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CRYOPRESERVATION OF REDWOOD (*SEQUOIA SEMPERVIRENS* (D. DON.) ENDL.) SHOOT APICES BY ENCAPSULATION-DEHYDRATION

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Abstract: Shoot tips excised from *in vitro* redwood (*Sequoia sempervirens* (D. Don) Endl.) plants were successfully cryopreserved by encapsulation-dehydration and subsequently regenerated shoots. The effects of osmoprotection and dehydration duration on regrowth of cryopreserved shoot tips were studied. The shoot tips (3–4 mm in length) were coated in alginate beads, osmoprotected and dehydrated (up to 6 h) under laminar air-flow at 24ºC prior to a direct plunge in liquid nitrogen (-196°C). After rewarming in a water bath at 38ºC for 2 min, encapsulated shoot tips were plated on a semisolid (5.5 g L^{-1} agar) medium supplemented with 1.0 mg L^{-1} 6-benzylaminopurine, 0.25 mg L^{-1} indole-3-butyric acid and 30 g L^{-1} sucrose for regrowth. Cryopreserved shoot tips resumed growth within 30 days and regenerated shoots within 45 days. The highest regrowth level of encapsulated apices after cryopreservation was 67% following 24 h osmoprotection in 0.5 M sucrose, 3 h dehydration and 27% moisture content of the beads.

Keywords: Encapsulation-dehydration, *Sequoia*, Shoot tips

Introduction

Redwood (*Sequoia sempervirens* (D. Don) Endl.), also called Coast Redwood to distinguish it from Giant Redwood (*Sequoidendron giganteum* (Lindley) Buchholz, of the Sierra Nevada, California) is a vulnerable tree species native to the central and northern California coast (IUCN Red List of Threatened Species, http://www.iucnredlist.org). Other areas of successful cultivation outside of the native range include Western Europe, New Zealand, Mexico and the south-eastern USA [1]. Redwood reproduces both sexually and asexually. Seed production begins at 10–15 years of age and large seed crops occur frequently but the viability of the seeds is low, below 15% [1]. Using seeds for the storage of plant germplasm is not always practical [2, 3]. Traditionally, genetic resources conservation for woody species of high economic value involves field collections [4]. This form of germplasm preservation requires large spaces, is expensive and carries the risk of losses due to biotic or abiotic stresses [5].

Cryopreservation, the storage of living cells and tissues at ultra-low temperatures in liquid nitrogen (-196°C), is now applied to a diverse range of plant species and tissues [6, 7]. Cryopreservation which completely arrests cell divisions and metabolism has been considered as an ideal means for the long-term storage of plant germplasm [8, 9] and it is useful to maintain woody species in a juvenile status [10].

Micro-propagation of tree material in combination with cryopreservation provides several major advantages for silviculture, as well as for research applications. It allows the effective multiplication of elite genotypes, propagation throughout the year and the possibility to produce pathogen-free plants [11]. Among the most interesting for practical applications, the following studies can be mentioned: biomass production and woody detritus in a coast redwood forest [12]; the establishment of an effective system of somatic embryogenesis and organogenesis from *in vitro* needles of redwood [13]; direct shoot organogenesis from needles of three genotypes of *Sequoia* [14]. Regarding woody species, cryopreservation of has been mostly applied to fruit

trees [15, 16] and only for a reduced number of forest woody species such as oaks (*Quercus*) [17], silver birch (*Betula pendula*) [18], *Eucalyptus* [10, 19], European elms (*Ulmus*) [20], *Pinus* [21] or *Ginkgo* [22].

