileense W. T. Wang, the only species of the *Paraisometrum* genus in the Gesneriaceae family is endemic to the limestone area of Southeast Yunnan, China. During his work for the Flora of China Project, Prof. Wen-Tsai Wang described this species on the basis of one gesneriad specimen, collected by a French missionary, F. Ducloux, in Southeast Yunnan in 1906 (23). *Paraisometrum mileense* was thought to be extinct until 2006, when a wild population (~320 plants) was rediscovered by Dr. Yuming Shui during a seed collection trip in Shilin County, Southeast Yunnan (http://www.kew.org /news/paraisometrum-mileense.htm). According to the International Union for Conservation of Nature (IUCN) criteria, *P. mileense* is critically endangered because of its restricted distribution and small population size (8). In 2012, the State Forestry Administration of China launched the Conservation Program for the Wild Plants with Extremely Small Population in China (2012–2015 Operational Plan); the natural habitat of *P. mileense* is protected under this program (8). However, for the long-term preservation, an efficient *ex situ* conservation methods, such as cryopreservation, are required to complement *in situ* conservation programs.

Cryopreservation, the storage of biological material at an ultra-low temperature (–196°C), is a method for long-term preservation of plant germplasm. At the low temperature, cell division and metabolic processes completely stops. The material can thus be stored without alteration for a theoretically unlimited period of time (2). Cryopreservation method for the genebanking of important crops, such as potato (18) and banana (11), are well established. To date, more than 100 plant species have been cryopreserved via

vitrification (13). Cryopreservation is also used in *ex situ* conservation of endangered plant species. Endangered species, such as *Cosmos atrosanguineus* (24) *Hypericum umbellatum* (1), *Emmenopterys henryi* (4) and *Teucrium polium* (12) have been successfully cryopreserved using various techniques. Conservation agencies, such as Kings Park and Botanic Garden in Perth, Australia (5), and the Royal Botanic Gardens, Kew, UK (17), are currently applying cryogenic approaches to preserve threatened plant species.

Droplet vitrification is derived from the droplet freezing method developed for the cryopreservation of potato shoot tips (18). In the droplet vitrification protocol, shoot tips are loaded, treated with plant vitrification solution 2 (PVS2) (15), transferred to a droplet of PVS2 on a strip of aluminium foil and plunged directly into liquid nitrogen. Given that there is direct contact between the explants/cryoprotective solution and liquid nitrogen, the cooling rate in the droplet vitrification procedure is higher than in vitrification procedures in which cryotubes are used (6). More rapid cooling and rewarming increases the chance of achieving a vitrified state during freezing, and avoids devitrification during rewarming; as such, they result in higher explant survival and regeneration following cryopreservation (11). Droplet vitrification was successfully applied to shoot tips of taro (16), yam (6), asparagus (9) and the model plant *Arabidopsis thaliana* (19).

The Gesneriaceae family has numerous species endemic to China (25), and many of them are listed as endangered. The need to develop a simple and efficient method for long term conservation of these species is urgent. However, the cryopreservation for Gesneriaceae plants has been scarcely reported. In the present study, we report a method for cryopreservation of adventitious shoot tips (ASTs) of *P. mileense* by droplet vitrification, in which a preculture of ASTs in liquid Murashige and Skoog (MS) medium with 0.3M sucrose for 24 h is sufficient to ensure high level of shoot regeneration following cryopreservation.

MATERIALS AND METHODS

Adventitious shoot tips from leaf explants

In vitro-grown plantlets of *P. mileense* were obtained from the Germplasm Bank of Wild Species, Kunming, China. Shoot cultures were transferred to a hormone-free MS medium with 0.09 M sucrose, and solidified with 2.5 g l^{-1} Phytagel. Culture jars were maintained in a room at 25°C under a 14/10 h (day/night) photoperiod with LED at 40 μmol m^{-2} s^{-1}. After four weeks of culture, leaves from *in vitro* shoots (Fig. 1a) were collected and used as explants to induce ASTs.

Leaves dissected from *in vitro* shoots of *P. mileense* were cut into 1.0 cm^2 explants and inoculated on induction medium in 6 cm diameter Petri dishes. The induction medium contained MS basal medium with 0.5 mg l^{-1} 6-benzylaminopurine (BA), 0.05 mg l^{-1} indole-3-butyric acid, and 0.09 M sucrose, and was solidified with 2.5 g l^{-1} Phytagel. After five weeks of culture, ASTs of about 2 mm formed on leaf explants (Fig. 1b).

