

## CRYOPRESERVATION OF STRAWBERRY SHOOT TIPS BY ENCAPSULATION-DEHYDRATION

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**Abstract.** *In vitro* grown strawberry (*Fragaria x ananassa* Duch.) shoot tips were successfully cryopreserved using an encapsulation-dehydration procedure. Encapsulated shoot tips were precultured on MS medium supplemented with different sucrose concentrations (0.25, 0.5, 0.75 and 1.0 M) for 24 h and dehydrated (1 to 5 hours) in a sterile laminar air flow prior to direct immersion in liquid nitrogen (-196°C). A maximum of 63% regrowth of cryopreserved shoot tips was obtained following preculture in 0.75 M sucrose solution and 4 h dehydration. Plants produced from control and cryopreserved shoot tips were phenotypically similar.

**Key words:** conservation, dehydration, *Fragaria*, liquid nitrogen, shoot tip

### INTRODUCTION

The recent progress in cryogenic technology is attracting much attention because of its significance as a safe approach to the preservation of plant biodiversity and promising results have been obtained in the last two decades for numerous crops, woody, ornamental and medicinal plants (Engelmann, 2004; Panis and Lambardi, 2005). It is essential to underline the complementarity of *in vitro* techniques with other strategies of plant genetic resources conservation. *In vitro* conservation does not intend to replace conventional approaches for *in situ* and *ex situ* conservation.

Cryopreservation is an attractive alternative for the storage of plant germplasm, consisting in the conservation of plant material (shoot tips, meristems, cells, somatic and zygotic embryos) at ultra-low temperature, in general the temperature of liquid nitrogen (-196°C). At this temperature cell divisions and all other biological activities are completely arrested. The transfer of cells from room temperature to -196°C must be done in such a way that the viability of the stored material is retained, so that their biological functions and growth can be reactivated after thawing and transfer to the regrowth medium (Towill, 1991).

Whilst some cryopreservation methods are dependent upon the use of expensive cryogenic facilities, recently developed techniques involve simplified procedures which permit plant material to be cryopreserved by direct immersion in liquid nitrogen. Among them is the encapsulation-dehydration procedure (Dereuddre et al., 1990). By using the encapsulation-dehydration protocol Clavero-Ramirez et al. (2005) obtained recovery rates between 23-63% depending on genotypes.

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pregrown in liquid medium enriched with sucrose, partially desiccated to a water content around 20% (on fresh weight basis), then frozen rapidly (Engelmann, 1997). The reduction of the water content of

synthetic seeds to a minimal level is a necessary step for a successful cryopreservation of the encapsulated shoot tips (Bouafia et al., 1996; González-Arno et al., 1996). This aim is achieved by combining the bead preculture in sucrose-containing medium with their dehydration in silica gel or under a sterile air flow.

## MATERIALS AND METHODS

*Plant material.* *In vitro* grown strawberry (*Fragaria x ananassa* Duch., cv. Regina) plants were selected for cryopreservation in this study. Stock cultures were cultured on Murashige and Skoog (1962) (MS) medium supplemented with 1 mg l<sup>-1</sup> thiamine, 1 mg l<sup>-1</sup> pyridoxine, 1 mg l<sup>-1</sup> nicotinic acid, 1 mg l<sup>-1</sup> 6-benzylaminopurine (BAP), 0.5 mg l<sup>-1</sup> naftalene-acetic acid (NAA), 0.08 M sucrose and 7 g l<sup>-1</sup> agar (Sigma) (noted S-medium). The pH was adjusted to 5.8 before autoclaving. The plants were grown at 24°C during a 16 h light photoperiod with a light intensity of 40 μmol m<sup>-2</sup> s<sup>-1</sup> photosynthetic active radiation (PAR) provided by cool white fluorescent tubes. For micro propagation, nodal segments consisting of a piece of stem about 10 mm in length with two leaves were transferred to S-medium and incubated as mentioned above. Subcultures of the plants were performed every 4 weeks.

