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CRYOPRESERVATION OF SEEDS AND *IN VITRO*-CULTURED PROTOCORMS OF *Oncidium bifolium* Sims. (ORCHIDACEAE) BY ENCAPSULATION-DEHYDRATION

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Abstract

Encapsulation-dehydration was employed for cryopreserving seeds and *in vitro*-cultured protocorms of *Oncidium bifolium*. Freshly harvested seeds, 120 days after pollination, were encapsulated in beads containing ½ MS medium with 3% sucrose and 3% calcium alginate and subsequently pretreated in agitated (80 rpm) liquid medium supplemented with 0.15 M sucrose (24 h) followed by 0.25 M sucrose (48 h), 0.5 M sucrose (24 h) and 0.75 M sucrose (24 h). The beads with seeds were dehydrated with silica gel for 5 h to 19.2% moisture content and immersed in liquid nitrogen for 1 h, thawed at 30 ºC for 2 min, post-treated using the same series of liquid media [0.5 M sucrose (24 h), 0.25 M sucrose (48 h), 0.15 M sucrose (24 h)], and recultured on ½ MS medium with 0.1M sucrose and 0.7% agar. As much as 4.8% of the cryopreserved seeds produced complete plants. *In-vitro* cultured protocorms were successfully cryopreserved following the same procedure, allowing 11.3% of them to produce plants.

Keywords: *Oncidium bifolium*, Orchidaceae, cryopreservation, encapsulation-dehydration, seeds, protocorms.

INTRODUCTION

Approximately 20,000 plant species are estimated to be endangered, rare, or threatened with extinction (11). The preservation of plant germplasm is presently being applied in many laboratories throughout the world, and biotechnology plays an important role for genetic preservation.

Cryopreservation, *i.e.* storage of living cells, tissues and organs at ultra low temperature (liquid nitrogen, -196ºC), is considered the best procedure for germplasm long-term preservation without genetic alterations (1,4,13,14). Cryopreservation techniques developed over the last 25 years have been successfully applied to well over 100 species (9,15,20). However, up to now there have been only a few reports on orchid cryopreservation. Wang *et al.* (19) have developed techniques for cryopreservation of seeds and protocorms of *Dendrobium candidum*. Pritchard *et al.* (12) have shown that seeds of *Eulophia gonychila, D.*

anosmun, Dactylorhiza fuchsii, Dactylorhiza majalis and *Paphiopedilum rothschildianum* could withstand 12 months storage at -196ºC. Thammasiri (17) cryopreserved seeds of *Doritis pulcherrima* by vitrification. Using the encapsulation-dehydration technique,Wood *et al.* (21) cryopreserved seeds of *Dactylorhiza fuchsii* and *Anacamptis morio* together with their fungal symbiont. Thinh and Takagi (18) cryopreserved *in vitro*-grown apical meristems of *Cymbidium* spp. by vitrification.

Encapsulation-dehydration is one of the new vitrification based cryopreservation techniques which was developed for shoot tips of pear (3), *Solanum* (5) and subsequently applied to a broad range of species (16). It entails encapsulation of explants in calcium alginate beads, pre-growing them in medium containing high levels of sucrose (0.3 - 1.5 M), desiccating them to around 20% moisture content (MC) and immersing them in liquid nitrogen (5).

The genus *Oncidium*, with more than 500 species, grows in subtropical and tropical areas of America. In Argentina there are 15 *Oncidium* species (2), some of them of great ornamental value such as *O. bifolium, O. cebolleta, O. jonesianum* and *O. fimbriatum.*

Oncidium bifolium, an epiphytic orchid, has a wide distribution in humid forests of Northern Argentina, Bolivia, Brazil, Paraguay and Uruguay (8). This species is threatened because of rapid deforestation and its excessive exploitation due to the beauty of its flowers. The seeds of this species remain viable only for 2 months after collecting (unpublished). Some attempts have been made in the past to protect this species by *in vitro* germination of the seeds and protocorm production (6). Protocorms and protocorm like-bodies have the potential to regenerate plants, and represent thus a source of material for germplasm conservation.

Our unpublished observations made on *O. bifolium* showed that it was possible to recover an efficient protocorm yield from seeds maintained at 4ºC for up to 6 months only. When seeds were stored for a longer period at this temperature, protocorm production decreased dramatically.

This paper describes an effective procedure for the cryopreservation of seeds and *in vitro*-growing protocorms of *O. bifolium* using the encapsulation-dehydration technique.