Droplet vitrification procedure

ASTs were harvested under a stereomicroscope in a laminar flow hood (Fig. 1c). ASTs were incubated in semi-solid MS medium (solidified with 2.5 g l^{-1} Phytagel) with 0.09 M sucrose or in liquid preculture solution containing MS basal medium with 0.3 M sucrose for 24 h at 25°C in darkness. Ten ASTs were then transferred to a 1.2 ml-capacity

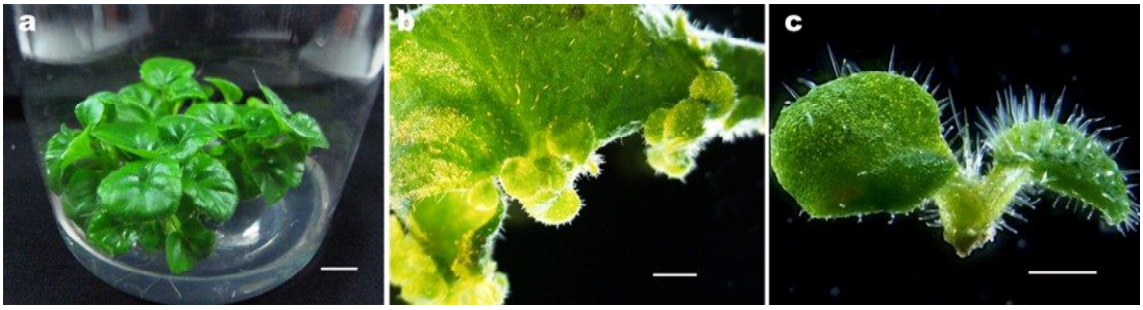

Figure 1. Induction of adventitious shoot tips of *Paraisometrum mileense*. (a) *In vitro* shoots of *P. mileense* cultured on hormone-free Murashige and Skoog medium (bar = 1 cm). (b) Adventitious shoot tips (ASTs) formed in leaf explants (bar = 1 mm). (c) Dissected ASTs of *P. mileense* (bar = 1 mm).

cryovial containing 1 ml of loading solution (MS basal medium with 2 M glycerol and 0.4 M sucrose) and incubated for 20 min at 25°C. After loading, the loading solution was replaced with 1 ml of ice-chilled PVS2 solution and kept for various times on ice. The PVS2 solution contained MS basal medium with 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), 15% (w/v) dimethylsulfoxide (DMSO), and 0.4 M sucrose. Approximately 1 min before the end of the PVS2 treatment, ASTs were transferred to a strip of sterile aluminium foil (8 × 25 mm) with a drop of PVS2 solution (15 µl). After the PVS2 treatment, the aluminium foil strip was plunged directly into liquid nitrogen (LN) using fine forceps. After the bubbling had subsided, the aluminium foil strip with drop was transferred to a cryovial in a holder placed in a Styrofoam box that contained LN, and held for at least 30 min. For rewarming, the aluminium foil was removed from the LN and quickly plunged into 10 ml of unloading solution in a 6 cm diameter Petri dish for 20 min at 25°C. The unloading solution contained MS basal medium with 1.2 M sucrose. The ASTs were then transferred to recovery medium, which contained MS basal medium with 0.5 mg l^{-1} BA and 0.09 M sucrose, and was solidified with 2.5 g l^{-1} Phytagel in a 6 cm Petri dish. Cultures were maintained at 25°C in darkness for seven days, and then transferred to the light conditions described above.

Sucrose concentration and AST regeneration following cryopreservation

The ASTs of *P. mileense* were dissected and incubated in one of the following preculture media for 24 h in darkness: (a) semi-solid MS medium (solidified with 2.5 g l^{-1} Phytagel) with 0.09 M sucrose, (b) liquid MS medium with 0.3 M sucrose, and (c) liquid MS medium with 0.6 M sucrose. The precultured ASTs were then treated with 1 ml of loading solution (MS medium with 2 M glycerol and 0.4 M sucrose) at 25 °C for 20 min. After loading, the loading solution was replaced with 1 ml of ice-chilled PVS2 solution and treated for 90 min on ice. Approximately 1 min before the end of PVS2 treatment, ASTs were transferred to a strip of sterile aluminium foil (8 × 25 mm) with a drop of PVS2 solution. ASTs were divided into two subsamples, one used for cryopreservation and the other for non-cryopreserved control. ASTs were cryopreserved and rewarmed as described above. The non-cryopreserved control ASTs on were directly unloaded in 10 ml of unloading solution in 6 cm diameter Petri dishes for 20 min at 25°C. ASTs were then transferred to recovery medium and cultured as described above.