Methods involving alginate encapsulation and dehydration have been used in many cryopreservation protocols for a wide variety of plant germplasm since their development by Fabre & Dereuddre [23]. During rapid cooling in liquid nitrogen, ice crystals are formed in cells. Thus, prior to freezing, free water in cells needs to be reduced to avoid crystallization [24]. Encapsulation-dehydration is based on the desiccation of alginate-coated plant material (meristems, somatic embryos, cell suspensions) in the presence of sucrose, followed by evaporative dehydration performed either under laminar air-flow or over silica gel [25]. This method was applied for producing synthetic seeds by encapsulation of explants in calcium alginate beads [26]. The encapsulation-dehydration method requires osmotic conditioning in high concentrations of sucrose. Encapsulation of the explants allows exposure to extreme treatments including pre-culture with high sucrose concentrations and desiccation to low moisture contents that could be damaging for non-encapsulated tissue. Data from literature suggest that successful cryopreservation is dependent on the nature of the plant sample, its water status and the protocol applied.

The aim of the present study was to determine the most suitable conditions that resulted in successful cryopreservation for redwood shoot tips.

Materials and Methods

Plant material and micro-propagation

In vitro-grown redwood (*Sequoia sempervirens* D. Don. (Endl.) plants were used in this study for cryopreservation using the encapsulation-dehydration method. The plants were multiplied by monthly subculture of nodal stem segments (1.5 cm in length). Stock cultures were grown in glass flasks on 20 ml sterilized basal Murashige and Skoog (MS) [27] medium supplemented with 1.0 mg L^{-1} 6-benzylaminopurine (BAP), 0.25 mg l^{-1} indole-3-butyric acid (IBA) and 30 g L^{-1} sucrose. The pH was adjusted to 5.7 and the medium was solidified with 7.5 g L^{-1} agar before autoclaving at 121ºC for 20 minutes. The plants were grown at 24°C during a 16 h light / 24 h photoperiod with a light intensity of 34 µmol m^{-2} s^{-1} provided by cool white fluorescent tubes.

Explants

Shoot tips (2–3 mm in length) consisting of the apical meristem covered by 2–3 leaf primordia were excised from 2 month-old *in vitro* mother plants under a stereo-microscope in sterile conditions.

Encapsulation and osmoprotection

The excised apices were individually encapsulated in alginate beads by transferring them from a solution of 3% sodium alginate (250 cps) in Ca^{2+}-free MS to a solution of 100 mM $CaCl_2$ plus MS mineral salts, where they remained for 20 minutes. After 20 min polymerization the beads (approximately 5–6 mm in diameter) were washed with liquid MS medium (pH 5.7). To increase the tolerance to liquid nitrogen, encapsulated shoot tips were incubated in MS medium containing sucrose in various concentrations (0.25, 0.5, 0.75 and 1.0 M) for 24 and 48 h on a rotary shaker (98 rpm) at 24°C.

Dehydration and cryopreservation

Osmoprotected beads were then dehydrated in a laminar air-flow for up to 6 hours. During desiccation the environmental conditions of the laboratory were monitored for temperature (24±1°C) and relative humidity (RH 65±4%). At 1-hourly intervals dehydrated beads were placed in 2 ml cryovials (10 beads/cryovial) and immersed in liquid nitrogen (LN) at -196°C contained in a 25 L Dewar flask. Cryotubes remained in liquid nitrogen for 24 h.

Moisture content of the beads

The moisture content of samples was determined as follows: the fresh weight of 10 beads/treatment was measured after 24 h sucrose osmoprotection and dehydration. For determination of the dry weight the beads were oven-dried at 60°C until constant weight was attained. Moisture content of the beads on each treatment was calculated from these values and expressed as the percentage of moisture content over fresh weight. Beads were weighed singly on an analytical balance. Moisture content determinations were related to the entire bead. Controls consisted of beads which were not osmoprotected in sucrose and were not dehydrated.

Growth recovery of shoot tips following cryopreservation

Thawing was performed by immersion of cryovials in a water bath at 38ºC for 2 minutes. Regrowing of shoot tips was performed on a semisolid medium with reduced (5.5 g L^{-1}) agar content under the same light and temperature conditions as mentioned for micro-propagation.

