RESEARCH CONCERNING THE MEDIUM AND LONG-TERM CONSERVATION OF THE COLLECTION OF OAK EMBRYOGENIC LINES

MAGDALENA PALADA-NICOLAU[1], LAURENT JOUVE[2], JEAN-FRANCOIS HAUSMAN [2]

[1]Institutul de Cercetări şi Amenajări Silvice, Staţiunea Simeria, Romania
[2]CRP Gabriel Lippmann, Luxembourg

Abstract

The aim of this paper is to review some practical methods of oak somatic embryos conservation, applied to the collection of embryogenic lines of our laboratory. Simple and not too expensive methods, based on encapsulation and cryo-techniques, have been compared with the classical ones, from the point of view of the efficiency of recovering and re-growth. The preservation of regeneration ability in recovered embryogenic tissues and embryos, the prevention of senescence and possible genetic drift have also been analyzed. The results of a combined method of encapsulation and cryopreservation of oak embryos (recovery, development, plant regeneration) are also presented

Keywords:conservation, encapsulation, cryopreservation, somatic embryogenesis, embryogenic lines, oak.

Rezumat

CERCETĂRI PRIVIND CONSERVAREA PE TERMEN MEDIU ŞI ÎNDELUNGAT A LINIILOR EMBRIOGENE DE STEJAR

Scopul acestei lucrări este de a sumariza câteva metode practice de conservare a embrionilor somatici de stejar, aplicate la colecţia de somaclone embriogene a laboratorului nostru. Metode simple şi puţin costisitoare, bazate pe încapsulare şi criotehnologii, au fost testate comparativ cu metodele clasice, din punctul de vedere al eficienţei reconstituirii şi al reluării creşterii. Au fost analizaţi următorii parametri: prezervarea capacităţii de regenerare a embrionilor conservaţi, prevenirea senescenţei somaclonelor şi posibila derivă genetică.

Sunt prezentate rezultatele unor experimente cu o metodă combinată de încapsulare şi crioprezervare a embrionilor somatici de stejar, din punctul de vedere al reconstituirii liniilor embriogene şi regenerării plantelor din embrioni conservaţi.

Cuvinte cheie: conservare, încapsulare, crioprezervare, embroigeneză somatică, linii embriogene, stejar

1. INTRODUCTION

Long-term preservation of embryogenic lines is important both for conservation of germplasm and biodiversity and also, for technical reasons of plant propagation.

The most common way for conservation is the cryopreservation, but it usually claims controlled prefreezing, and the cryostat is not affordable for all propagation laboratories.

Encapsulation of somatic embryos, initially developed as a basis for artificial seeds, was proved to be a tool for medium and long-term preservation and also a way to improve oak somatic embryo germination by dehydration

Comparisons between cryopreservation (with and without controlled freezing) and encapsulation were made, and the genetical implications of each procedure were analyzed. The long-term culture of embryogenic lines (living collection) is considered as reference for the physiological status of cultures and for the conformity of regenerated plants.

This paper is a revue of some conservation methods applied to embryogenic lines of oak. The experiments were performed in cooperation with CRP Gabriel Lippmann Luxembourg, under the IPGRI project "Genetic resources in broadleaved forest species fron the South-Eastern Europe"

2. MATERIAL AND METHODS

The biological material was represented by embryogenic lines of oak (*Quercus robur*) from the collection of the biotechnology lab of Forest Research and Management Institute, Research Station of Simeria (Palada-Nicolau, 1999; Palada-Nicolau and Hausman, 2000).

The cryopreservation of oak embryos was performed without controlled freezing, according to the method of Tutkova and Wilhelm (1999) with modifications.

The embedding in calcium alginate was performed according to the protocol proposed by Kinoshita and Saito (1990) for the conservation of buds.

The media used for the cultivation of embryogenic lines and for their reactivation after conservation were based on the P 24 formula (Patent no. 92902531.0. R. Teasdale/Forbio)

The recovery of embryos after cryopreservation was assessed by microscopical observation with the fluorescein-diacetate coloration.

3. RESULTS AND DISCUSIONS

A combined method of encapsulation and cryopreservation of oak somatic embryos

Three oak embryogenic lines were used in the experiment (Q.r.1., Q.r.3., and NL 100), all of them belonging to an embryogenic system based on multiplication by serial adventitious embryogenesis, on hormone-free medium.

