CryoLetters **32** (6), 498-505 (2011)

LONG-TERM CONSERVATION THROUGH CRYOPRESERVATION OF IMMATURE SEED OF *Mantisia spathulata* AND *Mantisia wengeri*; TWO ENDANGERED PLANTS OF NORTH-EAST INDIA

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Abstract

A successful protocol for long-term conservation of two endangered plants viz. *Mantisia spathulata* and *M. wengeri* has been devised through cryopreservation of immature seeds. Immature seeds of both the species were precultured in 0.6 M Sucrose and 2 M Glycerol for 3 h at 24 ± 2ºC. Precultured seeds were then desiccated under the airflow of 27 ± 3 m min^{-1} velocity inside laminar air flow cabinet for different time periods. The seeds were then cryostored in liquid nitrogen for an hour. A maximum of 40% and 36.6% seed germination was recorded after cryostorage at moisture contents of 26.0% and 16.2% for *M. spathulata* and *M. wengeri* respectively. To protect these rare plants against loss due to disease, insect damage, or natural disaster a back up collection has been established using the protocol and applied to a large number of immature seeds that were obtained from the *ex situ* plants growing in the experimental garden of the North-eastern Hill University, Shillong.

Keywords: long-term conservation, cryopreservation, germination, moisture content, dehydration

INTRODUCTION

Mantisia is the smallest genus of the Zingiberaceae family and comprises three species (19). Out of these few species, *Mantisia spathulata* and *Mantisia wengeri* are very vital plants which are on the verge of extinction and found only in two restricted areas of North-eastern India. A maximum of only 60 - 70 plants of both the species are found in their wild habitats. Due to the drastic reduction in the number of individuals, plants of both the species were rescued from extinction through *in vitro* multiplication and *ex situ* reintroduction in the Botanical Garden (3). Seeds of both the species are associated with inherent complications that restrict the natural enhancement of the plant populations. Seed-bearing capsules prematurely dehisce immediately after seed set. These seeds are then predated by insects and birds. The remaining seed gets infested by microorganisms in the wet soil, which is also prone to landslides after heavy rainfall. Therefore, no new seedlings are found in the location where the seeds are dispersed. Besides this, seeds become non-viable

after 6 - 7 month of its storage at 4ºC inside desiccators. Although the propensity for natural regeneration through seed germination in the soil is very poor, immediately after dehiscence the seeds can be fully germinated under *in vitro* conditions using the growth regulator GA_3 (4).

As seeds are heterogenous in most plant species, conservation of seeds can facilitate the preservation of genetic diversity of critically endangered and endemic plants (9) including those of North-east India. Cryopreservation is a technique which provides long-term preservation of germplasm by reducing metabolism to such a low level that all biochemical processes are significantly reduced and biological deterioration becomes negligible (18, 26, 27). Thus, it suspends ageing and provides a cost-effective means for the long-term storage of germplasm (15). Although several methods such as slow freezing, encapsulation-dehydration (11), vitrification etc. are available for the cryopreservation of plant germplasm, one of the most feasible, inexpensive and simple methods is the desiccation of plant material in sterile air or silica gel followed by plunging into LN. This has been successfully carried out using the seeds, embryos and embryonic axes of several plants (2, 5, 7, 8, 10, 12, 13, 17, 20, 27). It has been reported that cryopreserving intact seed averts the problems associated with the cryopreservation of excised embryonic axes, such as the development of appropriate embryo excision methods and *in vitro* culture techniques (6). Furthermore, problems of contamination by fungi and bacteria observed in embryonic axes of non-orthodox seeds can be overcome by cryopreserving the seeds (1, 21). There is no report on the seed storage biology of these species until now. In this paper we report for the first time the potential for long-term conservation of immature seeds *M. spathulata* and *M. wengeri* through desiccation and cryopreservation methods.