Preculture duration and AST regeneration following cryopreservation

ASTs of *P. mileense* were dissected, and precultured in liquid MS medium with 0.3 M sucrose for 24, 48 or 72 h at 25°C in darkness. Precultured ASTs were then treated with 1 ml of loading solution (MS medium with 2 M glycerol and 0.4 M sucrose) at 25°C for 20 min. After loading, the loading solution was replaced with 1 ml of ice-chilled PVS2 solution and treated for 90 min on ice. ASTs were then cryopreserved and rewarmed as described above.

PVS2 treatment time and AST regeneration following cryopreservation

The ASTs of *P. mileense* were dissected, and then incubated in liquid preculture solution containing MS basal medium with 0.3 M sucrose for 24 h at 25°C in darkness. The precultured ASTs were then treated with 1 ml of loading solution (MS medium with 2 M glycerol + 0.4 M sucrose) at 25°C for 20 min. After loading, the solution was replaced with 1 ml of ice-chilled PVS2 solution and treated for 30, 60, 90, 120, or 150 min on ice. ASTs were then cryopreserved and rewarmed as described above.

Cold acclimation and AST regeneration following cryopreservation

Leaf explants (1.0 cm^2) from *in vitro* shoots of *P. mileense* were inoculated on induction medium in 6 cm diameter Petri dishes in order to induce ASTs, as described above. After five weeks of culture, ASTs of about 2 mm formed on the leaf explants. For treatments of cold acclimation, ASTs in the 6 cm Petri dishes were incubated at 4°C with a 8/16 h (day/night) photoperiod with light at an intensity of 20 µmol m^{-2} s^{-1} for 7 or 14 days. Cold-acclimated ASTs were dissected under a stereomicroscope in a laminar flow hood. Dissected ASTs were either used directly in cryopreservation experiments or precultured with liquid MS medium containing 0.3 M sucrose for 24 h at 25°C before cryopreservation. Ten ASTs were treated with 1 ml of loading solution (MS medium with 2 M glycerol and 0.4 M sucrose) at 25°C for 20 min. After loading, the loading solution was replaced with 1 ml of ice-chilled PVS2 solution and treated for 90 min on ice. After PVS2 treatment, ASTs were cryopreserved and rewarmed.

Data collection and analysis

Regeneration was determined by formation of normal shoots 30 days after rewarming. For all experiments, at least 10 shoot tips were used per treatment. All experiments were repeated at least three times. Data were subjected to the analysis of variance (ANOVA) with mean separation (P <0.05) by Duncan's new multiple range test using SPSS.

RESULTS

Preculture sucrose concentration and AST regeneration following cryopreservation

ASTs of *P. mileense* on the semi-solid MS medium with 0.09 M sucrose showed 45% shoot regeneration following cryopreservation, and the non-cryopreserved control had 68% shoot regeneration (Fig. 2). AST regeneration was significantly higher following 24-h preculture with 0.3 M sucrose (86%) (Fig. 2). ASTs precultured with 0.3 M sucrose also showed more rapid regrowth after cryopreservation than ASTs incubated on semi-solid MS medium only with 0.09 M sucrose. When preculture sucrose concentration was elevated to 0.6 M, however, a significant reduction in the regeneration of cryopreserved ASTs was observed (Fig. 2). Non-cryopreserved ASTs that had been precultured with 0.6 M sucrose and dehydrated with PVS2 also had a very low level of regeneration (7%) (Fig.2). Thus, the preculture medium with 0.3 M sucrose was found to be optimal for shoot regeneration of ASTs of *P. mileense* following cryopreservation

Preculture Duration and AST regeneration following cryopreservation

ASTs that were not precultured showed 45% regeneration following cryopreservation. Regeneration of cryopreserved ASTs was increased to 86% by 24 h preculture in liquid MS medium with 0.3 M sucrose (Fig. 3). However, preculture for an extended duration was detrimental to ASTs. When ASTs were precultured with liquid MS medium containing 0.3 M sucrose for 48 or 72 h, shoot regeneration following cryopreservation decreased to 49% and 10%, respectively (Fig. 3).