The lines Q.r.1.and Q.r.3 were selected from embryogenic cultures obtained by direct embryo induction from immature zygotic embryos, but the line NL100 was selected from somatic embryos formed on the callus resulted as a secondary product in the process of NL100 oak micropropagation by cuttings.

Before starting the procedure, the cultures with a good proliferation rate and good dispersion of embryos were partially synchronized (Fig. 1 and 2) and the embryos were separated in 4 development stages (Palada-Nicolau and Hausman, 2001).

Fig. 1. Partially synchronized oak embryogenic culture

Cultură embriogenă de stejar parțial sincronizată

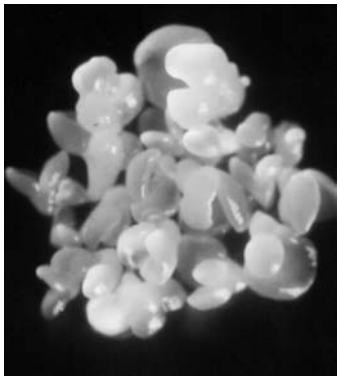

Fig. 2. Collection of embryos in the same developmental stage extracted from a synchronized culture

Colecție de embrioni în acelaşi stadiu de dezvoltare, extraşi dintr-o cultură parțial sincronizată

Only the stages 1 and 4 were used for encapsulation and cryopreservation (Fig. 3 and 4).

Two embedding methods were used: the classical one, based on immersion in Na-alginate gel and hardening in a CaCl solution (Fig. 5), and the so-called "hallow-beads" method: embedding in a polymer of carboxymethyl-cellulose hardened with an envelope of Ca-alginate (Fig. 6). The alginate beads containing each one embryo (or a 1 mm embryo cluster in the case of stage 1) were conserved for a short to medium time period (one to three months) at 4^{o}C, in refrigerator. The recovery and re-growth in this case was in the range of 85% to 100%, depending of time and conservation conditions (Fig. 7a). We found that t is very important to maintain a constant humidity inside the plastic Petri dishes containing the beads, otherwise, the dried beads will not re-growth.

After one-month cultivation of beads on P24 hormone-free medium, the small

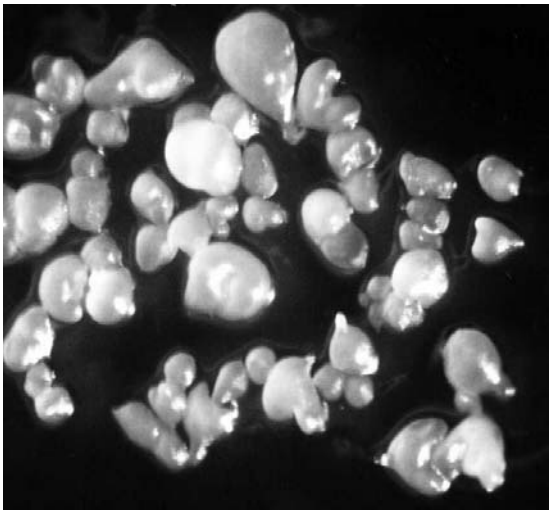

Fig. 3. Stage 1 oak somatic embryos selected for encapsulation and cryopreservation

Embrioni în stadiul 1, selectionați pentru încapsulare şi crioprezervare

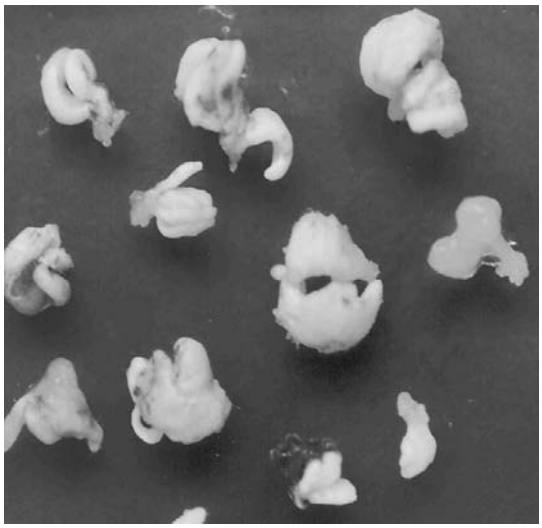

Fig. 4 - Stage 4 oak somatic embryos selected for encapsulation

Embrioni în stadiul 4, selectionați pentru încapsulare

embryos (stage 1) proliferated by serial embryogenesis, forming embryo clusters or colonies, and the big (stage 4) ones contiued naturation and started to germinate (Fig. 7b). Emblings were obtained after the conversion of germinated embryos (Fig. 8a, b). In some conditions, the rate of germination and conversion was improved by the encapsulation of stage 4 embryos (table 1). These results were in concordance with those of Eva Wilhelm et al. (Tutkova & Wilhelm, 1999)

