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A simple way to overcome the recalcitrance of the water fern *Ceratopteris thalictroides* (L.) Brongn. to cryopreservation

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Abstract

Ceratopteris thalictroides is a water fern very sensitive to both dehydration and low temperature. This study focuses on the cryopreservation of this species by encapsulation-dehydration technique, in particular on the effects of pre-culture step, alginate bead size and the physical conditions of culture on the cryopreservation efficiency. Encapsulated and non-pre-cultured gametophytes did not survive cooling with liquid nitrogen. When cryopreservation was preceded by a 2-week period of pre-culture, regrowth reached 42.1%. Reduction in the size of the alginate bead, and culture in total darkness resulted in improved gametophyte regrowth capacity (75.5% or 81.7%, respectively). The best results (91.3%) were obtained when all factors tested occurred simultaneously. The gametophytes recovered very quickly and sporophytes were formed within 4 weeks after rewarming. These simple improvements can be used, not only for the cryopreservation of gametophytes in cryptogams but also for some recalcitrant species of seed plants.

Keywords: abscisic acid; alginate bead size; encapsulation-dehydration; darkness; pre-culture; regrowth

Introduction

Cryopreservation is currently the best method for long-term, ex situ conservation of plant genetic resources and involves the use of liquid nitrogen (LN; −196°C). This technology has been applied to a wide range of high-yielding, vegetatively propagated crops and their wild relatives, i.e., a group of plants whose developmental biology and in vitro multiplication strategies are well-known [1]. Differences in resistance to low temperature are genetically determined and thus affect viability during cryopreservation. The tolerance of plants to cryopreservation varies from very cold-hardy species that can be cryopreserved with little effort, as well as highly viable species, such as *Malus*, *Pyrus* and *Diospyros* [2], to very sensitive, recalcitrant species, such as many major tropical crops, e.g., bananas and plantains [3], whose cryo-conservation requires greater care. The induction of dehydration tolerance is a key to the successful cryopreservation of recalcitrant species [3]. Several methods can be employed to overcome recalcitrance during cryopreservation. These include one- or two-step freezing protocols and the use of various cryoprotectants (to minimize ice crystal formation and cell damage) or modification of cryopreservation protocols, e.g., by using: the most effective but least toxic vitrification solutions; pre-culture treatment involving sugars, abscisic acid or proline; progressive osmotic dehydration at high sucrose concentrations; and increased cooling/warming rates [3–7]. Sometimes, these protocols are lengthy, and this restricts their use to small-scale cryopreservation of plants/cell lines. As a result, a much simpler method would be most welcome [6]. The effective cryopreservation of ferns has been described for the gametophytes of 18 species [5,8–10] and the young sporophytes of a single species of epiphytic fern, namely *Platycerium ridleyi* H. Christ. [11]. These reports suggest that pre-culture and encapsulation are necessary for increasing the cellular resistance of gametophytes and sporophytes to cryopreservation.

Ceratopteris thalictroides (L.) Brongn. is an aquatic, annual, homosporous and tetraploid fern species, distributed widely from Africa and Asia to America and Australia. This species has been used as an experimental model for sporophyte development and, because of its great phenotypic plasticity, for investigating morphological variations resulting from its response to day length and temperature [12]. We chose *C. thalictroides* as the subject for this study because of its recalcitrant nature. The present paper describes a simple method for the successful cryopreservation of this highly sensitive to low temperature and dehydration stress fern species.

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Material and methods

Plant material

Spores of *C. thalictroides* were collected from the aquatic collection of the Botanical Garden of the University of Wrocław (Poland). Spores were prepared according to the protocol of Mikuła et al. [9]. In brief, spores were surface-sterilized by wrapping sporangia in filter paper and immersing the packages in 70% (v/v) ethanol for 30 s, followed by 5% (v/v) commercial bleach (Domestos) for 20 min. The packages with spores were then washed three times in sterile water and the spores subsequently spread onto the surface of half-strength Murashige and Skoog [13] micro- and macro-nutrients (½ MS) medium, with full complement of vitamins and containing 2% (w/v) sucrose and 0.8% (w/v) Difco agar, at pH 5.8. The spore, gametophyte and sporophyte cultures were maintained in a growth chamber at 21 ±1°C with 16-h illumination provided by 50 μM m^{-2} s^{-1} daylight fluorescent tubes.