Statistical analysis of results

Survival of shoot tips was evaluated 45 days after liquid nitrogen treatment and was expressed as the percentage of single apices that developed into shoots. Encapsulated explants treated with sucrose and dehydrated but not frozen were used as a control. Between 13 and 15 shoot tips were used for each of the three replicates per treatment. The results were expressed as the mean ± standard error (SE). Data were analysed by ANOVA using the Tukey test for data comparison.

Results and Discussion

In a preliminary experiment the regrowth capacity of non-encapsulated *Sequoia* shoot tips was determined. After 4–5 days the excised shoot tips started to grow and within two weeks shoot formation was 93% (data not shown). Similarly, encapsulation did not influence survival and regrowth, although shoot growth was slower (data not shown).

Effects of osmoprotection and dehydration duration on regrowth of shoot tips

The benefit of osmoprotection for 24 and 48 h of encapsulated (control and cryopreserved) shoot tips was examined and the effects on the regrowth of shoots are shown in Fig. 1a, b. A progressive decrease in regeneration of non-cryopreserved shoot tips with increasing sucrose concentrations was noted for both of the tested sucrose treatments, 24 h and 48 h. Treatment with sucrose for 48 h does not always significantly reduce the number of regrowing shoots but a sucrose concentration of 1.0 M decreased significantly the number of non-cryopreserved shoot tips. This can be explained by the fact that high sucrose concentrations resulted in excessive cell dehydration with consequences on the viability of explants. The sucrose concentration exerted a strong effect on the shoot regrowth especially of the cryopreserved shoot tips. When cryopreservation followed osmoprotection low shoot formation rate (23% for 24 h osmoprotection in 0.5 M sucrose and 11% for 48 h osmoprotection in 0.25 M sucrose) was achieved (Fig. 1a, b). For the other sucrose concentrations the regrowth level was even lower to none depending on the treatment duration.

This experiment, however, shows that osmoprotection in sucrose without additional dehydration did not induce tolerance of shoot tips against liquid nitrogen. Following osmotic dehydration, extensive evaporative dehydration is necessary for encapsulated shoot tips to reach a moisture content compatible with cryopreservation [28]. High levels of desiccation tolerance have been induced with osmoticum treatment by a single sucrose treatment [29] or by successive pre-culturing in a medium containing increasing sucrose concentration (0.1 – 1.0 M) [30]. In cryopreservation protocols using dehydration, survival was increased by encapsulation of the sample, as for oilseed rape [31] or apple [32]. In dehydration-cryopreservation methods it is important to induce a certain level of desiccation tolerance in the cells [33]. It was demonstrated that sucrose was efficient for the induction of tolerance to dehydration in *Pelargonium* meristems [28]. However, some species are sensitive to the high sucrose concentrations and require

progressive sucrose treatments [34, 19]. Pre-culture was made overnight on solid medium [35] or for one to several days on a solid medium with a high sucrose concentration [36, 37, 38].

