Contribuţii Botanice, XLIII, **2008**
Grădina Botanică "Alexandru Borza"
Cluj-Napoca

THE EFFECTS OF PRECULTURE ON PLANT REGENERATION FROM CRYOPRESERVED SHOOT TIPS OF REDWOOD (*Sequoia sempervirens* (D. Don.) Endl.)

Adela HALMÁGYI
Biological Research Institute, 48, Republicii str., **RO-400015 Cluj-Napoca, Romania**
e-mail: adela_halmagyi@yahoo.com

Abstract: The aim of the present study was to investigate the importance of the preculture conditions on the survival of redwood (*Sequoia sempervirens* (D. Don.) Endl.) **s**hoot tips cryopreserved using a combined droplet-vitrification procedure. In vitro grown *Sequoia* plants were used for cryopreservation. Preculture was critical for the survival of shoot tips after cryopreservation. Among the different tested sugars and sugar alcohols (glucose, sucrose, mannitol and sorbitol), treatment with 0.5 M sucrose for 24 h was the most efficient one. One-step vitrification protocol with PVS2 for 30 minutes was efficient for shoot regeneration following cryopreservation. After rapid thawing and transfer of shoot tips on recovery medium, 67% growth recovery was observed. Plants produced from control and cryopreserved shoot tips were phenotypically similar.

Key words: cryopreservation, droplet-vitrification, *Sequoia*

Introduction

The continuous loss and degradation of important plant genetic resources having as effect the diminuation of the biodiversity led to the conservation of plant germplasm [5]. As the maintenance of field gene banks is problematic and costly, in vitro conservation is increasingly being considered as a safer, more practical option [1]. Cryopreservation in liquid nitrogen (LN) therefore offers great potential for the long-term conservation of plant germplasm. Cryopreservation techniques which promise a high genetic stability and long-term storage of plant material have been developed for many species [4]. Cryopreservation has an important role in conservation strategies for woody species. Among woody species white spruce and interior spruce [8], silver birch [9] or pinus [6] were successfully cryopreserved until now. Vitrification protocols have been first used to crypreserve shoot apices [10] and it has become the preferred method for cryopreservation over the last decade, for more than 100 species and cultivars [10].

In the present study the optimal preculture conditions for successful cryopreservation of *Sequoia* shoot tips using a combined droplet-vitrification method were determined.

Materials and Methods

Plant material and micropropagation

In vitro grown redwood (*Sequoia sempervirens* D. Don. (Endl.) plants were selected in this study for cryopreservation using the droplet-vitrification method. The plants were multiplied by montly subculturing of nodal stem segments (approx. 1.5 cm in length). Stock cultures were grown in Erlenmeyer flasks on 25 ml sterilized basal MS [7] medium, supplemented with 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 0.1 mg l^{-1} indole-1-acetic acid (IAA) and 30 g l^{-1} sucrose (noted S medium). The pH was adjusted to 5.7 and the medium was solidified with 7.5 g l^{-1} agar before autoclaving at 121ºC. 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. For plant regeneration following cryopreservation a modified S medium with reduced agar (4 g l^{-1}) content was used (noted S1).

Explants and preculture

Shoot tips (1-2 mm in length) with 2 to 3 leaf primordia were excised from 1-to 2-months old axillary shoots using two hypodermic needles under a stereo microscope in sterile conditions. Shoot tips were transferred to MS liquid basal medium enriched with 0.5 M glucose, sucrose mannitol or sorbitol. Sterile filter paper were placed in Petri dishes (5 mm diameter) and were soaked with 2.5 ml of MS medium supplemented with sugars and sugar alcohols mentioned above. The incubation took place at 24°C for 24 h and 48 h.

Vitrification and cryopreservation

Following preculture for 24 h or 48 h in sugar and sugar alcohol solutions, shoot tips were dehydrated in a 4 µl droplet of plant vitrification solution - PVS2 [11] placed on sterilized aluminium foil strips (0.5 x 2 cm in length) for 10-30 minutes at room temperature in sterile conditions. After dehydration with the vitrification solution the aluminium foils with shoot tips were transferred directly in liquid nitrogen (–196°C) contained in a 10 l Dewar flask. Samples remained in LN for 24 h.

Growth recovery after cryopreservation

Thawing of the samples was performed in liquid MS medium at room temperature. Recovery of cryopreserved shoot tips took place on semisolid (with 5 g/l agar) S medium. 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 deviation (s.d.). Data were analysed by ANOVA using the Tukey test for data comparison.

Root formation medium

Shoots (approx. 3-4 cm in length) regenerated from cryopreserved shoot tips were used for rooting and were transferred to MS medium supplemented with 1-naphtyl-acetic acid (NAA) in different concentrations (0.1, 0.25, 0.5, 1.0, 1.5 and 2 mg l^{-1}) to improve rooting. After 1 month in culture, the percentage of root formation, the number and length of the formed roots were recorded.