Cryopreservation

Axenic, secondary young gametophytes (about 1 mm in size) were used as a source of explants for experiments. These were prepared as previously described [9] and cryopreserved using the encapsulation–dehydration method [14]. Briefly, solutions containing 3% (w/v) sodium alginate (Sigma) and 0.1 M $CaCl_2$ were prepared using a 2% (w/v) aqueous sucrose solution. Gametophytes were embedded in sodium alginate for 10 min, and hardened in $CaCl_2$ solution for 45 min, at room temperature. Beads 2.5 mm (small) and 5 mm (standard) in diameter (made using micropipette equipped with a tip modified to a diameter of about 2.5 and 5 mm) were pre-cultured for 2 weeks on ½ MS agar medium supplemented with 0.25 M sucrose and 10 μM ABA [9]. Beads were then immersed in ½ MS liquid medium and osmotically dehydrated by increasing the sucrose concentration in daily increments from 0.5 to 0.75 to 1.0 M. After 3 d, the beads were harvested and surface-dried for 5 h in a laminar flow cabinet at room temperature, before being placed in 2 ml cryovials (15 beads per vial). The latter were immersed directly into LN, where they remained for 1 day. Vials were removed from LN and rapidly rewarmed by immersing them in water bath at 38°C for 3 min. Following rewarming, beads (in the dehydrated form) were immediately transferred to ½ MS agar medium and maintained for the first 7 d in darkness or at a 16/8 h photoperiod of 50 μM m^{-2} s^{-1}. Encapsulated gametophytes that had not been pre-cultured, but had been osmotically dehydrated, air-desiccated for 5 h and exposed to LN and, following rewarming, had been recovered under light conditions, were used as a control (henceforth referred to as minus pre-culture).

Explant survival was assessed by its capacity to regrow gametophytes. Percentage gametophyte regrowth was calculated following 7 days after rewarming.

Statistical analyses

Statistical analyses were performed on two independent experiments of 5 repetitions; each studied treatment comprising 75 gametophytes. Data were expressed as the mean ±standard deviation. Results were analyzed by means of a one-way ANOVA analysis of variance and Fisher's least significant difference (LSD) procedure using Statgraphics Plus software. Significance was set at the 0.05 confidence level.

Results and discussion

Our previous studies showed that fern gametophytes of different genera vary in their tolerance of cryopreservation [5]. Gametophytes of species from areas with mild frosts, but low risk of water loss, appeared to be less tolerant of cryopreservation stress compared with those species from tropical environments that may be subjected to regular wetting and drying cycles. Our present study focused on a fern species which is particularly sensitive to both low temperature and dehydration. As far as we know, this is the first study assessing the effect of cryopreservation on the survival of an aquatic, vascular plant species. It is also the first experiment for which the effect of the size of the alginate bead and lighting conditions of recovery culture on explant viability following cryopreservation has been proven.

In control culture, encapsulated and non-pre-cultured gametophytes of *C. thalictroides* did not survive the standard protocol of cryopreservation by encapsulation/dehydration (Fig. 1). Similar results were obtained for the tree-fern *Cyathea dealbata* (G. Forster) Swartz, growing in the wild, where it was subjected to high humidity and tropical temperatures [5]. Gametophyte viability of another six tree-ferns, i.e., *Cibotium glaucum* (Sm.) Hook. & Arn., *Ci. schiedei* Schlecht. & Cham., *Cyathea delgadii* Sternb., *C. australis* (R. Br.) Domin, *C. smithii* Hook. and *Dicksonia fibrosa* Col., and two herbaceous ferns, i.e., *Osmunda regalis* L. and *Phyllitis scolopendrium* (L.) Newman ranged from 12–80% following exposure to LN [5]. The encapsulation/dehydration protocol preceded by 2-week-long pre-culture in the presence of 0.25 M sucrose and 10 μM ABA was sufficient to provide protection against cryopreservation treatments in all previously studied species [5,9]. Moreover, this protocol consistently protected the gametophytes resulting in >70% survival [5]. For *C. thalictroides*, however, this protocol resulted in only 42.1% viability. Nevertheless, pre-culture was essential (Fig. 1), since rewarmed explants showed extensive necrosis of gametophyte tissue following rapid cooling with LN.