MATERIALS AND METHODS

Optimization of germination/regrowth medium

The unopened capsules (two weeks after seed set) of *M. spathulata* and *M. wengeri* were surface disinfected by treating with 0.2% Cetrimide (Shalaks Pharm Industrial Ltd., New Delhi) for 10 min followed by washing several times with sterile distilled water. Finally, the capsules were surface sterilized with 0.075% mercuric chloride for 5 min and rinsed several times with sterile distilled water. The immature seeds (1 – 2 mm) were taken out from the aseptic capsules and inoculated in 150 ml conical flask containing 50 ml of sterilized Murashige and Skoog (MS) medium supplemented with 3% sucrose and gibberellic acid (GA_3) at various concentrations (1.4 - 28.9 µM). Cultures maintained in MS medium without GA_3 were treated as a control. The medium was solidified with 0.8% extra pure agar (Hi-media, India) and pH was adjusted to 5.8, prior to autoclaving for 15 min at 1.06 kg cm^{-2} (121ºC). The cultures were incubated at 25 ± 2ºC under a 12 h photoperiod with photosynthetic photon flux density (PPFD) of 40.5 µmol m^{-2} s^{-1}, provided by cool white fluorescent lamps. To determine the best germination/regrowth medium the percentage seed germination was assessed after 2 weeks of culture. The experiments were repeated thrice with ten replicates per treatment. Statistical analysis was done by analysis of variance (ANOVA) at $P<0.05$ and the means compared using Tukeys test (PC version Origin 7.0.NORTHAMPTON, MA, USA).

Preculture and dehydration of seeds

To protect injury during liquid nitrogen (LN) treatment and to optimize the osmotolerance of seeds against dehydration, aseptic seeds were precultured in liquid MS medium supplemented with three different combinations of sucrose and glycerol (0.4 M

sucrose and 1 M Sucrose; 0.6 M sucrose and 2 M glycerol; 0.8 M sucrose and 3 M glycerol) at different time intervals (0 to 4 h) at 25 ± 2ºC under 12 h photoperiod with PPFD of 40.5 µmol m^{-2} s^{-1}. MS medium supplemented without sucrose and glycerol was considered as the negative control. The precultured seeds were then taken into the sterile laminar flow and gradually dehydrated under the airflow of 27 ± 3 m min^{-1} velocity over a period of 0 to 8 h. Desiccated seeds were removed at periodic intervals (2 h) and implanted on germination medium to study the germination after exposure to different moisture levels. The moisture content (MC) of the seeds was determined by drying in the oven at 102°C for 48 h (11). The percentage of MC was expressed on fresh weight (FW) basis using the formula,

MC (%) = [(Fresh weight – Dry weight) / Fresh weight] x 100

Storage in liquid nitrogen (LN)

Seeds (10 per sample) were removed from the laminar air-flow at periodic intervals and placed in 2 ml cryovials (Polypropelene, Polylab India Ltd.). After fixing the cryotubes in 20 inches long cryocanes, the canes were rapidly dipped into a liquid nitrogen in a 35 l narrow neck storage Dewar and finally stored for 1 h at -196°C. The cryocanes, and seeds, were rapidly thawed at 40 ± 2°C by shaking the tubes for 2 min inside a water bath. The seeds were removed from the cryotubes and placed to germinate in their optimized germination/regrowth medium. After 4 weeks, germination was assessed based on the emergence of new shoots and roots from the seed embryo. The experiments were repeated three times with ten replicates per treatment. Statistical analysis was done by ANOVA ($P<0.05$) and the means compared using Tukeys test (PC version Origin 7.0.NORTHAMPTON, MA, USA).

Hardening of seedlings

About 8 weeks-old, *in vitro*-raised seedlings (recovered after cryopreservation) measuring 2.5 - 3.0 cm in size were removed from the culture vessels and washed with water to remove the agar-containing medium. The plantlets were then potted into paper cups containing mixtures of soil and compost (1:1). The cups were covered with perforated polybags and sprinkled with water at two days interval for the initial two weeks and acclimatized at 30 ± 2ºC and 70 ± 5% RH under glass-house conditions.

