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CRYOPRESERVATION OF GEMMAE OF *Marchantia polymorpha* L. (Marchantiophyta, Marchantiaceae) WITHOUT PRIOR PRETREATMENT

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Abstract

BACKGROUND: Successful cryopreservation of gametophytic material of bryophytes requires pretreatment with sucrose or abscisic acid. Compared to gametophyte materials, spore and gemmae cryopreservation may be more efficient, simple and stable systems for storing large amounts of genetic diversity of bryophytes within a small space. However, there has still been no attempt at cryopreserving bryophyte gemmae. **OBJECTIVE:** The aim of this study is to determine whether bryophyte gemmae with differing levels of desiccation tolerance could survive and germinate after cryopreservation without prior encapsulation and pretreatment. **MATERIALS AND METHODS:** Gemmae of *Marchantia polymorpha* L. were dried with silica gel for different times and then rapidly cooled in liquid nitrogen. **RESULTS:** The germination level of fresh gemmae was 95%. After 3 h predrying and 1 d in LN, germination was 68%, and was still up to 59% after storage for 75 days. **CONCLUSION:** We conclude that the natural desiccation tolerance of bryophyte gemmae permits cryopreservation without prior pretreatment other than drying.

Keywords: liverwort; gemmae; *Marchantia polymorpha*; cryopreservation

INTRODUCTION

Cryopreservation techniques are advocated for the long-term storage of plant material when conventional methods (e.g. seed banking) are inappropriate (1). As all cell metabolism is in a state of inactivity at the temperature of liquid nitrogen (LN, –196°C), cryopreservation has been proved to be an effective and stable long-term storage technique for vascular plants (3, 12).

Bryophytes (mosses, liverworts and hornworts), although the second largest group of terrestrial plants, are often overlooked in conservation initiatives in comparison to vascular plants as they are diminutive and difficult to identify and deemed unimportant (20). Actually, bryophytes are essential to a number of large scale ecosystem processes and play an important role in nutrient, water and carbon cycles (16, 23). At the same time, bryophytes have several characteristics that make them particularly good candidates for cryopreservation and which provide more

flexibility in approach than with seed plants (18). For example, the gametophyte stages are highly regenerative and can reproduce vegetatively from fragments, and some bryophytes have vegetative tissues that are naturally desiccation tolerant.

Although not as widely studied as vascular plants, there are still a number of reports of cryopreservation of bryophyte gametophytic material (4, 5, 14, 21) and spores (11). Apart from freezing spores, methods for cryopreserving bryophytes have focused almost entirely on *in vitro*-grown gametophytes, which require several steps of pretreatment and tedious aseptic techniques for culturing the material. Compared to gametophyte materials, spores and gemmae may be an efficient, simple and stable method for storing large amounts of genetic diversity of bryophytes within a small space. Furthermore, asexual propagules have been shown to be more tolerant of wider ranges of temperature and humidity, and to result in the reproduction of new gametophytes at a faster rate than via sexual reproduction in the moss *Syntrichia ruralis* (9, 13). Most bryophyte propagules can easily survive dry periods longer than one year (2). However, as bryophyte gemmae are much smaller and less conspicuous, there has so far been no attempt at cryopreserving them.

Many dioecious bryophyte species, including a number of threatened species (20), never or rarely produce spores and thus a variety of asexual propagule types, such as gemmae, tubers or brood cells, have evolved among these bryophytes (15, 20). For the effective cryopreservation of rare and endangered bryophyte species the development of methods for cryopreservation of gemmae are especially important. The aim of this study is to investigate to what extent bryophyte gemmae with different levels of rapid desiccation tolerance can be cryopreserved without cryoprotectants and to find a simple approach to cryopreserving large amounts of bryophyte material at a time. The study was motivated by the need to obtain information for the development of more and efficient methods and materials for the *ex situ* conservation of bryophytes. To achieve this goal, the cryopreservation protocols were explored using a common species, *Marchantia polymorpha* L. The purpose of this study was to obtain a simple protocol for cryopreserving a range of threatened bryophyte species in the future.

MATERIALS AND METHODS

Plant material

Gametophytes with gemmae of *M. polymorpha* were collected from East China Normal University, Shanghai, China. Voucher specimens were deposited in East China Normal University Herbarium (HSNU). Freshly collected material, with soil substrate, was placed in a Petri dish and maintained in an artificial climate chamber with a 12 h light/12 h dark regime for 2 weeks before drying.

Optimum rapid drying time for gemmae

Fresh gemmae with higher water content would produce ice crystals during the cryopreservation process and damage the cells. Therefore, it was important to explore the best drying time of gemmae. Time gradients were designed from 0–10 h, using a total of 11 groups. About 10 fresh gemmae were put into a small filter paper envelopes, and the top opening sealed with transparent plastic. Eleven strips were fully immersed in boxes filled with silica gel. After drying, the gemmae were then transferred to a slide and examined under the dissecting microscope. The shape, color and degree of curl were observed and recorded.