In order to conserve the oak embryogenic lines for a long time period, and perform the cryopreservation without controlled pre-freezing, the alginate beads were gradually immerged in liquid nitrogen.

Cryoprotection treatments with sucrose were applied to embryos, before encapsulation.

After one week in liquid nitrogen, the beads were cultivated on hormone-free medium at 25^{o}C for one month, in order to test the viability and re-growth.

The following results were obtained:

Only the small stage 1 embryos recovered after cryotreatment (Fig. 9). The surviving rate was around 10 - 15 % (maximum 27 % and minimum 6,7 %) and the re-growth rate was around 5%, with a maximum of 6,7 % and a minimum of 2,8 %.(Table 2). The embryo clusters recovered better than the isolated embryos.

There were differences among genotypes: Q.r.1 and NL 100 gave better results than Q.r.3 (Fig. 10)

Fig. 5. Ca-alginate beads containing oak somatic embryos

Capsule de alginat de calciu cu embrioni somatici de stejar

Fig. 6. "hallow-beads" of carboxymethyl -cellulose containing oak somatic embryos

Capsule de carboximetil-celuloză cu interior lichid, conţinând embrioni somatici de stejar

Fig. 7 a. Encapsulated stage 4 embryos conserved for 60 days at 4°C, starting to germinate

Embrionii încapsulaţi în stadiul 4 şi conservaţi timp de 60 de zile la 4°C, la începutul germinării

Fig. 7 b. The germination of a encapsulated stage 4 embryo after conservation

Germinarea unui embrion încapsulat în stadiul 4, după conservare

The cryoprotection treatments were not benefic moreover, the immersion of embryos in sucrose solution before encapsulation was harmful (Fig. 11).

As a consequence of the recovery and re-growth of cryopreserved oak embryos, 16 new embryogenic lines were obtained out of a single cryopreserved embryo. Their behavior in culture was different, regarding the shape and dimension of embryos, the maturation ability and the germination rate, showing the possibility of a genetic drift or somaclonal genetic instability.

Fig. 8. The conversion of oak somatic embryos into plants after encapsulation and conservation:
a) - *in vitro*; b) - transplanted

Conversia embrionilor somatici de stejar în plantule, după încapsualre şi conservare:
a)- *in vitro*; b) - după transplantare

Table 1. The effect of encapsulation upon the germination and conversion ability

Efectul încapsulării asupra capacității de germinare şi conversie

	% viability	% germination	% conversion
Non-encapsulated embryos (control)	100	20	3,3
Alginate beads (classical method)	90	23,3	6,6
Hallow beads	83,3	16,6	3,3

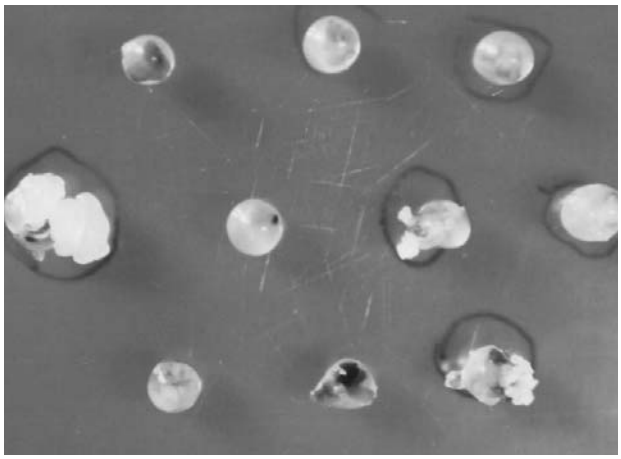

Fig. 9. Stage 1 oak embryos recovered after cryotreatment

Embrioni somatici de stejar în stadiul 1, recuperați după crioprezervare

Table 2. Results of encapsulated oak embryo cryopreservation without controlled pre-freezing