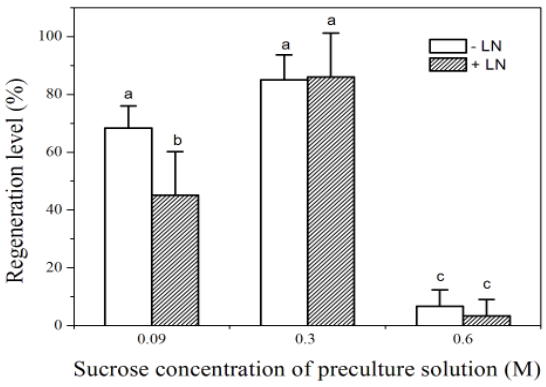

Figure 2. Effects of preculture sucrose concentration on the regeneration of adventitious shoot tips of *P. mileense* following cryopreservation. Vertical bars represent standard errors. Values with different letters are significantly different (P < 0.05).

PVS2 treatment time and AST regeneration following cryopreservation

To determine the optimal duration of PVS2 treatment at 0°C, precultured ASTs were exposed to PVS2 for 30, 60, 90, 120 and 150 min prior to treatment with LN. Treatment with

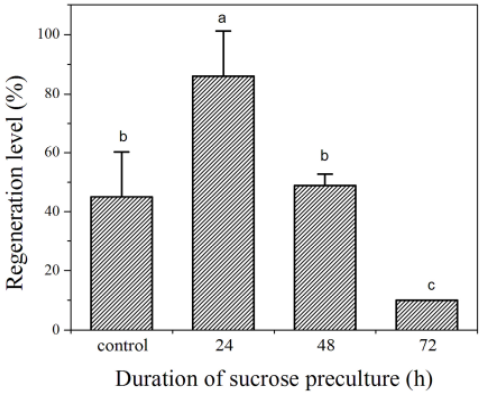

Figure 3. Effects of the preculture duration on adventitious shoot regeneration of *Paraisometrum mileense* following cryopreservation. ASTs were precultured in semi-solid MS medium with 0.09 M sucrose for 24 h (control) or precultured in 0.3 M sucrose for 24, 48 or 72 h. Vertical bars represent standard errors. Values with different letters are significantly different (P < 0.05).

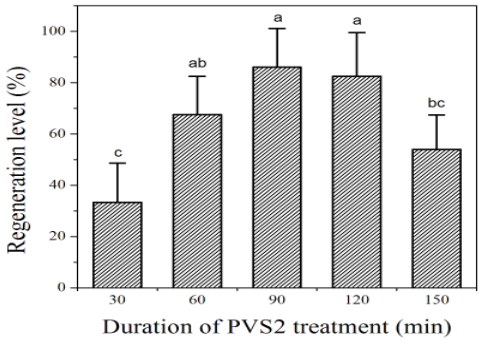

Figure 4. Effects of duration of plant vitrification solution 2 (PVS2) treatment on AST regeneration of *P. mileense* after cryopreservation. Precultured ASTs were loaded and exposed to PVS2 at 0°C for various lengths of time before liquid nitrogen immersion. Vertical bars represent standard errors. Values with different letters are significantly different (P < 0.05).

PVS2 for 30 min resulted in 33.3% regeneration (Fig. 4). When PVS2 treatment was prolonged, the AST regeneration increased to a maximum of 86.0% at 90 min, and then decreased to 54.0% at 150 min (Fig. 4). Therefore, PVS2 treatment for 90 min at 0°C was considered to be the optimal for shoot regeneration of ASTs of *P. mileense* following cryopreservation.

Cold acclimation and AST regeneration following cryopreservation

Seven days of cold acclimation resulted in a slightly lower regeneration of cryopreserved ASTs of *P. mileense* compared with that in the control that did not undergo cold acclimation (Table 1). Although the regeneration of ASTs acclimated to the cold for 14 days was 63%, whereas that for the control was 45%, the difference was not significant different (Table 1). After 21-day cold acclimation, the explant turned brown and died.