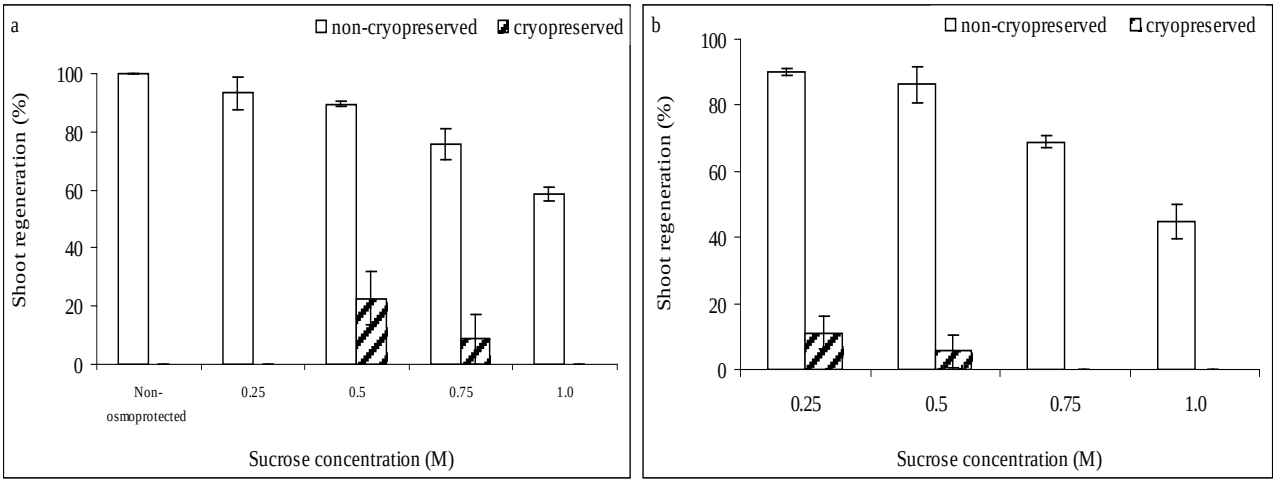

Fig. 1: Regeneration of encapsulated shoot tips following sucrose treatment. Vertical bars represent SE.

The effects of osmoprotection and dehydration duration on regrowth of control shoot tips can offer information about their efficiency in the induction of tolerance to low temperatures. Longer dehydration duration (6 h) and higher sucrose concentrations (0.75 M and 1.0 M) had a generally negative effect on the survival of shoot tips and lower recovery levels were obtained for both of the treatment periods examined (Fig. 2a, b). A 6 h dehydration period resulted in no regeneration when the sucrose treatment was performed in 1.0 M sucrose for 24 or 48 h. It was reported that sucrose (0.5-0.75 M) in culture medium for 4 days increased survival of alfalfa encapsulated cells [39]. Due to desiccation of explants most or all freezable water is removed from cells and vitrification of internal solutes takes place during rapid exposure to liquid nitrogen, avoiding lethal intracellular ice crystallization [8].

As a consequence, a large part of the frozen explant is kept intact after rewarming, which results in high survival, rapid and direct regrowth after cryopreservation [40].

Tolerance of encapsulated shoot tips to cryopreservation

Fig. 3 presents the moisture content of the beads following a 24 h osmoprotection in sucrose and dehydration of up to 6 h in laminar air-flow. The moisture content of beads decreased (without dehydration) with increasing sucrose concentration from 90% in beads treated with 0.25 M sucrose to 61% in beads treated with 1.0 M sucrose. The decrease in water content of the beads before dehydration seemed to indicate that water loss was accompanied by an uptake of sucrose by the beads. Shoot development from cryopreserved shoot tips (osmoprotected with 0.5 M sucrose for 24 h) increased up to 3 h dehydration (67% regrowth) when the moisture content of the beads was 27% (Fig. 4). When a 3 h dehydration time followed the osmoprotection in 0.75 M sucrose for 24 h not significantly lower percentages (58%) of shoot formation were achieved (Fig. 5a). The prolonged osmoprotection period (48 h) had a negative effect on shoot regeneration after cryopreservation. The application of sucrose treatment reduced the water content prior cooling and enhanced post-cryopreservation survival.

The use of 0.5 M sucrose treatment has been shown to be effective for shoot regeneration after cryopreservation also for other species [41, 42]. Thermal analysis of alginate beads showed that the process of removing the fraction of water that would freeze is dependent upon the initial content of water content, type of plant material and its mass related to the bead volume [43]. The water content of alginate beads was critical for survival after cryopreservation for apple shoot tips, when survival after exposure to liquid nitrogen was attained with beads having a water content of 25% [30] or between 25% and 35% for cork oak beads [44].