Optimal cryopreservation condition for gemmae

Based on the results of the rapid drying experiment, 0 h, 3 h, 5 h, 7 h were chosen as gemmae pre-conditions before cryopreservation in LN. Gemmae were prepared for cryopreservation according to the methods developed for spores (11). The same shape, color and size gemmae were selected and placed in the bottom of small filter paper envelopes. After drying for 0 h

(control), 3 h, 5 h and 7 h in silica gel, the envelopes were put into 10 ml cryovials. Cryovials were rapidly cooled to –196°C by direct immersion in liquid nitrogen. After cryopreserved for 24 h, the vials were thawed by immersing in running water (15–20°C) for 15 min. Thawed samples were disinfected for 1 min with 0.05% solution of sodium hypochlorite (NaClO) and washed 6–8 times in distilled water, for a few minutes each, transferred onto Knop medium and maintained under standard growth conditions (16/8 h of light / darkness, at 25/18°C). Light at 2200–3200 lx was supplied by cool white fluorescent tubes. The number of germinative gemmae was recorded under the microscope (Motic: SFC-28) every day until the germination percentage became almost constant.

Comparison of gemmae cryopreserved for different times

After drying for 3 h and 5 h, gemmae were put into 10 ml cryovials and then immersed in liquid nitrogen for 1 d, 15 d, 30 d and 75 d. Gemmae were removed from liquid nitrogen at different times, thawed, disinfected, and then transferred onto Knop medium for culture. The number of germinative gemmae was recorded under the microscope every day until the germination level became almost constant.

Data analysis

Each treatment was repeated three times. Three fields per dish were selected for the observation of each repeat. The test results were analyzed using SPSS19.0. LSD and Duncan multi-comparisons tests were used for significance comparisons, test level $\alpha = 0.05$.

RESULTS

The general appearance of gemmae growing *in vitro* is shown in Fig. 1.

Optimum rapid drying time of gemmae

As shown in Table 1, gemmae became slightly curled, darker in colour and became less sticky (i.e. not adhere to tweezers) after being dried for 3 h. As most gemmae were broken after drying for 8 h, a gradient of times from 3 h to 7 h were chosen for pre-conditioning gemmae before cryopreserving in LN.

Cryopreservation of gemmae after different drying times

As there was little difference between the appearance of gemmae after dried for 3 h and 5 h, these two drying times were

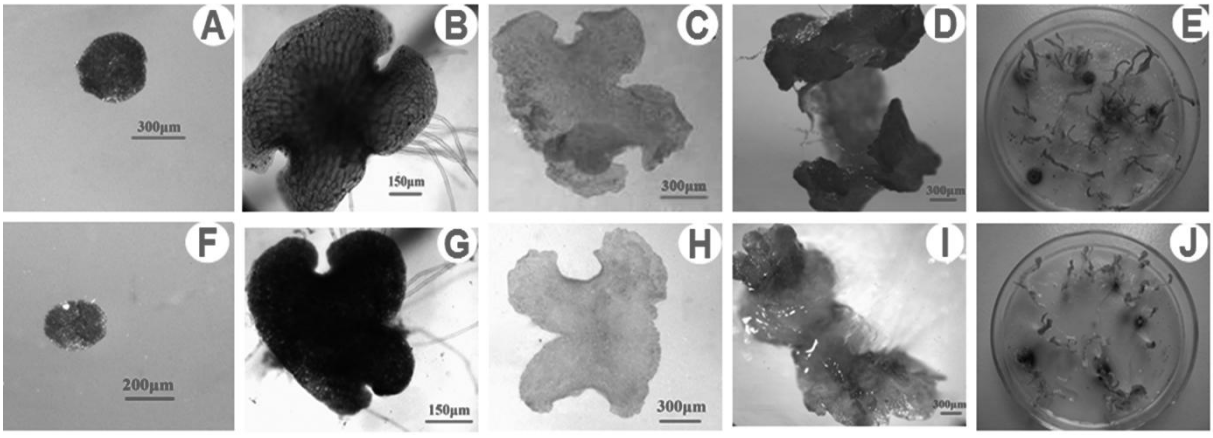

Figure1. Comparison of the regeneration of gemmae before and after cryopreservation. A, F: Gemmae. B, G: Gemmae after culturing for 4 d. C, H: Gemmae culturing for 10 d. D, I: Gemmae culturing for 26 d. E, J: Gemmae culturing for 45 d. A-E are gemmae without cryopreservation; F-J are gemmae after cryopreservation.

