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Cryopreservation of *Cyrtopodium hatschbachii* Pabst (Orchidaceae) immature seeds by encapsulation-dehydration

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ABSTRACT: The aim of the present study was to investigate the efficiency of the encapsulation-dehydration technique for cryopreservation of *Cyrtopodium hastchbachii* Pabst seeds. Immature seeds of this species were cryopreserved by an encapsulation-dehydration technique. Seeds of five immature pods, 120 days after pollination, were encapsulated in 3% calcium alginate matrix and pretreated in liquid medium supplemented with 0.08 M sucrose (24 h), 0.15 M sucrose (24 h), 0.25 M sucrose (48 h), 0.5 M sucrose (24 h) and 0.75 M sucrose (24 h) in shaker at 60 rpm. Alginate beads were dehydrated 5 h in silicagel and immersed in liquid nitrogen for 12 h. Cryopreserved beads were thawed at 30°C for 1 min, rehydrated using the same liquid mediums [0.75 M sucrose (24 h), 0.5 M sucrose (24 h), 0.25 M sucrose (48 h) and 0.15 M sucrose (24 h)] and cultivated in half strength Murashige & Skoog medium (1962) with the addition of 2 g/L activated charcoal. Sixty four percent of seeds survived and developed into acclimatized plants after being cryopreserved. In this work, the encapsulation-dehydration technique was employed for first time in *Cyrtopodium hatschbachii*.

Introduction

Orchidaceae Juss. is one of the most numerous families of Angiosperms with more than 30,000 species (Dressler, 1990). The destruction and alteration of natural habitats are the main problems that threaten the conservation of orchid diversity. The increasing interest in orchids shown by collectors and growers translates into millions of plants collected and sold each year Johnson (2001).

At the moment, cryopreservation is considered one of the best alternatives for long term conservation of plant germplasm. This tool allows the conservation of plant material in a limited space with less maintenance and risk of contamination (Ashmore, 1997; Engelmann, 2000). It has been demonstrated that cryopreservation protocols are able to preserve orchid germplasm (Popova *et al.*, 2003). Several orchid explants were successfully cryopreserved; for example, zygotic embryo and immature seeds of *Bletilla striata* (Ishikawa *et al.*, 1997), seeds of *Doritis pulcherrima* (Thammasiri, 2000), cells in suspension of *Doritaenopsis* (Tsukazaki *et al.*, 2000), protocorms and protocorm-like bodies of *Dendrobium candidum* (Brian *et al.*, 2002), shoot tips of *Dendrobium* Walter Oumae (Lurswijidjarus and Thammasiri, 2004), seeds of *Bratonia* hybrids (Popov *et al.*, 2004) and immature seeds of *Ponerorchis graminifolia* (Hirano *et al.*, 2005).

The encapsulation-dehydration procedure is a new cryopreservation technique based on the vitrification concept. It has been described by Fabre and Dereuddre (1990), and consists in the encapsulation of plant material in a calcium alginate matrix followed by a pretreatment in high sucrose liquid media and fast freezing of

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explants in liquid nitrogen. As a result, the intracellular solutes vitrify and ice formation is prevented. This technique has been successfully used in different plant species (Takagi, 2000). Recently, Flachsland and coworkers (2006) have applied it to preserve seeds and protocorms of *Oncidium bifolium*.

Cyrtopodium hatschbachii is an orchid species grows in the Misiones province in Northeastern Argentina (Schinini *et al*., 2008). It has been discovered in Jataí, Goiás (Brasil) at 400 m over the sea level (Menezes, 2000). Its pseudobulbs are small, 8 cm long, prolonged and fusifoms. The inflorescence is simple, 8 – 15 flowers of 3 – 3.5 cm in diameter and reddish, pink or pinkish with a yellowish labelum. The callus labelum is used to identify the species of this genus (Fig. 1 A, B, C, D and E).