*Explants.* Shoot tips with 2 to 4 leaf primordia were excised from 1-to 2-months old *in vitro* grown plants using two hypodermic needles under a stereo microscope in sterile conditions. Shoot tips were incubated over night at 24°C on sterile filter paper humidified with 2.5 ml of liquid S-medium in Petri dishes (5 cm diameter).

*Encapsulation, dehydration, cryopreservation.* The shoot tips were individually encapsulated in alginate beads by transferring them with a pipette from a 3% solution of sodium alginate in Ca<sup>2+</sup>-free MS to a solution of 1 M CaCl<sub>2</sub> plus MS mineral salts. After 20 min of gentle stirring, the beads (about 3 mm in diameter) were washed with MS medium (pH 5.8). The beads were then either (i) dehydrated for 1 to 5 h or (ii) incubated in MS medium containing various sucrose concentrations: 0.1 M, 0.25 M, 0.5 M, 0.75 M and 1,0 M for 24 h (and 48 h only for control shoot tips) on a rotary shaker (98 rpm) at 24°C. Subsequently the beads were desiccated in sterile laminar air flow. At 1-h intervals dehydrated beads were placed in 2 ml cryovials and directly immersed into liquid nitrogen contained in a 20 l Dewar flask. Samples remained in liquid nitrogen for at least 2 h. In a parallel experiment a drying curve was obtained by weighing ten beads each hour.

*Growth recovery after cryopreservation.* Rewarming of samples was performed in liquid MS medium at room temperature by transfer of the beads into 5 ml of the mentioned medium. Recovery of cryopreserved encapsulated shoot tips took place in Petri dishes (5 cm diameter) on a modified S-medium (with 3 g/l agar) under standard illumination conditions. Encapsulated and dehydrated but not frozen explants were used as control.

*Analysis of results.* Shoot formation was assessed 30 days after thawing. For evaluation of the regrowth level after cryopreservation, only direct shoot regeneration was considered. A number between 10 and 12 shoot tips were used for each of the three replications per treatment. The results were expressed as the mean ± standard error (SEM).

## RESULTS AND DISCUSSION

### *Fresh weight of beads*

After excision the shoot tips were precultured for 24 h at 24°C in liquid MS medium, encapsulated in alginate beads, dehydrated in laminar air flow and then directly plated on semi-solid culture medium. Figure 1, compares the fresh weight of control and dehydrated

beads as a result of sucrose molarity in the preculture medium. Before dehydration the fresh weight of the beads increased with the increase of sucrose concentration in the preculture medium. Following drying pretreated beads, especially those pretreated with higher sucrose concentrations (0.75 M and 1.0 M) lost water at lower rates than the non pretreated beads. These results indicate that during pretreatment there was an uptake of sucrose into the alginate beads with a concomitant reduction in water content.

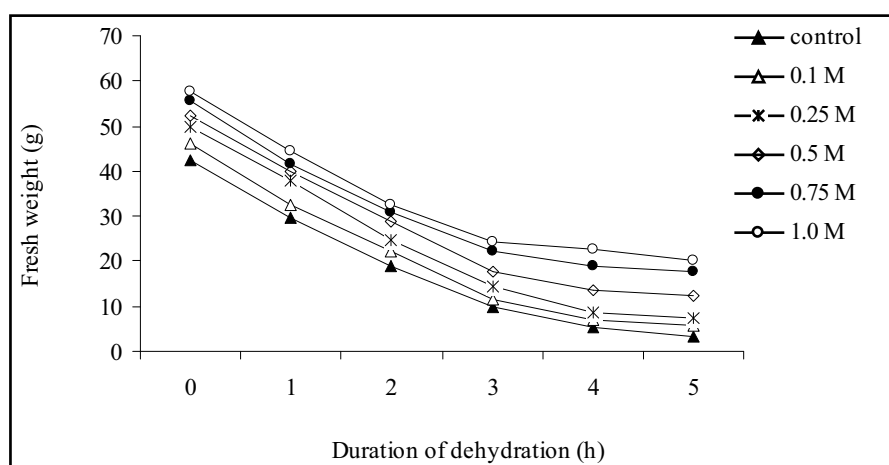


Figure 1. Effects of various dehydration times in laminar air flow on the encapsulated shoot tips following 24 h pretreatment in sucrose (each point is the mean of ten beads)