Rezultate ale experimentelor de crioprezervare a embrionilor somatici de stejar fără precongelare controlată

	maximum	average	minimum
Viability of cryopreserved embryos (%)	27,5	14,5	6,7
Re-growth of cryopreserved embryos (%)	16,7	5	2,8
Control (%)	100	100	100

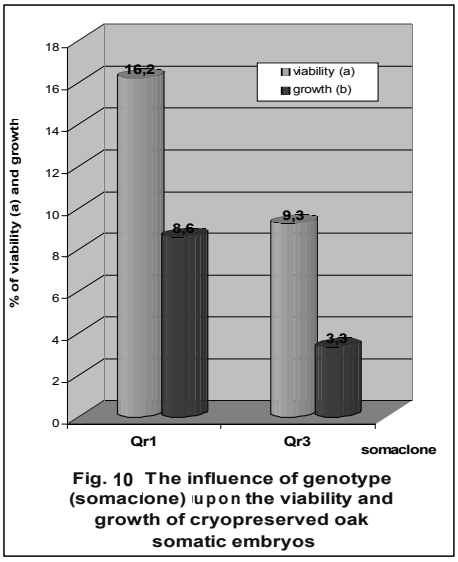

Fig. 10 The influence of genotype (somaclone) upon the viability and growth of cryopreserved oak somatic embryos

Influența genotipului (somaclonei) asupra viabilității şi creşterii embrionilor de stejar crioprezervați

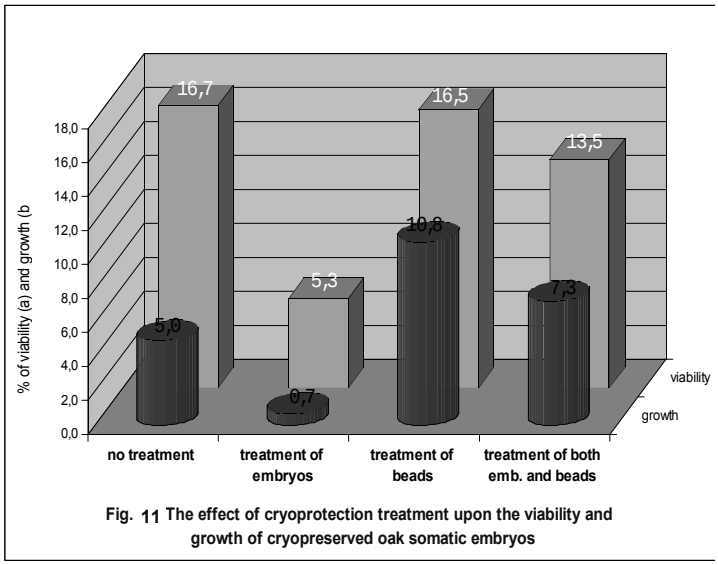

Fig. 11 The effect of cryoprotection treatment upon the viability and growth of cryopreserved oak somatic embryos

Efectul tratamentelor de rioprotecție cu sucroză asupra viabilității şi creşterii embrionilor de stejar crioprezervați

The germination and conversion ability of conserved embryos was not affected by the cryo-treatment.

Regenerated oak plants were obtained out of cryopreserved embryos derived from the somaclones Q.r.1 and NL 100.

4. CONCLUSIONS

A combined method of encapsulation and cryopreservation of oak somatic

embryos, in order to conserve them for a long period.

Alginate beads containing each one embryo or a small embryo cluster were conserved for a short to a medium time period (one to three months) at 4°C, into refrigerator. The recovery and re-growth in this case was in the range of 85% to100%.

Only the small stage 1 embryos recovered after cryotreatment. The surviving rate was around 14,5 % (maximum 27 % and minimum 6,7 %) and the re-growth rate was around 5%, with a maximum of 6,7 % and a minimum of 2,8 %.. The embryo clusters recovered better than the isolated embryos.

There were differences among genotypes.

The cryoprotection treatment of the beads with sucrose was not benefic. Moreover, the immersion of embryos in sucrose solution before encapsulation was harmful.

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The results presented in this paper were obtained in cooperation with CRP Gabriel Lippmann Luxembourg, under the IPGRI project "Genetic resources in broadleaved forest species fron the South-Eastern Europe"

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Corespondență/Corrspondence to: Magdalena Palada Nicolau magdalena.palada@rdslink.ro