Table 1. Effects of drying time on appearance and properties of *M. polymorpha* gemmae

Drying time (h)	Shape	Colour	Degree of curl	Remarks
control	Stretch	Bright green +	-	Stick to tweezers
1, 2	Most stretch	Dark green ++	+	Stick to tweezers
3, 4	Small part curly (one side)	Dark green ++	++	Not stick to tweezers
5, 6, 7	Most curly (two sides), a few roll-folded	Black green +++	+++	Not stick to tweezers
8, 9, 10	Most roll-folded and became thinner, broken	Black green +++	++++	Not stick to tweezers

Table 2. Post-cryopreservation germination level of *M. polymorpha* gemmae after different drying times. The germination level of fresh gemmae was 94.5%.

Drying time (h)	Germination level (%)
0	0 ± 0
3	68.3 ± 7.5[a]
5	60.1 ± 8.9[a]
7	29.2 ± 4.3[b]

Note: Experimental data were expressed as means ± SD. Within a treatment, data followed by the same letter is not significantly different.

compared in the cryopreservation experiment. Drying time is the most critical step in the procedure of cryopreservation and different materials require different optimum drying times. As shown in Table 2, the germination level of gemmae after being dried for 3 h was the highest (68.3%), followed by 5 h (60.1%). The lowest survival (29.2%) occurred after 7 h pre-drying (Table 2). The intermediate drying times (3 h and 5 h) resulted in germination levels that were not significantly different.

Effect of cryopreservation storage time

When 5 h pre-drying was used, the germination level declined significantly over 75 d of cryostorage (Table 3). However, a gemmae germination level of up to 58.5 % was observed after storage in LN for 75 d following 3 h of pre-drying. This level of germination was not significantly different to that recorded after 1 d of storage.

Regeneration of gemmae before and after cryopreservation

As can be seen in Fig. 1, there is no difference between the process of regeneration of gemmae of *M. polymorpha* before and after cryopreserved for 1 d. Gemmae all began to germinate after culturing for 2 d and the germination level reached the highest value of 94.5% for the control group (without cryopreservation) and 68.3% for cryopreserved gemmae. Gemmae began to differentiate into immature gametophytes after been cultured for 26 d and differentiated into mature gametophytes after 45 d.

Table 3. Effect of cryopreservation storage time (d) on the germination of gemmae of *M. polymorpha* after different pre-drying times. The germination level of fresh gemmae was 94.5%.

Storage time (d)	Germination level (%)	
	3 h drying	5 h drying
1	68.3 ± 7.5a	67.8 ± 12.3a
15	57.3 ± 6.5ab	54.6 ± 14.5ab
30	61.3 ± 13.9a	38.9 ± 8.0bc
75	58.5 ± 2.7ab	18.9 ± 1.9c

Note: Note: Experimental data were expressed as means ± SD. Within a treatment, data followed by the same letter is not significantly different.

DISCUSSION

Many studies have shown that cryopreservation is a suitable method for the storage of protonemata and gametophytes of bryophytes, although there is often a requirement for pretreatment with sucrose or ABA (6, 10, 14, 19). However, the protonemata of some naturally desiccation tolerant mosses can survive rapid dehydration and cryopreservation without prior pretreatment (4, 5). The ability to undergo rapid dehydration and rapidly reduce the water potential of cells during periods of water stress has been suggested as the mean by which bryophytes minimize tissue damage during desiccation (4, 17). As the failure of some species to regenerate after cryopreservation may be primarily due to the lack of desiccation tolerance and the inability to repair the damage sustained during the dehydration phase (4), the key point of success of cryopreservation is to select the materials tolerant to natural desiccation.

A previous study has shown that asexual propagules are more tolerant of wider ranges of temperature and humidity (9). As a result, the current study selected gemmae of *Marchantia polymorpha* to test the possibility of cryopreservation following rapid dehydration and without any other pretreatment. The results show that the gemmae could survive after rapid dehydration and be conserved directly in LN.

The phenomenon of variable germination level of gemmae during storage was also shown in spores of *Haplocladium microphyllum* (Hedw.) Broth and *Alsophila gigantean* Wall. Ex Hook (11, 24). This may be due to physiological differences in sub-populations to cryopreservation stresses.

Cryopreserving spores is a fairly straightforward procedure which requires drying the material, followed by freezing directly in LN (18). Spores of *Haplocladium microphyllum* (Hedw.) Broth. were successfully cryopreserved after having been dried for 180 days (11). Similar to spores, we have shown that gemmae cryopreservation is also an efficient and stable method. One important feature of gemmae survival, under conditions of cold or dehydration, is their ability to maintain dormancy (7).

As many dioecious bryophyte species, including a number of threatened species (16), reproduce asexually and never or rarely produce spores (e.g., *Acrolejeunea* subg. *Acrolejeunea*) (8, 15, 20, 22), developing a method for the cryopreservation of bryophyte gemmae can underpin conservation efforts for these important taxa, including *Caudalejeunea tridentata* R.L. Zhu *et al.* (25), the most intriguing Chinese

taxon that lacks sporophytes but bears abundant gemmae.

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