*Effects of encapsulation and preculture on control shoot tips*

In a preliminary experiment following excision the shoot tips were encapsulated in alginate beads, precultured in medium containing various sucrose concentrations (0.25 M, 0.5 M, 0.75 M and 1.0 M) for 24 h and 48 h and plated on the modified S-medium. The shoot tips resumed growth within 7 days after plating and developed normal shoots without callus formation within 15 days. The preculture in sucrose was tested in order to improve survival after dehydration and storage in liquid nitrogen. As shown in Table 1, the encapsulation and preculture in sucrose for 24 h did not influence survival.

Table 1

Shoot formation from encapsulated and precultured (24h and 48 h) non-frozen (control) shoot tips of strawberry

Duration of preculture (h)	Shoot formation (% ± SE)			
	Sucrose concentration (M)			
	0.25	0.5	0.75	1.0
24	93.3 ± 0.57	90.0 ± 1.73	86.6 ± 1.52	86.6 ± 1.15
48	76.6 ± 2.08	73.3 ± 1.52	56.6 ± 2.51	40.0 ± 2.64

The shoot formation following a 24 h preculture of synthetic seeds in liquid MS medium containing various sucrose concentrations was higher than the shoot formation following 48 h preculture.

As shown in Table 1, the percentages of shoot formation after 48 h of preculture were situated between 40% after preculture in 1.0 M sucrose and respectively 76% after preculture in 0.25 M sucrose. The extension of preculture to 48 h was detrimental for the shoot regrowth, therefore for the following experiments only the 24 h preculture was used.

*Effects of encapsulation, preculture and dehydration on cryopreserved shoot tips*

The highest percentage of shoot formation (63%) was obtained following the combination of preculture in 0.75 M sucrose containing medium with 4 hours of dehydration under the sterile air of a laminar flow hood (Figure 2C, Figure 3).