Due to the restricted distribution of C. *hatschbachii* natural populations, its conservation acquires ecological significance. Our recent studies have demonstrated that immature seeds of this species exhibit a higher *in vitro* germination than the mature ones (date not shown). Since immature seeds are kept under sterile conditions, they represent a suitable material for cryopreservation. Up to date, there are not reported cryopreservation protocols for the genus *Cyrtopodium*. The aim of the present study was to adjust a cryopreservation protocol for immature seeds for *C. hatschbachii* using the encapsulation-dehydration technique.

FIGURE 1. *Cyrtopodium hatschbachii* Pabst. (A) Flower, front side. (B) Inflorescence. (C) Adult plant in summer (high: 60 cm). (D) Pseudobulb of an adult plant in autumn. (E) Immature fruit, pod at 120 d after pollination. Bar = 1 cm.

Plant Material

Five adult plants of *Cyrtopodium hatschbachii* Pabst were collected from the University Campus of the Universidad Nacional de Misiones (UNaM) in Posadas, Misiones, Argentina. They were cultivated at the greenhouse of the IBONE, in Corrientes, Argentina. Flowers were manually self pollinated and immature pods were collected 120 d after pollination (Fig. 1E). Pods were disinfected in 70% ethanol (1 min), submerged in 1% NaOCl solution with 0.1% Triton 100® (30 min) and washed twice in sterile water. Immature seeds were removed from the pods and maintained at 4ºC in 4.5 mL cryotubes.

Encapsulation of immature seeds

Sterile immature seeds of *Cyrtopodium hatschbachii* were suspended in a sterile 3% (w/v) sodium alginate solution in a shaker and dropped into 0.1 M $CaCl_2$ solution using 5 mL micropipette. After 1 min in the $CaCl_2$ solution, each bead (4–5 mm in diameter) containing a mean of 50 seeds was recovered. To establish the number of seeds by bead, 50 drops of seed solution were dropped on a Petry-dish and counted under a stereo-microscope. The alginate beads were kept in sterile 4.5 mL cryotubes at 4°C.

Cryopreservation and culture conditions

Immature seeds were cryopreserved using the encapsulation-dehydration technique. Beads with encapsulated sterile seeds were pretreated in half strength Murashige and Skoog (1962) liquid medium (˚Murashige & Skoog medium (1962)) enriched with a progressively increasing sucrose concentration, using the following sequence: 0.08 M (24 h); 0.15 M (24 h); 0.25 M (48 h); 0.5 M (24 h) and 0.75 M (24 h) in a shaker at 60 rpm. The encapsulated seeds were dehydrated using 30 g silica gel per container (5 h) until 18% moisture content was achieved (calculated using the equation [fresh weight (FW)-dry weight (DW) / FW] x 100). Beads were then put into sterile cryotubes (Nalgene® 4.5 mL capacity) and submerged in liquid nitrogen (12 h). Cryotubes which had been immersed in liquid nitrogen were rapidly rewarmed in a water-bath at 30ºC for 1 min. Following, beads were precultured with sucrose and postcultured on a rotary shaker (60 rpm) in liquid medium (˚Murashige & Skoog medium (1962)) containing progressively decreasing sucrose concentrations using the following sequence: 0.75 M (24 h); 0.5 M (24 h); 0.25 M (48 h); 0.15 M (24 h) and 0.08 M (24 h). Explants were then transferred to germination medium: ˚Murashige & Skoog medium (1962) + 0.08M sucrose + 2 g/L activated charcoal + 0.7% agar Sigma® A-1296. The pH was adjusted to 5.8 before the addition of agar. The 125 mL containers with 30 mL culture medium were sterilized at 1.45 kg cm^{-2} and 121ºC for 20 min. Explants from all treatments were incubated at 27 ± 2ºC under 116 μmol m^{-2} s^{-1} of light intensity and a 14 h photoperiod.