Results and Disscusion

Effect of preculture medium on the survival of cryopreserved shoot tips

The results obtained after preculture in sucrose, glucose, mannitol and sorbitol were variable. It was possible to obtain high survival rates, 64% after 24 h preculture and 42% after 48 h preculture in sucrose and dehydration treatment with PVS2, which indicated that preculture in sucrose protected the cells against freezing in liquid nitrogen (Table 1).

Table 1: Effect of preculture in 0.5 M sugars and sugar alcohols on regrowth of non-cryopreserved (control) and cryopreserved shoot tips

Shoot tips	Preculture duration (h)	Shoot regeneration (%±s.d.)			
		sucrose	mannitol	sorbitol	glucose
non-cryopreserved	24	97.7±0,5[a]	93.3±1.0[a]	82.2±1,1[ab]	53.3±1.7[b]
	48	88.8±0.5[a]	77.7±1.5[ab]	60.0±2.0[b]	35.5±1.1[c]
cryopreserved	24	64.4±1.5[a]	40.0±3.4[b]	28.8±1.5[c]	22.2±1.5[c]
	48	42.2±2.3[a]	33.3±3.4[ab]	31.1±2.0[ab]	17.7±2.8[b]

Values followed by the same letter in the same row are not significantly different at p=0.05.

The lowest regeneration percentages for both non-cryopreserved and cryopreserved shoot tips were obtained when glucose was used in the preculture medium (Table 1).

It is known that sucrose stabilized membranes and proteins during dehydration [2]. It is possible that the cells may absorb sugars with different rates during preculture and may have different quantities of crystallizable water. It was demonstrated that sucrose was efficient for the induction of tolerance to dehydration of meristems [3]. Additional experiments including measurement of sucrose uptake during pregrowth for various durations and the analysis of

thermal behaviour of apices during the freeze/thaw cycle may contribute to explain the differences in survival after cryopreservation.

Effect of vitrification solution on the survival of cryopreserved shoot tips

After the preculture with different sugars and sugar alcohols described above, the cell content was sufficiently concentrated to tolerate the exposure to cryoprotective solutions for a more marked dehydration.

In the case of cryopreservation by vitrification it is important to determine the toxicity of the cryoprotective mixture intending to ensure cellular vitrification, without endocellular crystallization, at the time of the immersion of tissues in liquid nitrogen, as well as to find the optimal time for the exposure of the plant material to this mixtures. In order to find the optimal time for the vitrification of *Sequoia* shoot tips different exposure durations were tested (Table 2).

Table 2: Effect of the cryoprotective treatment duration on regrowth of non-cryopreserved and cryopreserved shoot tips

Duration of treatment (min.)	Shoot regeneration (%±s.d.)	
	non-cryopreserved	cryopreserved
0	98±0.5^{a}	0
10	93±1.0^{a}	11±1.5^{c}
15	89±1.1^{a}	15±2.5^{c}
20	84±1.5^{ab}	47±2.0^{b}
25	80±2.6^{b}	60±1.7^{a}
30	82±0.5^{b}	67±1.7^{a}
40	75±2.0^{bc}	42±3.7^{b}

Values followed by the same letter in the same column are not significantly different at the 0.05 probability level.

The regeneration rate of non-cryopreserved shoot tips was higher than 75% regardless of the treatment duration (Table 2). The highest regrowth rate (67%) following cryopreservation was found for a 30 min. treatment duration. The fact that the best results regarding shoot regeneration following cryopreservation were obtained using high cryoprotective mixture concentrations was emphasized by Yamada and Steponkus [13, 12]. The authors considered that as much as 90% of the water responding to osmotic dehydration can be eliminated by this treatment. The combined vitrification-droplet method has the advantage that practical application and handling seem to be easier than for other methods.

Rooting

Initiation of rooting in the shoots obtained from cryopreserved shoot tips occurred within 3 weeks. Different NAA concentrations showed differences on rooting (Table 3). The highest number of roots was observed when shoots were cultured on MS medium supplemented with 0.75 mg/l NAA.

Table 3. The effect of NAA concentration in MS medium on the root formation of *Sequoia* shoots regenerated from cryopreserved shoot tips

NAA (mg/l)	Rooting rate (%±s.d.)	Number of roots±s.d.	Root length (cm±s.d.)
0.1	28.8±0.5^{c}	2.6±1.1^{c}	0.6±0.4^{c}
0.25	37.7±1.5^{bc}	6.3±0.5^{a}	1.2±0.3^{b}
0.5	44.4±1.1^{b}	3.3±1.5^{b}	1.8±0.3^{b}
0.75	62.2±0.5^{a}	3.0±0.0^{b}	2.9±0.2^{a}
1.0	46.6±1.0^{b}	2.3±0.5^{c}	1.7±0.8^{b}
1.5	31.1±1.5^{c}	1.3±1.5^{d}	0.4±0.1^{c}

Values followed by the same letter in the same column are not significantly different at p=0.05.