RESULTS

Optimization of germination/regrowth medium

A maximum of 90% of *M. spathulata* seeds germinated in MS media supplemented with 4.3 µM of GA_3 which was higher than the control and other GA_3 concentrations tested (Table1). This medium was therefore considered to be the best regrowth/germination medium for seed germination in this species. Similarly, seed germination was significantly enhanced to a maximum of 96.7% for *M. wengeri* in MS medium supplemented with 7.2 µM of GA_3 compared to the control and other concentrations of GA_3 and subsequently used as the best regrowth/germination medium (Table 1).

Effect of preculture solutions on cryotolerance

Of the three different preculture solutions tested to enable seed cryopreservation, a preculture solution containing MS medium supplemented with 0.6 M sucrose and 2 M glycerol was found to be exclusively suitable for withstanding LN. Preculturing the seeds in this solution for 3 h before dehydration played a vital role in successful recovery of seed germination after LN exposure. Although seeds tested in other preculture solutions could withstand dehydration at different moisture levels they were unable to survive after exposure

to LN. The effects resulting from the other preculture solutions were statistically non significant and are therefore not mentioned further in this paper. The results mentioned below are for the seeds precultured in 0.6 M Sucrose and 2 M Glycerol.

Table 1. Mean seed germination percentage (±SEM) of *M. spathulata* and *M. wengeri* *in vitro* in MS medium supplemented with GA_3

GA_3 (µM)	% Germination *M. spathulata*	% Germination *M. wengeri*
0.0	16.7 ± 3.3[a]	13.3 ± 3.3[a]
1.4	53.3 ± 3.3[b]	30.0 ± 5.7[a]
4.3	90.0 ± 5.7[c]	56.6 ± 3.3[b]
7.2	83.3 ± 6.7[c]	96.6 ± 3.3[d]
14.2	63.3 ± 3.3[bc]	73.3 ± 3.3[c]
28.9	36.7 ± 3.3[a]	46.6 ± 6.6[b]

Values are mean ± SEM of three experiments with ten replicates/experiment. ANOVA test shows a highly significant (5% level) effect of GA_3 treatment on seed germination. Tukey's test (p = 0.05) shows that concentrations of GA_3 except 28.9 µM and 1.4 µM have significant effects on seed germination in *M. spathulata* and *M. wengeri* respectively compared to the controls. The data were recorded after 45 days of culture.

Table 2. Mean seed germination percentage (±SEM) in *M. spathulata* after cryopreservation following dehydration for different time intervals

Dehydration time (h)	Moisture content (%)	Germination (%) - LN	Germination (%) + LN
0.0	79.6 ± 0.6[a]	70.0 ± 5.7[a]	0.0 ± 0.0
2.0	26.0 ± 1.5[b]	63.3 ± 3.3[a]	40.0 ± 5.7[a]
4.0	19.0 ± 0.9[c]	46.6 ± 3.3[b]	26.6 ± 3.3[b]
6.0	9.3 ± 0.7[d]	36.6 ± 3.3[c]	20.0 ± 5.7[b]
8.0	3.8 ± 0.1[d]	0.0 ± 0.0	0.0 ± 0.0

Values are mean ± SEM of three experiments with ten replicates/experiment. ANOVA test shows that seed germination after cryopreservation is highly significant (5% level) after drying for 2 h. Means followed by the same letter are not significantly different according to Tukey's test (p = 0.05). Seeds were pretreated with 0.6 M sucrose and 2 M glycerol for 3 h before drying. Data were recorded after 45 days of culture

Effect of dehydration time on moisture content and germination without exposing to liquid nitrogen (LN)

A maximum of 70% seed germination was recorded on the regrowth medium for *M. spathulata* with an initial moisture level of 79.6%, i.e. without dehydration (Table 2). With subsequent increase in the dehydration time, seed germination significantly reduced. With the decline in MC from an initial 79.6% to 26.0% after 2 h, a maximum of 63% of seeds germinated on the regrowth medium. As dehydration periods increased the MC reduced sharply. About 37% of seeds germinated after 6 h of dehydration. However, germination was completely hindered following 8 h desiccation in the laminar air-flow to 3.8% MC.