The cryopreservation experiment included the following treatments:

T1 – encapsulated seeds + sucrose + silica gel + liquid nitrogen + rehydration
T2 – encapsulated seeds + silica gel + liquid nitrogen
T3 – encapsulated seeds + liquid nitrogen
T4 – non encapsulated seeds
T5 – encapsulated seeds
T6 – encapsulated seeds + sucrose + silica gel

Statistical analysis

Seed survival was registered 45 d after culture by counting the seeds with hypertrophied embryos and those with protocorms. The survival values are presented as standard error of means (±SEM) based in 5 repetitions (30 beads/repetition). Means were compared using the Tukey´s test ($P \leq 0.05$).

Results

The self-pollination of *C. hatschbachii* plants was successfully performed and each plant produced 4 pods (120 days after pollination). Based on fresh weight, it was calculated an average of 250,000 seeds/pod for this species. Seeds used for experimental studies had normal embryos visible at stereoscopic microscope and a yellowish appearance, characteristic of the tribe Cymbidieae.

Encapsulation of immature seeds

Alginate beads containing an average of 50 seeds/bead were put into cryotubes (4.5 mL) and maintained at 4ºC. Seed viability was not affected by cold-storage. Seeds with embryos notably bigger than the harvested ones and those that produced protocorms were consider as germinated seeds (Fig. 3B).

Cryopreservation of immature seeds

Previous studies have shown that the survival of seeds was optimal at 5 h dehydration in silica gel (data not shown). The treatment 1 (T1) has resulted in the highest survival percentage, thereby 64% of seeds survived the sucrose pretreatment (Fig. 2A), dehydration in silica gel (5 h), direct immersion in liquid nitrogen (12 h) and rehydration. In T1, the mean of survival percentage was significantly higher ($P \leq 0.05$) than means obtained in other treatments, even compared with controls (Table 1).

It was effectively demonstrated the positive effect of sucrose pretreatment. Only 8% of seeds without sucrose pretreatment survived to liquid nitrogen after dehydration in silica gel (T2) and there was evident tissue damage (Fig. 2B). The pretreatment and rehydration in sucrose solutions has not affected seed germination as we observed an average of 49% survival in controls (Table 1, T4 – T6).

A high percentage of protocorms (data not shown) from both cryopreserved and non cryopreserved seeds developed normal plants *in vitro* (Fig. 3C-D). The development of plants in the greenhouse was evaluated in 10 month old plants. There were no phenotypic differences when we compared plants coming from cryopreserved treatments against not cryopreserved material (Fig. 3E).

Discussion

Cyrtopodium hatschbachii is a terrestrial orchid described for Argentina (Schinini *et al*., 2008). Even though this species has ornamental value, it is not culti-

TABLE 1.

Seed germination of *Cyrtopodium hatschbachii*, (% ± SEM) cryopreserved (T1 – T3) and not cryopreserved (T4 – T6). Values followed by different letters indicate significant differences. Tukey's test ($P < 0.05$).

	Treatments				% Seed germination
N°	Encapsulation	Sucrose pretreatment	Silica gel	Liquid nitrogen	%
T1	+	+	+	+	64 (± 1.01)[a]
T2	+	-	+	+	8 (± 0.99)[c]
T3	+	-	-	+	0[d]
T4	-	-	-	-	48 (± 2.03)[b]
T5	+	-	-	-	49 (± 1.15)[b]
T6	+	+	+	-	50 (± 1.89)[b]