MATERIALS AND METHODS

Plant material

Flowers from the same inflorescence of *O. bifolium* Sims. were hand-pollinated and the green capsules containing the mature seeds, were collected 120 days after pollination. They were surface sterilized by immersion in 70% ethanol (2 min), then in a solution of commercial bleach containing 2.75% NaOCl (30 min). The capsules were rinsed three times with sterile distilled water.

Desiccation and cryopreservation of seeds

Mature seeds of *O. bifolium* were collected from the sterilized capsules and introduced *in vitro*. It was necessary to determine the number of seeds per alginate bead in order to enable counting of seeds, evaluation of germination and conversion into plants. Successive dilutions of 0.015 mg of seeds in 1 ml, 2 ml, 4ml and 6 ml of liquid ½ strength Murashige and Skoog (MS) medium (10) containing 0.1M sucrose (6) and 3% alginate were made. To obtain ± 70 seeds/alginate bead, the adequate dilution was 0.015 mg seeds / 2 ml alginate medium. To determine the number of seeds in each alginate bead, 30 randomly chosen beads were opened and the number of seeds counted under a stereomicroscope. The same procedure was employed to control the number of protocorms suspended in the alginate medium. Beads (4 -

5 mm in diameter) were formed by dropping the alginate medium in a 0.1 M $CaCl_2$ solution to form the alginate beads.

Encapsulated seeds (approximately 70 seeds per bead) were pretreated with sucrose on a rotary shaker (80 rpm) using the following sequence: 0.15 M sucrose (24 h) followed by 0.25 M sucrose (48 h), 0.5 M sucrose (24 h) and 0.75 M sucrose (24h). Each time encapsulated seeds were submitted to a sucrose pretreatment, and whether they were or not cryopreserved, they were also post-treated on a rotary shaker (80 rpm) consisting of the inverse sequence of sucrose media, *i.e*. 0.5 M sucrose (24 h), followed by 0.25 M sucrose (48 h), 0.15 M sucrose (24 h), before transfer to standard culture medium including ½ MS macro- and micro-elements, 0.1 M sucrose and 0.7% agar (Sigma A1296). The pH of the medium was adjusted to 5.8 before the addition of agar. The flasks (125 ml capacity), containing 25 ml of culture medium, were covered with aluminium foil and autoclaved at 1.45 kg cm^{-2} for 20 min.

Moisture content (MC) of beads was determined gravimetrically and expressed as the percentage of the initial fresh weight (7). It was measured on 30 pretreated beads at 60 min intervals between 0 and 6 h. Beads were dehydrated with silica gel (30g per flask with 150 ml capacity).

The cryopreservation experiments included the 10 following treatments:

C1- Non-encapsulated seeds
C2- Encapsulated seeds
C3- Encapsulated seeds + sucrose pretreatment
C4- Encapsulated seeds + silica gel
C5- Encapsulated seeds + sucrose pretreatment + silica gel
T6- Non encapsulated seeds + LN
T7- Encapsulated seeds + LN
T8- Encapsulated seeds + sucrose pretreatment + LN
T9- Encapsulated seeds + silica gel + LN
T10- Encapsulated seeds + sucrose pretreatment + silica gel + LN

The encapsulated seeds were placed in cryotubes Nalgene with 5 ml capacity (30 beads/cryotube) aand cryopreserved by direct immersion of the cryotubes in LN and then rewarmed after 1 h in a 30ºC (2 min) water-bath. In cases where seeds had been pretreated, they were post-treated as described previously and transferred to standard recovery medium. In cases where no pretreatment was performed, seeds were transferred directly onto recovery medium.

The seeds were incubated at 27 ± 2ºC under a photoperiod of 14 h light/10 h dark, with a light intensity of 116 µmol s^{-1} m^{-2} provided by cool fluorescent lamps.

Survival was evaluated at various periods: after 30 days, by counting the number of seeds that had turned green; after 90 days, by counting the number of seeds which formed protocorms and after 180 days by counting the number of protocorms that developed into plantlets. Values for survival of seeds, seeds forming protocorms and protocorms forming plants are presented as means with standard errors (± SEM) based on three replications of the experiment. Comparisons of means were made by Tukey's Studentized Range (MSD) ($P < 0.01$).

Cryopreservation of protocorms

Fresh seeds, 120 days after pollination, were used to obtain the protocorms. Seeds were germinated *in vitro* in ½ MS agitated (80 rpm) liquid medium with 3% sucrose, incubated at 27 ± 2ºC under a photoperiod of 14 h light/10 h dark, with a light intensity of 116 µmol s^{-1} m^{-2} provided by coolluorescent lamps. After 60 days of culture it was possible to obtain protocorms of.3 mm iniameter which were used for the preservation experiments.