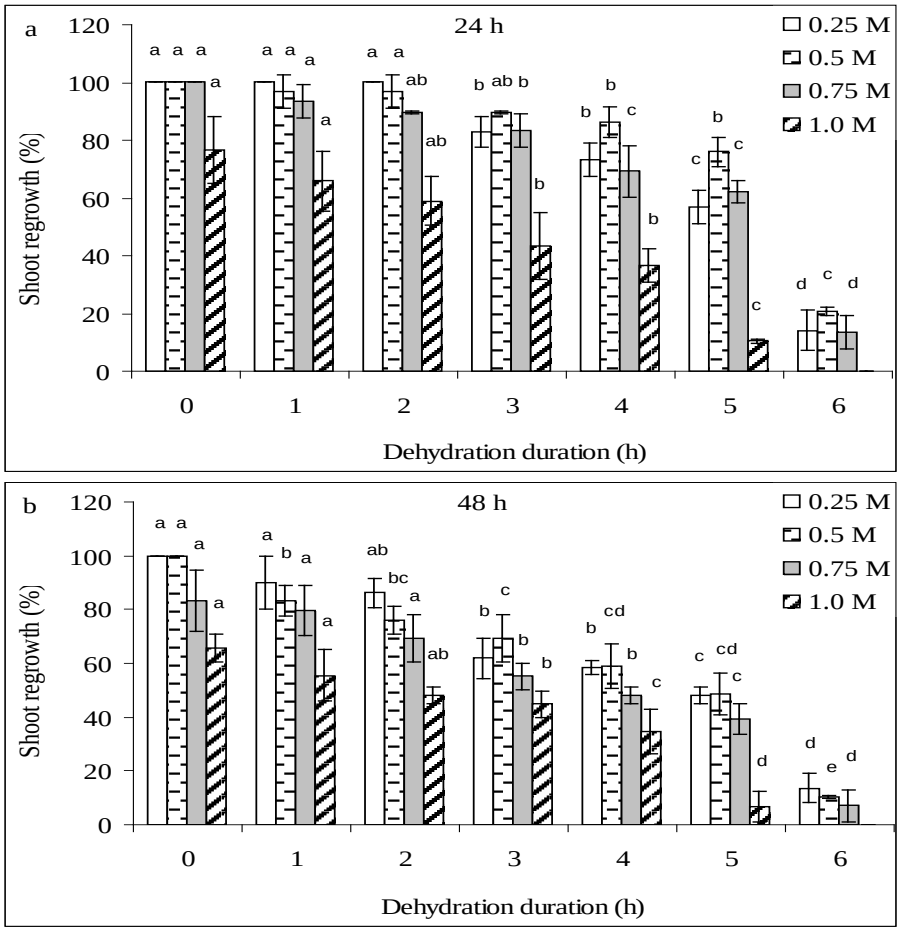

Fig. 2: Effect of dehydration duration on shoot formation from osmoprotected, dehydrated, non-cryopreserved shoot tips. Vertical bars represent SE.

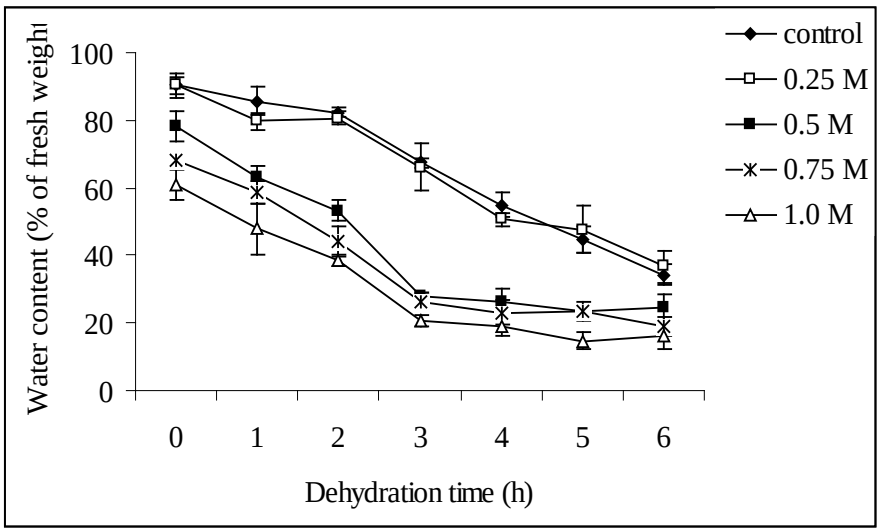

Fig. 3. Effect of various dehydration times on the moisture content of the beads. Vertical bars represent SE.