Similarly, around 73% seed germination was recorded for *M. wengeri* for control seeds (without drying) at an initial moisture level of 85% (Table 2). About 57% of seeds germinated after 2 h desiccation, with a fall to 21% MC. As dehydration was extended to 6 h, seed germination progressively reduced to a minimum of 10% at 6.5% MC. Following 8 h desiccation in the laminar air-flow, the MC reached 2.7% and germination was completely inhibited.

Effect of storage in liquid nitrogen (LN)

When directly immersed in LN seeds of *M. spathulata* at their initial moisture content of 79.6% were unable to germinate in the regrowth medium. However, seed germination was significantly improved after cryostorage in LN with gradual declining MC. The highest survival recorded was 40% germination when seeds at 26% MC (2 h dehydration) were cryopreservevd (Table 2). However, the germination of seeds progressively decreased with further reduction in the MC at 4 h and 6 h and consequently so did recovery from liquid nitrogen. For the time intervals 2 to 6 h and 26 to 9% MC, the effect of cryopreservation was a reduction in germination by about 20% compared with seeds that had been dried only.

Similarly, when *M. wengeri* were exposed to liquid nitrogen at their initial moisture level of 85% the seeds failed to germinate (Table 3). However, as moisture level fell following 2 h, 4 h and 6 h dehydration, seeds had significantly improved germination after cryoexposure to LN. After drying to 16% MC (4 h dehydration) a maximum of 37% seed germination was recorded after cryopreservation; this was significantly higher than 0, 6 and 8 h treatments but not significantly greater than the 2 h treatment (21% MC). For the 2 h and 4 h drying treatments, cryopreservation reduced germination by ~13 to 27% compared with seeds that had been dried only. However, most of the seeds that tolerated drying for 6 h (7% MC) survived cryopreservation. Ultimately, seed germination was completely inhibited with the decline in MC to 3% after 8 h dehydration.

Table 3. Mean seed germination percentage (±SEM) of *M. wengeri* after cryopreservation through dehydration at different time intervals

Dehydration time (h)	Moisture content (%)	Germination (%)	
		- LN	+ LN
0.0	85.3 ± 1.4^{a}	73.3 ± 3.3^{a}	0.0 ± 0.0
2.0	20.8 ± 0.9^{b}	56.6 ± 3.3^{ab}	20.0 ± 5.7^{a}
4.0	16.2 ± 0.4^{b}	50.0 ± 0.0^{b}	36.6 ± 3.3^{a}
6.0	6.5 ± 0.6^{c}	10.0 ± 5.7^{c}	6.6 ± 3.3^{c}
8.0	2.7 ± 0.3^{c}	0.0 ± 0.0	0.0 ± 0.0

Values are mean ± SEM of three experiments with ten replicates/experiment.
ANOVA test shows that seed germination was significantly (5% level) affected by drying and cryopreservation. Means followed by the same letter are not significantly different according to Tukey's test (p = 0.05). Seeds were pretreated with 0.6 M sucrose and 2 M glycerol for 3 h. Data were recorded after 45 days of culture.

Hardening of seedlings

Following cryopreservation > 90% seedlings of both species could be hardened and acclimatized within 8 weeks. The seedlings are being maintained inside the glass-house for further assessment of growth and elongation of plantlets.

DISCUSSION

Seed moisture content is the most critical factor for the successful development of a cryopreservation protocol. Importantly, seed moisture content is dependent on seed chemical composition, particularly lipid level. Generally, oilseeds can withstand higher desiccation levels, but may be susceptible to liquid nitrogen treatment (26). Thus determining moisture content before cryopreservation is very important.