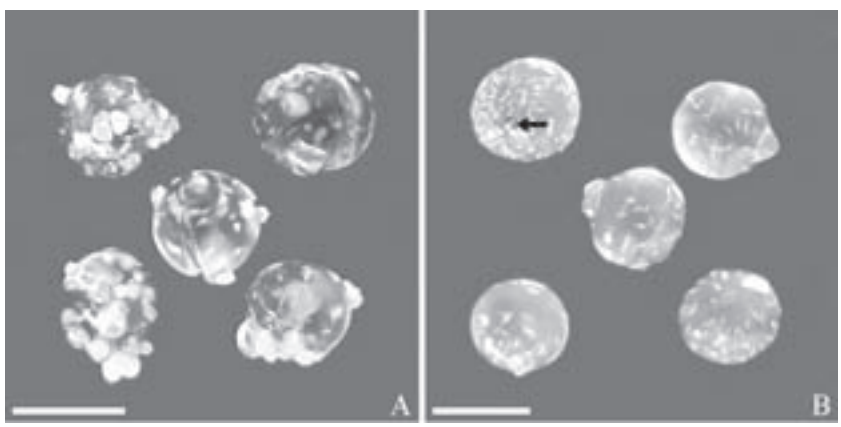

FIGURE 2. Effect of sucrose pretreatment on encapsulated immature seeds of *Cyrtopodium hatschbachii*. (A) Pretreated beads with high sucrose concentrations before immersion in liquid nitrogen, T1. (B) Not pretreated beads, T2. Arrow show embryo with tissue damage. Numbers indicate survival percentage. Bar = 5 mm.

vated commercially at the moment. Populations of this species have been reduced due to the indiscriminate destruction and alteration of its natural habitats. For this reason, this resource should be preserved *in situ* and *ex situ*, propagated and reintroduced in its natural environments. The known natural populations of *C. hatschbachii* are restricted to the northeast of Argentina and have been used in the present study as representative genotypes.

Orchids have small seeds that are hard to manipulate, but this problem can be resolved by encapsulating them in calcium alginate matrix beads (Fig. 3A). In our experiments, we obtained alginate beads containing 50 seeds that represent a good sample of the gene pool of the species. Moreover, each 4.5 mL cryotube can contain as many as 450 beads, which would represent 22,500 genotypes. As a consequence, this method is an excellent alternative to long term conservation of genetic variability of *C. hatschbachii* and orchid germplasm.

Our results show that the *in vitro* germination of *Cyrtopodium hastchbachii* seeds is not affected by alginate beads, sucrose pretreatment or dehydration in silica gel (Table 1, T5 and T6) as there is no difference with the germination rate of control uncoated seeds (Table 1, T4). These results are consistent with those found by Flachsland *et al.* (2006), who observed that encapsulated and control seeds of *Oncidium bifolium* had a similar germinability.

Orchid embryo is composed of a few homogeneous small parenchymatic cells and has, in most species, a lipidic reserve (Hadley, 1982; Pritchard, 1984). Due to its reduced size (0.1 to 1 mm), it can be dehydrated enough to get a successful cryopreservation (Berjak *et al.*, 1996). In the present research, the dehydration seems to be an important factor in seed survival after immersion in liquid nitrogen. Seeds that were not dehydrated before immersion in liquid nitrogen could not resist the exposure to ultra low temperatures (Table 1, T3).

Cryopreservation must allow the regeneration of acclimatized plants in the greenhouse, which are stable at the genotypic and phenotypic level. The genetic stability of 190 days *in vitro* plants of *C. hatschbachii* from cryopreserved seeds has been confirmed cytogenetically (Surenciski *et al*., 2007). In the present study, 10 month old plants of *Cyrtopodium hatschbachii* originated from cryopreserved material have shown normal development and shape under greenhouse conditions (Fig. 3E).