By combining osmoprotection of the beads in sucrose-containing media with their dehydration a reduction of the water content of alginate beads was achieved to a level which allowed shoot regrowth following cryopreservation. When sucrose concentration was stepwise,

100% of encapsulated *Cichorium* shoot tips resumed growth [45] and an increment in survival of *Coffea* embryos was reported [46]. Sucrose treatment and dehydration were essential to achieve glass transition during cooling [47] and a direct relationship between the formation of a glassy state and survival after cryopreservation was noted [48]. Due to the extreme dehydration of explants most freezable water is removed from cells, and vitrification of internal solutes takes place during rapid exposure to liquid nitrogen avoiding lethal intracellular ice crystallization [8].

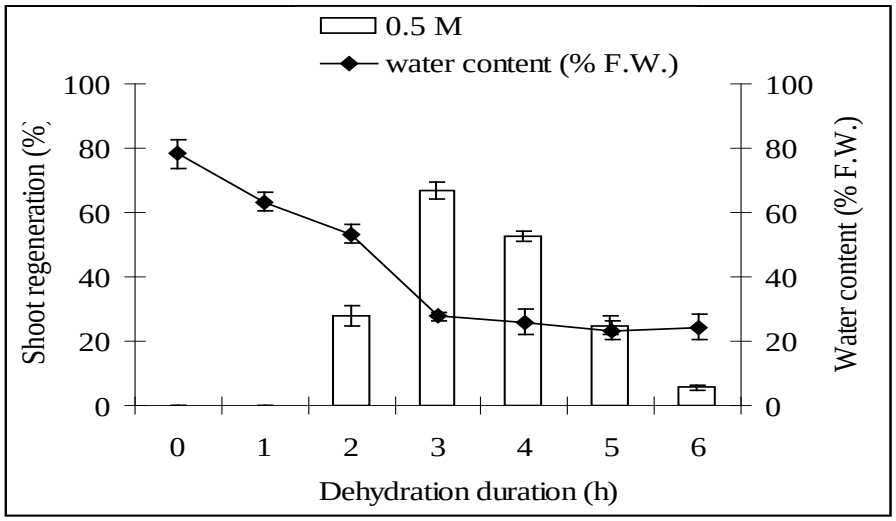

Fig. 4. Effect of water content on shoot formation of alginate-coated shoot tips. Vertical bars represent SE.