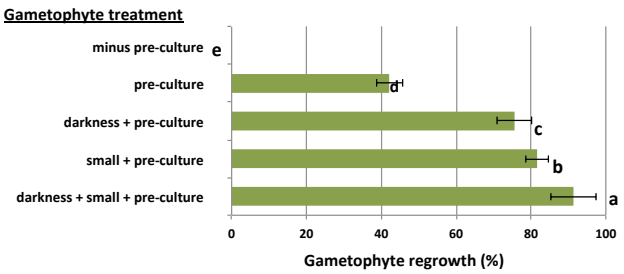

Fig. 1 Effect of 2 weeks pre-culture (with 0.25 M sucrose and 10 μM ABA), size of beads (small and standard – 2.5 and 5 mm in size, respectively), and 7 d darkness during recovery culture on the regrowth of *C. thalictroides* gametophytes cryopreserved by encapsulation-dehydration. Control treatment (minus pre-culture) – encapsulated, non-pre-cultured, dehydrated and cooled with LN gametophytes.

Gametophytes of *C. thalictroides* cultured in darkness for the first 7 d following rewarming exhibited 75.5 % regrowth (Fig. 1). Similarly, Bui et al. [4] reported that the use of low level of light intensity (~8 μM m^{-2} s^{-1}) during the recovery phase improved the cryopreservation protocol of chlorophyte microalgae. Light at high intensities resulted in the formation of reactive oxygen species, photo-inhibition, and damage to photosystem I [15]. For chlorophyllous explants, such as the gametophytes of *C. thalictroides* or microalgae, darkness or light at very low levels help mitigate the unfavorable effects of cryopreservation-induced stress. The use of darkness for recovery may provide a promising possibility for improving cryopreservation protocols by reducing photosynthesis and photo-oxidative damage [4].

In studies of *C. thalictroides*, a reduction in the size of the alginate beads was the most effective factor in protecting gametophyte regrowth (81.7%; Fig. 1). The alginate bead supports the cells, and prevents both mechanical and physical injury during dehydration and cooling [7,10]. However, it also may slow down the rate of cooling and warming. Rapid cooling is essential to achieve the glassy state of intracellular ice crystals (vitrification), and rapid warming is necessary to prevent recrystallization on rewarming [16]. The effect of various protocols involving different cooling and warming rates on the survival of mouse oocytes following cryopreservation has been documented by Mazur and Seki [16]. Similarly, ultra-rapid cooling and rewarming rates proved to be essential for high and reproducible regeneration rates in "difficult to cryopreserve" cultivars of Musa [3].

Protecting gametophytes of *C. thalictroides* with small alginate coat during cooling with LN and, following rewarming, culturing them in darkness, resulted in a >90 % regrowth rate (Fig. 1). Under such conditions, a reduction in the occurrence of necrosis in recovering explants was observed (Fig. 2a). The cultures of gametophytes were recovered very quickly (Fig. 2b), and sporophytes were formed during the 4 weeks period of rewarming culture (Fig. 2c,d). Furthermore, the epidermal cells of the sporophyte lamina margin divided to produce bulbils – propagules of vegetative reproduction (Fig. 2e). Our experience suggests that sporophyte production following cryopreservation occurs more quickly in *C. thalictroides* than in most fern species, the life cycle of *Ceratopteris* being completed in just 120 d [17].

In conclusion, a number of factors, such as the pre-culture of gametophytes, a reduction in the size of the alginate beads, and a 7 d period of recovery culture in darkness greatly improved the regrowth rate of *C. thalictroides* gametophytes. These simple modifications provide a very good example of how small changes to the cryopreservation protocol can significantly increase the survival rate of cryptogams, such as ferns or mosses, as well as that of certain recalcitrant species of seed plants.