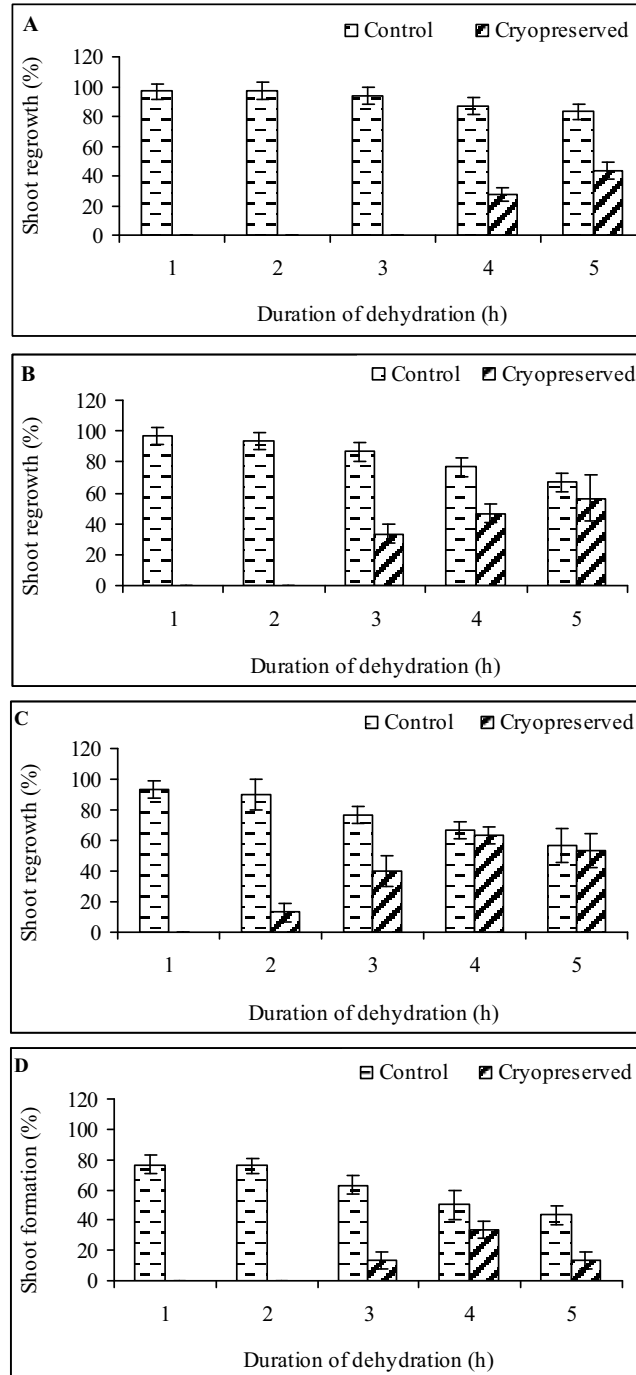


Figure 2, A-D. Shoot formation from strawberry shoot tips, following a 24 h preculture of synthetic seeds in liquid MS medium containing sucrose, various times of dehydration under sterile laminar air flow, direct immersion in liquid nitrogen, thawing and plating on semi-solid culture medium.

A) preculture in 0.25 M sucrose; B) preculture in 0.5 M sucrose; C) preculture in 0.75 M sucrose; D) preculture in 1.0 M sucrose; (bars represents standard errors).

When this dehydration time followed the preculture of synthetic seeds in the medium with sucrose in higher concentration (1.0 M), lower percentage of shoot formation (33%) was achieved (Figure 2D). The extension of the dehydration time to 5 hours lead to a shoot regrowth of 43% following preculture in 0.25 M sucrose and 56% following preculture in 0.5 M sucrose.

A dehydration time for 1 to 3 h was not effective to induce cell dehydration and to promote subsequent shoot regeneration. In this case the highest percentage of shoot formation was 40% after preculture in 0.75 M sucrose and 3 h of dehydration (Figure 2C). These observations lead to the conclusion that, with the combination of 0.75 M sucrose preculture and 4 h dehydration an optimum of cell dehydration of strawberry shoot tips was reached in order to achieve maximum survival after cryopreservation.

It is well known that sucrose in addition to its osmotic effect is able during preculturing to permeate into the cells in large quantity (Finkle et al., 1985). The accumulation of sugar within the tissue is associated with freezing tolerance but it is not a guarantee for the viability of the tissue after cryopreservation (González-Arno et al., 1996).



Figure 3. Shoot development from encapsulated shoot tips after cryopreservation.

## CONCLUSIONS

The present study demonstrates that the combined encapsulation-dehydration method can be efficiently utilized to cryopreserve strawberry shoot tips and to promote their subsequent regeneration. This method could be applicable to the long-term preservation in liquid nitrogen of *Fragaria* germplasm. Moreover, the procedure is easy to perform and there is no need for sophisticated equipment. No morphological abnormalities were observed in the plants developed from cryopreserved shoot tips by encapsulation-dehydration. The results shown that resistance to freezing could be induced by 24 h preculture of the beads in medium with 0.75 M sucrose followed by a 4 h of dehydration.

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## REZUMAT

### CRIOCONSERVAREA APEXURILOR DE CĂPȘUN PRIN ÎNCAPSULARE-DESHIDRATARE

Plante de căpșun (*Fragaria x ananassa* Duch.) *in vitro* au fost crioconservate cu succes prin metoda de încapsulare-deshidratare. Apexurile încapsulate în gel de alginat au fost supuse unor tratamente prealabile crioconservării. Aceste tratamente au constat din incubarea apexurilor încapsulate în soluții de zaharoză de diferite concentrații (0,25, 0,5, 0,75 și 1,0 M) timp de 24 h urmate de deshidratare în flux laminar de aer steril timp de 1 până la 5 h după care au fost imersate în azot lichid (-196°C). Cel mai ridicat procent de regenerare după crioconservare, de 63% a fost obținut ca urmare a unei preculturi în 0,75 M zaharoză și deshidratare în flux laminar de aer steril timp de 4 h. Plantele regenerate din apexurile control și crioconservate nu au prezentat diferențe fenotipice.