Cryopreserved seeds had the highest germination percentage (64%). This phenomenon could be attributed to seed coat damage during freezing-defrost cycles (Tikhonova *et al.*, 1997) that enhances seed permeability, allowing the uptake of nutrients from the culture

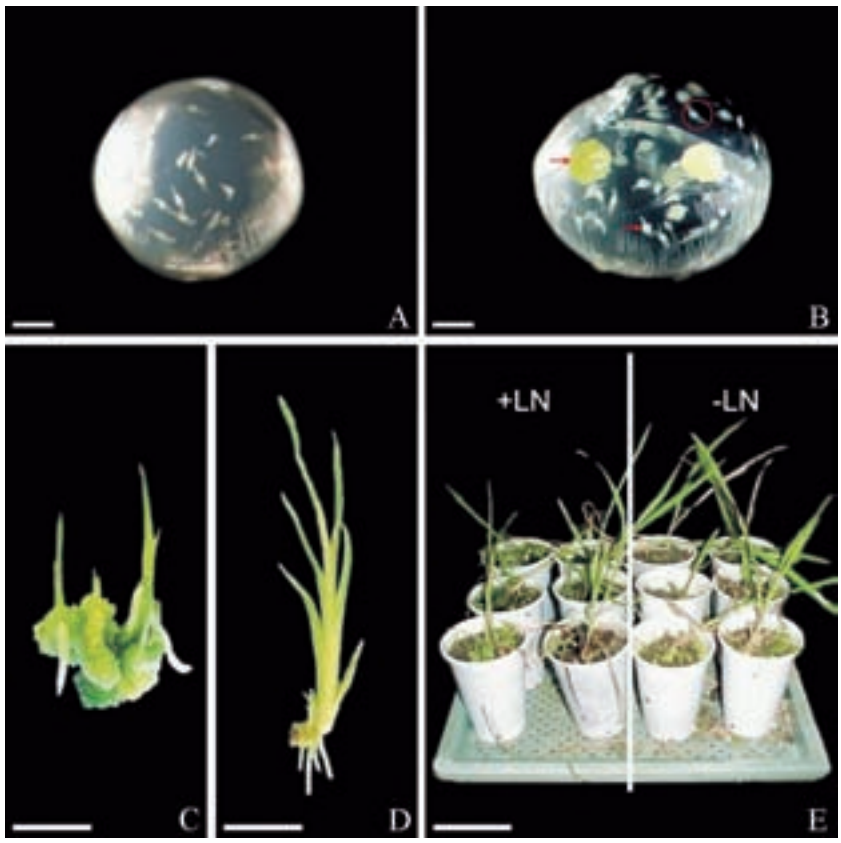

FIGURE 3. Plant regeneration of *Cyrtopodium hatschbachii* from encapsulated immature seeds. (A) Alginate beads with 50 seeds. (B) Cryopreserved bead with germinated seed (arrow) and non germinated seeds (circle) 45 d after sown. (C) 90 d age plants. (D) 195 d age *in vitro* plants from cryopreserved seeds. (E) Acclimatized plants at greenhouse, 300 d age. Bars = 1 mm (A, B), 10 mm (C, D), 60 mm (E).

medium. Our results agree with those obtained by Popova *et al.* (2003) and Popov *et al.* (2004) who also observed a rapid growth of cryopreserved seeds and the subsequent growth of protocorms in the orchid hybrid *Bratonia*. In *Oncidium bifolium,* a reduction in the germination rate was observed after the exposure of seeds to -196ºC (Flachsland *et al.,* 2006). These results are not in agreement with those obtained in this work with *Cyrtopodium hastchbachii*, where the liquid nitrogen treatment enhances germination.

In this work, the encapsulation-dehydration technique was applied for the first time in the *Cyrtopodium* genus in order to achieve the long term conservation of immature seeds of *C. hatschbachii*. This technique does not require toxic cryoprotectors like dimetilsulfoxid (DMSO) and ethilenglycol, used in other vitrification techniques such as the PVS2 solution that has been applied to cryopreserv *Dendrobium* Walter Oumae meristems (Lurswijidjarus and Thammasiri, 2004). This work opens new research topics such as the understanding of physiological processes implicated in the germination and development of plants from cryopreserved seeds as well as the possibility of using the encapsulation-dehydration technique in other *Cyrtopodium* species and Orchidaceae members.

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