When cold acclimation treatment for 7 or 14 days was combined with sucrose preculture, regeneration was increased to 93% and 85%, respectively (Table 1). Given that precultured ASTs without cold acclimation showed a regeneration level of 86%, these increases in regeneration could be attributed primarily to preculture in the presence of sucrose.

Plant regeneration

Three weeks after rewarming, new shoot tips formed from the meristematic dome of surviving AST of *P. mileense* (Fig. 5a). The surviving ASTs regenerated into clusters of shoots without intermediary callus formation after eight weeks of culture on recovery medium containing MS basal medium with 0.5 mg l^{-1} BA and 0.09 M sucrose, solidified with 2.5 g l^{-1} Phytagel, in a 6 cm diameter Petri dish (Fig. 5b). The regenerated shoots were dissected and transferred to culture jars that contained rooting medium composed of MS basal medium with 0.25 mg l^{-1} 1-naphthylacetic acid and 0.09 M sucrose, and solidified with 2.5 g l^{-1} Phytagel. The shoots formed a normal root system after four weeks (Fig. 5c). The rooted plantlets were transferred to plastic pots containing sterile greenhouse compost and moved to greenhouse conditions. No morphological abnormalities were observed during one year's growth of the plantlets developing from ASTs cryopreserved by droplet vitrification (Fig. 5d).

Table 1. Effects of cold acclimation on adventitious shoot tip regeneration of *Paraisometrum mileense* following cryopreservation. In Exp A, ASTs were precultured on semi-solid MS medium with 0.09 M sucrose for 24 h. In Exp B, ASTs were precultured in liquid MS medium with 0.3 M sucrose for 24 h. In Exp C, ASTs were cold-acclimated for 7 or 14 days. In Exp D, ASTs were cold-acclimated for 7 or 14 days and precultured in liquid MS medium with 0.3 M sucrose for 24 h. Values with different letters are significantly different ($P < 0.05$).

Exp	Acclimation (days)	Sucrose preculture	Regeneration (%)
A	–	–	45.1 ± 15.1^{cd}
B	–	+	86.0 ± 15.2^{ab}
C	7	–	30.0 ± 10.0^{d}
	14	–	63.3 ± 19.3^{bc}
D	7	+	93.3 ± 5.8^{a}
	14	+	85.0 ± 1.7^{ab}

DISCUSSION

In the present study, a simple and efficient droplet vitrification procedure was established for the cryopreservation of ASTs of *P. mileense*. The procedure consisted of preculture of ASTs in liquid MS medium with 0.3 M sucrose for 24 h and followed by droplet vitrification. With the optimized parameters, 86% ASTs regenerated into shoots following cryopreservation. This is the first report of successful cryopreservation of *P. mileense* ASTs.

The keys to success in cryopreservation by vitrification are careful optimization of the time and temperature of PVS2 treatment (14). According to previous studies, PVS2 treatment at 0°C was less toxic than treatment at 25°C (20). Dehydration by exposure to PVS2 at 0°C usually results in higher survival than dehydration at 25°C (21). Therefore, we chose 0°C as PVS2 treatment temperature in all the experiments. As for the optimized time of PVS2 treatment, PVS2 treatment for 90 min could achieve 86% regeneration for *P.mileense* ASTs (Fig. 4). ASTs treated with PVS2 at 0°C for 60 min and 120 min also maintained high levels of shoot regeneration following cryopreservation (Fig. 4). These results are consistent with a previous report that PVS2 incubation time could be extended at 0°C (22).

The use of sucrose preculture in plant cryopreservation has been widely reported (13). The optimal preculture conditions vary with the plant species and the type of explant. For ASTs of *P. mileense*, preculturing with 0.3 M sucrose