Both the pretreatment of protocorms, and experiments to determine the optimal dehydration period and number of protocorms per bead, were as described above for seeds. Dehydration periods tested varied between 0 and 10 h.

After dehydration of the encapsulated protocorms (for 7 hours) to 18.5% MC, alginate beads containing 30 protocorms were transferred to sterile 5 ml cryotubes (30 beads/cryotube) and immersed rapidly in LN for 1 h. After warming (2 min in a water-bath at 30ºC) protocorms were post-treated following the procedure established for seeds, transferred to the same recovery medium and placed under the same culture conditions. The cryopreservation experiments performed with protocorms included the same 10 treatments as those carried out with seeds.

Protocorm survival was determined after 10 days as the percentage of protocorms remaining green, and the percentage of protocorms forming plantlets was evaluated after 110 days of culture. Values for survival of protocorms and protocorms forming plants are shown as means with standard errors (± SEM) based on three replications of the experiment. Comparisons of means were made by Tukey's Studentized Range (MSD) ($P < 0.01$).

RESULTS

Cryopreservation of O. bifolium seeds

Seed MC varied from an initial 72.0% to 13.3% after 6 h dehydration (Fig. 1). Survival of control seeds was not affected by dehydration and always remained very high, above 88%. After freezing, survival was nil for up to 2 h dehydration; it increased progressively to reach 89% after 5 h (19.2% seed MC) and decreased again to 67% after 6 h (13.3% seed MC).

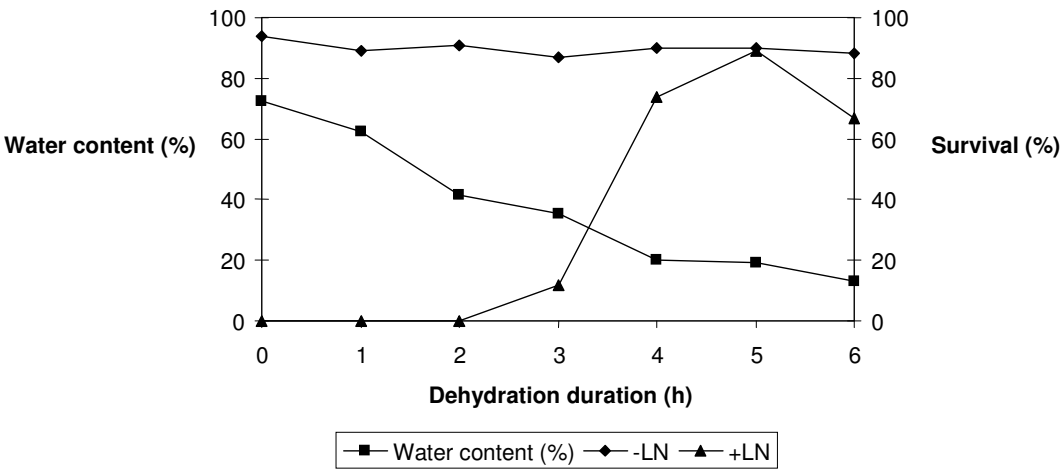

Fig. 1: Effect of dehydration duration (h) on the water content (%, FWB) and the survival (%) of control (-LN) and cryopreserved (+NL) *O. bifolium* seeds.

In all control treatments (C1-C5), seed survival and protocorm formation were high; however the percentage of protocorms forming plantlets was low, between 2.3% (C1) and 8.1% (C2), which was significantly higher than in the other control treatments (Table 1). Freezing was lethal for seeds without encapsulation (T6), when the encapsulated explants were not submitted to sucrose pretreatment and dehydration (T7), when encapsulated explants were dehydrated but not pretreated with sucrose (T9) and when they were pretreated with sucrose but not dehydrated (T8). After cryopreservation, positive results were obtained only

when encapsulated seeds were both sucrose pretreated and dehydrated (T10). Seed survival and protocorm formation were significantly lower than in all control treatments. However, 4.8% of the protocorms originating from frozen seeds produced plantlets, which was comparable to the results achieved with three control treatments (C3-5). There was no need to extract the control and cryopreserved seeds from the beads prior to culture on standard recovery medium, since they grew and emerged from the alginate beads without difficulty (Fig. 2 a & b). The protocorms developed into normal plantlets and were transferred to the greenhouse for further growth (Fig. 2 c).

Table 1: *O. bifolium* seed survival, seeds forming protocorms and protocorms forming plantlets (% ± SEM) without cryopreservation (C1-C5) and with cryopreservation (T6-T10).