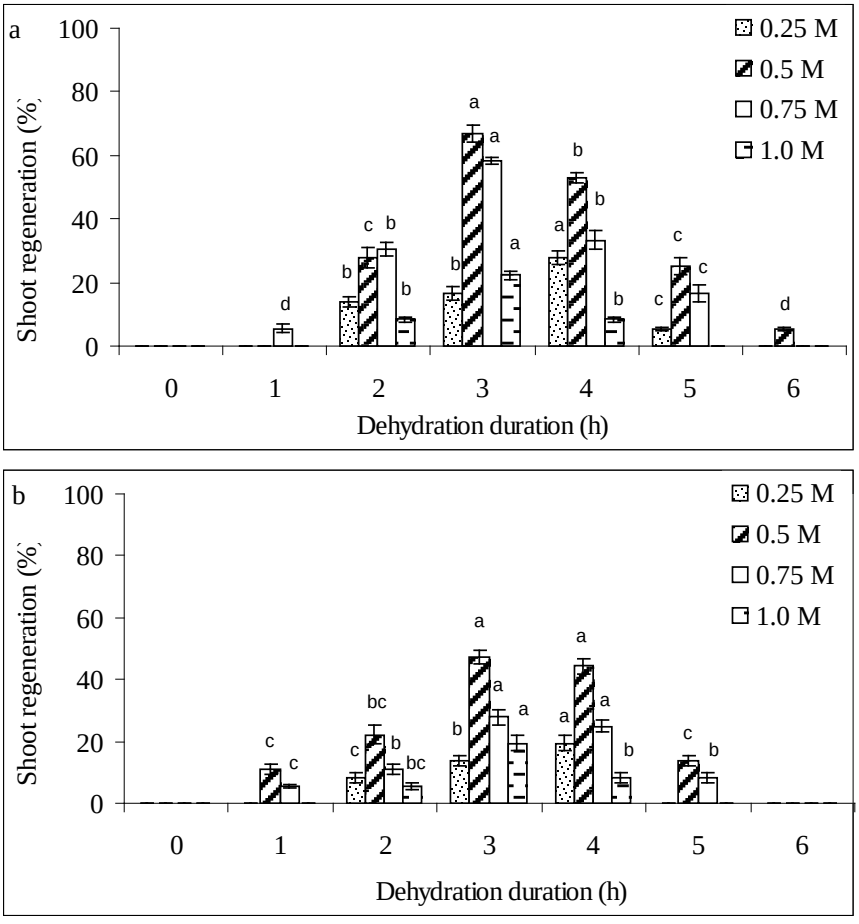

Fig. 5: Effect of osmoprotection 24 h (a) and 48 (b) and dehydration on shoot formation of encapsulated shoot tips after liquid nitrogen exposure. Vertical bars represent SE.

The success of encapsulation-dehydration for cryopreservation of *Sequoia* shoot tips depends on lowering the water content to as low a level as is possible that can sustain cell viability. In order to elucidate the complex mechanism involved in the acquisition of tolerance to freezing in liquid nitrogen and the processes involved in the role of sucrose further research is needed. These results indicate that *Sequoia* shoot tips can withstand deep-freezing in liquid nitrogen and the shoot formation could perhaps be improved by modification of the procedure and further experimentation.

Conclusions

The studies performed demonstrate that the encapsulation-dehydration method can be used as an effective and useful technique for the cryopreservation of *Sequoia* shoot tips and to promote their high regeneration.

In order to elucidate the complex mechanism involved in the acquisition of tolerance to freezing in liquid nitrogen and the processes involved in the role of sucrose further research is needed.

The results led to the conclusion that the ability of *Sequoia* shoot tips to tolerate cooling in liquid nitrogen and to regenerate whole plants after cryopreservation depends upon the preculture conditions.

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CRIOCONSERVAREA APEXURILOR DE *SEQUOIA SEMPERVIRENS* (D. DON.) ENDL.) PRIN ÎNCAPSULARE-DESHIDRATARE

(Rezumat)

Apexuri caulinare de *Sequoia* au fost crioconservate prin metoda de încapsulare-deshidratare. Apexurile (3-4 mm în lungime) au fost încapsulate în bile de alginat, incubate (24 şi 48 h) în soluţii de zaharoză de diferite concentraţii (0,25, 0,5, 0,75, şi 1,0 M) şi deshidratate (între 0-6 h) în flux laminar de aer steril. Congelarea s-a realizat prin imersia directă a bilelor de alginat în azot lichid (-196°C). Decongelarea s-a realizat după 24 h in baie de apă la temperatura de 38°C timp de 2 min iar regenerarea după crioconservare s-a realizat pe mediu de cultură semisolid (5,5 g l^{-1} agar) cu adaos de 1,0 mg l^{-1} 6-benzilaminopurină, 0.25 mg l^{-1} acid 3-indolilbutiric şi 30 g l^{-1} zaharoză.

Cel mai ridicat procent de regenerare a apexurilor încapsulate după crioconservare a fost de 67% în urma incubării timp de 24 h în soluţie de zaharoză de 0,5 M şi deshidratare timp de 3 h în flux laminar de aer steril.

Studiile efectuate au arătat că metoda încapsulării-deshidratării poate fi utilizată ca metodă eficientă în tehnica conservării pe termen lung a speciei *Sequoia sempervirens*.

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