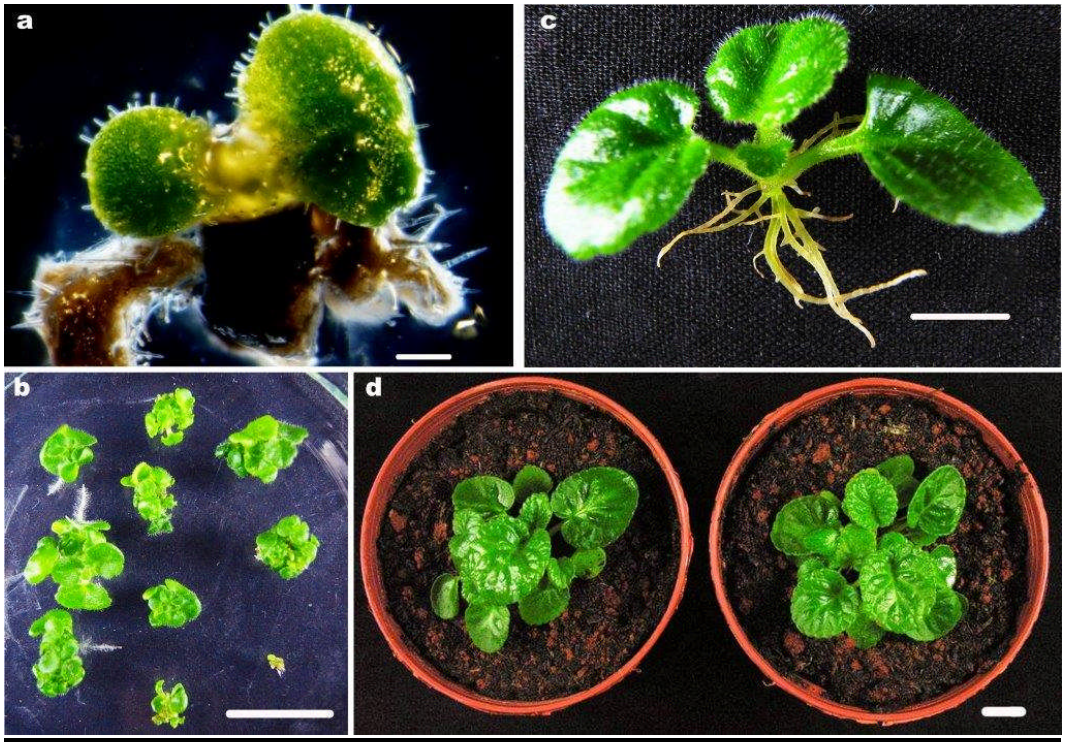

Figure 5. Regeneration of adventitious shoot tips (ASTs) of *Paraisometrum mileense* following cryopreservation. (a) Growth of cryopreserved ASTs on recovery medium, 3 weeks after rewarming (bar = 1 mm). (b) Cryopreserved ASTs formed clusters of shoots on recovery medium, 8 weeks after rewarming (bar = 1 cm). (c) Regenerated shoot formed a root system after 4 weeks of culture on rooting medium. The photography was taken 12 weeks after rewarming (bar = 1 cm). (d) Tissue-cultured plantlets 12 weeks after transfer to soil (left) and plantlets developed from cryopreserved ASTs 12 weeks after transfer to soil. The photography was taken 24 weeks after rewarming (right) (bar = 1 cm).

for 24 h produced the highest level of shoot regeneration following cryopreservation. The level of shoot regeneration decreased when the duration of preculture was prolonged to 48 h or the sucrose concentration was elevated to 0.6 M. In the present study, ASTs of *P. mileense* were precultured in liquid preculture medium. Tissues cultured in liquid medium were exposed to more severe osmotic stress and took up sucrose more rapidly than those cultured on solid medium. This was a possible explanation for the fact that dehydration tolerance of ASTs of *P. mileense* was induced in a relatively short time (24 h) by preculturing in 0.3 M sucrose.

Cold acclimation is essential to successful cryopreservation of many temperate plants The recovery of shoot tips of blackberry (3), apple, and pear (10) was significantly improved by different regimes of cold acclimation. In the case of tropical plants like yam (7), recovery following cryopreservation was improved after preconditioning at a low temperature. In the present study, however, cold treatment at 4 °C for 7 or 14 days did not improve the level of shoot regeneration from cryopreserved ASTs. Explant browning after 21-day cold treatment indicates that cold treatment is detrimental to *P. mileense* ASTs. Given that sucrose preculture alone ensures a high level of shoot regeneration (86.0%) of cryopreserved ASTs of *P. mileense*, other cold treatment regimens were not tested to optimise the protocol.

In conclusion, the droplet vitrification procedure provided a simple and efficient way for cryopreservation of the critically endangered species *P. mileense*. Further studies should examine the applicability of this technique for other gesneriad species.

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