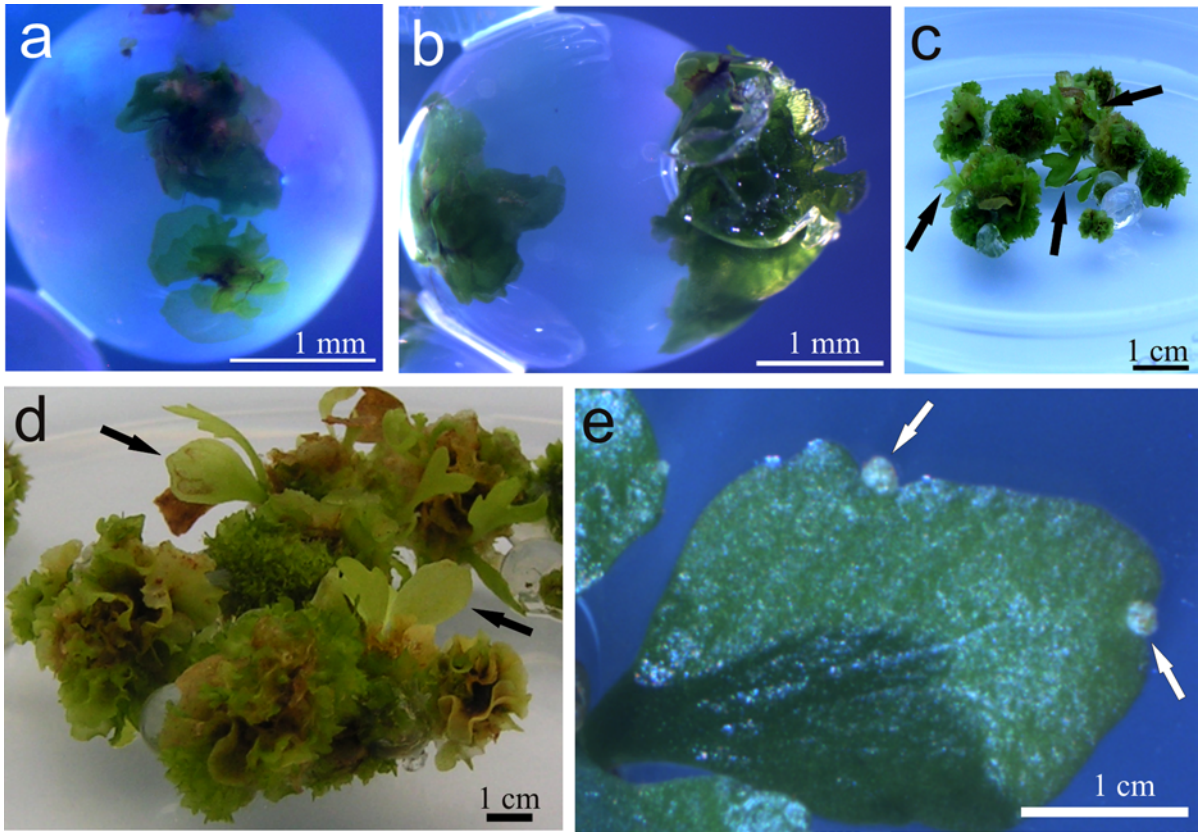

Fig. 2 Recovery of *Ceratopteris thalictroides* gametophyte culture and sporophytes following cryopreservation by means of the optimal protocol of encapsulation-dehydration. **a** Gametophytes without visible necrosis 3 days after rewarming. **b** Gametophyte multiplication 2 weeks after rewarming. **c,d** Mass sporophyte production (arrows) 4 weeks after rewarming. **e** Small bulbils on margin of lamina.

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Authors' contributions
The following declarations about authors' contributions to the research have been made: conducting experiments: DM; helping to interpret the results and discussion: JJR; research designing and writing the manuscript: AM.

Competing interests
The following declarations about authors' competing interests have been made: JJR is associate editor and a member of the *Acta Societatis Botanicorum Poloniae* Editorial Council; other authors: no competing interests.

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