Treatments					Seed survival	Seeds forming protocorms	Protocorms forming plantlets
Treatment (Nº)	Encapsulation	Sucrose pretreatment	Dehydration	LN	(%)	(%)	(%)
C1	-	-	-	-	90 (± 8.72)^{a}	90.3 (± 7.81)^{a}	2.3 (± 0.5)^{c}
C2	+	-	-	-	90 (± 7.64)^{a}	94 (± 5.81)^{a}	8.1 (± 0.60)^{a}
C3	+	+	-	-	94 (± 5.85)^{a}	94.6(± 6.73)^{a}	6.3(± 0.78)^{b}
C4	+	-	+	-	85 (± 6.75)^{a}	94.7(± 5.10)^{a}	5.4 (± 0.92)^{b}
C5	+	+	+	-	90 (± 6.89)^{a}	95.2(± 4.33)^{a}	5.7 (± 1.02)^{b}
T6	-	-	-	+	0^{c}	0^{c}	0^{d}
T7	+	-	-	+	0^{c}	0^{c}	0^{d}
T8	+	+	-	+	0^{c}	0^{c}	0^{d}
T9	+	-	+	+	0^{c}	0^{c}	0^{d}
T10	+	+	+	+	67 (± 6.88)^{b}	22.2 (± 4.57)^{b}	4.8 (± 0.85)^{b}

Similar letters within columns mean no significant difference according to the Tukey's Studentized Range (MSD) test ($P < 0.01$).

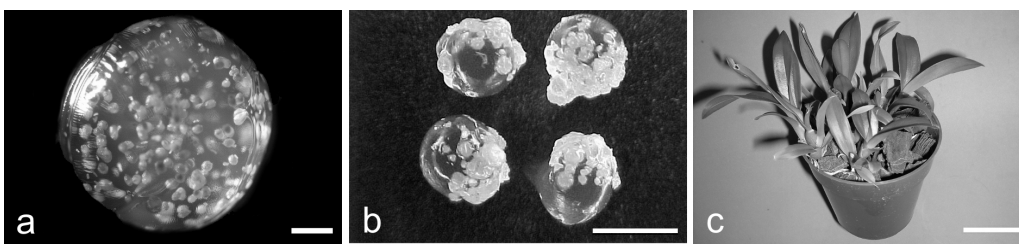

Figure 2: **a**: Cryopreserved encapsulated seeds of *Oncidium bifolium* (bar = 1 mm). **b**: Protocorms grown from cryopreserved seeds 90 days after warming from LN (bar = 5 mm). **c**: Plants transferred to the greenhouse 190 days after warming from LN (bar = 3 cm).

Cryopreservation of O. bifolium *protocorms*

Bead MC varied from 83.2% without desiccation to 5.7% after 10 h desiccation (Fig. 3). Survival of dehydration controls remained high (±80%) up to 7 h dehydration and then dropped rapidly to reach 20% after 10 h dehydration. Survival of cryopreserved protocorms was nil for 0 to 3 h dehydration. It increased progressively afterwards to reach 80% after 7 h dehydration (21.2% MC), *i.e.* a value similar to that of control protocorms. Survival then decreased progressively and reached 0% after 10 h dehydration. As for seeds, there was no

need to extract control and cryopreserved protocorms from the beads prior to culture on standard recovery medium, since they grew and emerged from the alginate beads without difficulty.

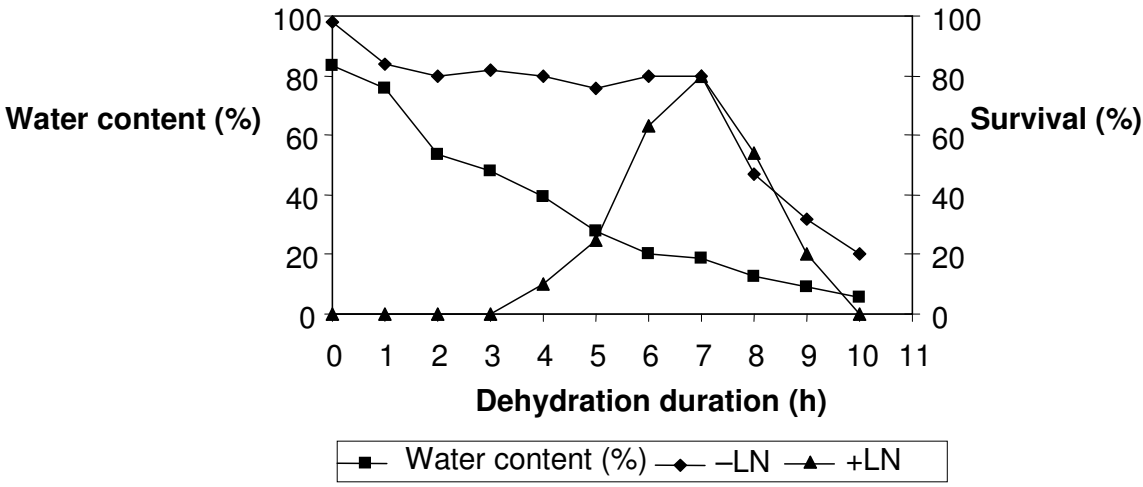

Fig. 3: Effect of desiccation duration (h) on the water content (%, FWB) and the survival (%) of control (-LN) and cryopreserved (+LN) *O. bifolium* protocorms.