In the present study, precultured immature seeds of *M. spathulata* and *M. wengeri* were subjected to dehydration for different time intervals to investigate the optimal MC for cryopreservation. The present study reveals that the immature seeds of *M. spathulata* and *M. wengeri* are unable to withstand LN at their initial MCs, of 80% and 85% respectively. However, these seeds can tolerate desiccation to a minimum of 9% and 7% MC for *M. spathulata* and *M. wengeri* respectively and survive cryopreservation; beyond these critical MCs the seeds fail to survive drying, i.e. to ~3 to 4% MC.

Studies on the seed biology and cryopreservation of rare species of Zingiberaceae family is extremely limited even though the family is known to contain many species with medicinal properties (14). It has been presumed that cryopreservation protocols that are effective for diverse species or cultivars can be directly applied to new species in some cases (23). Thus, in this study it has been found that preculture solutions tried in citrus (7, 8) are effective in enhancing the osmotolerance in immature seeds of two *Mantisia* species. Preculturing the seeds in high osmoticum is clearly crucial for cryopreservation success as no survival was possible without this step in the cryopreservation procedure. Immature seeds of *M. spathulata* and *M. wengeri* could withstand dehydration to ~16 to 26% MC and be cryostored in LN with a maximum of 40% and 37% recovery *in vitro*. The recovered plants could be successfully hardened and raised to maturity for complete plant development within few months.

In recent years a number of endangered plants, such as *Zizania texana* and *Lilium iedebourii*, have been successfully conserved through cryopreservation with a maximum of 75% recovery (16, 28). Similarly, cryopreservation recovery to a maximum of 41% and 65% has been achieved for seeds of *Citrus aurantifolia* and *Citrus madurensis* respectively (7, 8). Recovery of plants following cryopreservation is dependent on a variety of factors including the age of the mother plants, pretreatments, the length and type of cold acclimatization, the technique used and the recovery process (25). This is the first attempt to cryopreserve the endangered plants with limited experimental materials. Further trails with other methods, such as encapsulation-dehydration of seeds, vitrification etc., would augment the recovery of seed germination after cryopreservation. The present protocol has opened up the possibilities to cryopreserve other related rare and endangered species of the Zingiberaceae family.

Depletion of germplasm resources throughout the world due to loss of natural areas, plant diseases, and changes in agricultural practices mandates a move toward conserving as much genetic material as soon as possible (22). We have reported earlier the *ex situ* reintroduction of thousands of tissue culture-raised plants of the critically endangered *M. spathulata* and *M. wengeri* in the experimental garden of North Eastern Hill University, Shillong, India (3). Sufficient seed bearing capsules have been obtained from these plants. However, under natural conditions the dispersed seeds appear unable to sustain regeneration as the protrusion of new seedlings at a reintroduction site has not been observed for the past three years. The

number of introduced plants has remained constant and the plants increased in size and shoot number every year through clonal multiplication from the same cluster of rhizome. Thus, these plants represent an active (working) *in vivo* gene banks collection. Cryopreservation provides a secure, low maintenance, reliable means to back up these active collections (24). The storage of immature seeds of both these endangered *Mantisia* species through cryopreservation forms the backup (base) collection to insure against loss due to disease, insect damage, or natural disaster. In addition, it also confirms the possibility of shipping cryopreserved materials to remote locations in near future. The initiation of seed germplasm as base collections in LN is an important step in developing safe, efficient long-term storage for these critically endangered species. The protocols developed for the cryopreservation of immature seeds of both *Mantisia* species is a first step to maximizing the opportunities for the cryostorage of large numbers of seeds of these species, thereby enhancing the long-term *ex situ* genetic diversity conservation of two critically endangered and endemic plants.

Acknowledgements: Financial support received from the Department of Biotechnology, Government of India vide grant no. BT/PR-7055/BCE/08/437/2006 is gratefully acknowledged. The authors thank Mr. K.M. Lalhmachlwana (Forest Department, Mizoram), Dr. Lingaraj Sahoo (Department of Biotechnology, IIT Guwahati) and The Botanical Survey of India (Shillong) for their generous help.

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Accepted for publication 15/7/2011