Conclusions

The preculture of apices in a medium containing 0.5 M sucrose for 24 h and dehydration with PVS2 for 30 min. at room temperature were found beneficial for survival and regeneration of shoot tips after freezing in liquid nitrogen. The results presented here confirm that the combined vitrification-droplet procedure is a practical method for cryopreservation of *Sequoia*.

REFERENCES

1. Ashmore, S.E., 1997, *Status Report on the Development and Application of In vitro Techniques for the Conservation and use of Plant Genetic Resources*, Engelmann, F. (ed.) IPGRI, Rome.
2. Crowe, J.H., Carpenter, J.F., Crowe, L.M., Anchordoguy, T.J., 1990, Are freezing and dehydration similar stress vectors? A comparison of modes of interaction of stabilizing solutes with biomolecules, *Cryobiology*, **27**: 219-231.
3. Dumet, D., Grapin, A., Bailly, C., Dorion, N., 2002, Revisiting crucial steps of an encapsulation / dessication based cryopreservation process: importance of thawing method in the case of *Pelargonium* meristems, *Plant Science*, **163**: 1121-1127.
4. Engelmann, F., 2004, Plant cryopreservation: Progress and prospects. *In Vitro Cellular and Developmental Biology–Plant*, **40**: 427-433.
5. FAO, 1996, Romania: *Country Report to the FAO International Technical Conference on Plant genetic Resources*.
6. Lainé, E., Bade, P., David, A., 1992, Recovery of plants from cryopreserved embryogenic cell suspensions of *Pinus caribaea*. *Plant Cell Reports*, **1**: 295- 298.
7. Murashige, T., Skoog, F., 1962, A revised medium for rapid growth and bioassays with tobacco cell cultures, *Physiol. Plant*., **15**: 473-497.
8. Percy, R.E., Livingston ,N.J., Moran, J.A., Von Aderkas, P., 2001, Desiccation, cryopreservation and water relations parameters of white spruce (*Picea glauca*) and interior spruce (*Picea glauca x engelmannii* complex) somatic embryos, *Tree Physiol.*, **21**: 1303-1310.
9. Ryynänen, L.A., Häggman, H.M., 2001, Recovery of cryopreserved silver birch shoot tips is affected by the pre-freezing age of the cultures and ammonium substitution, *Plant Cell Rep.*, **20**: 354–360.
10. Sakai, A., 2000, Development of cryopreservation techniques. In: Engelmann F, Takagi H (eds.) *Cryopreservation of tropical plant germplasm*. Japan International Research Centre for Agricultural Sciences, Tsukuba/International.
11. Sakai, S., Kobayashi, S., Oiyama, I., 1990, Cryopreservation of nucellar cells of navel orange (*Citrus sinensis* Osb. var. brasiliensis Tanaka) by vitrification, *Plant Cell Rep.*, **9**: 30–33.
12. Steponkus, P.L., Langis, R., Fujikawa, S., 1992, Cryopreservation of plant tissues by vitrification. In: Steponkus PL (ed.) *Advances in Low-Temperature Biology*, JAI Press Ltd.
13. Yamada, T., Sakai, A., Matsumura, T., Higuchi, S., 1991, Cryopreservation of apical meristems of white clover (*Trifolium repens* L.) by vitrification, *Plant Sci.*, **73**: 111–116.

INFLUENȚA PRECULTURII ASUPRA REGENERĂRII PLANTELOR DIN APEXURI CRIOCONSERVATE DE *SEQUOIA SEMPERVIRENS* (D. DON.) ENDL.

(Rezumat)

Pentru experimentele de crioconservare prin vitrificare în picătură au fost utilizate apexuri caulinare de *Sequoia sempervirens* (D. Don.) Endl., provenite de la plante crescute in vitro. Rezultatele obținute au demonstrat faptul că, precultura în soluție de zaharoză 0,5 M este esențială pentru regenerarea de plante după congelare în azot lichid (-196°C). Cel mai ridicat procent de regenerare de 67% a fost obținut ca urmare a unui tratament de 30 minute cu soluția de vitrificare, prealabil crioconservării. Plantele regenerate din apexuri conservate nu au prezentat diferențe fenotipice față de cele care nu au fost supuse crioconservării.

Received: 28.05.2008; Accepted: 10.09.2008