Survival of protocorms after control treatments (C1-C5) was very high, ranging between 92.3 (C4) and 95.6% (C2) (Table 2). However, the percentage of protocorms forming plantlets was much lower, between 17.8 (C3) and 37.0% (C2). Freezing was lethal for protocorms after the same treatments as for seeds (T6-T9). Only when encapsulated protocorms were pretreated with sucrose and dehydrated before freezing (T10) did survival (81.7%) and plantlet formation (11.3%) occur.

Table 2: *O. bifolium* protocorm survival and protocorms forming plantlets (%± SEM) without cryopreservation (C1-C5) and with cryopreservation (T6-T10).

Treatments					Protocorm survival	Protocorms forming plantlets
Treatment (Nº)	Encapsulation	Sucrose pretreatment	Dehydration	LN	(%)	(%)
C1	-	-	-	-	94.6 (± 5.10)^{a}	24.7 (± 2.34)^{b}
C2	+	-	-	-	95.6 (± 4.23)^{a}	37 (± 3.2)^{a}
C3	+	+	-	-	94.4 (± 3.21)^{a}	17.8 (± 2.87)^{c}
C4	+	-	+	-	92.3 (± 4.75)^{a}	15.5 (± 2.34)^{c}
C5	+	+	+	-	95 (± 3.87)^{a}	18.3 (± 1.28)^{c}
T6	-	-	-	+	0^{c}	0^{e}
T7	+	-	-	+	0^{c}	0^{e}
T8	+	+	-	+	0^{c}	0^{e}
T9	+	-	+	+	0^{c}	0^{e}
T10	+	+	+	+	81.7 (±3.47)^{b}	11.3 (± 1.31)^{d}

Similar letters within columns mean no significant difference using Tukey's Studentized Range (MSD) test ($P < 0.01$).

DISCUSSION

For successful cryopreservation, it is essential to avoid lethal intracellular freezing, which occurs during cooling in liquid nitrogen (7). In our experiments, this was achieved by extending the dehydration duration, which resulted in a decrease in seed and protocorm MC and a concomitant increase in survival.

Preculture with a high concentration of sugar to increase desiccation and freezing tolerance has been reported to be critical for survival of cryopreserved tissues (4,16) including Orchids (21). By contrast, Wang *et al.* (19) found that another species of Orchids (*Dendrobium candidum*) was sensitive to high concentration of sucrose in an unexpected manner. In this work, we report a positive effect of sucrose on survival, of protocorms and plant production. However, the high sucrose concentrations employed during pre-treatment were diluted during post-treatment, thereby eliminating the toxic effect of high sucrose concentrations.

Encapsulation (C2) improved the *in vitro* response of seeds and protocorms in comparison with non-encapsulated seeds or protocorms (C1). A possible explanation for the positive effect of encapsulation on the percentage of explants forming plantlets could be a greater availability of mineral and hormonal nutrients (5). It is also possible that endogenous substances might be more slowly released to the medium because of the presence of the bead, thus remaining around the explants for a longer time and affecting favourably the *in vitro* response.

After cryopreservation, the percentage of seeds forming plants (4.8%) was lower than the values obtained in other orchids when seeds (17,19) were cryopreserved using the vitrification procedure; however, no survival was obtained when we tested the vitrification technique with *O. bifolium* seeds (unpublished results). The optimal survival of cryopreserved protocorms achieved in our study (81.7%) was similar to that reported for protocorms of *Dendrobium candidum* cryopreserved by vitrification (19).

Our results demonstrate for the first time the recovery of whole plants after cryopreservation of seeds and protocorms of *O. bifolium* by the encapsulation-dehydration technique. This cryogenic procedure does not require any special equipment and is simpler than vitrification with the material studied, i.e. minute explants. This report also opens up the possibility of recovering plants from seeds and protocorms from other *Oncidium* species after cryopreservation using the encapsulation/dehydration technique, and may be applicable to other species in the Orchidaceae.

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