EFFECT OF *IN VITRO* COLD STORAGE ON SURVIVING AND PROLIFERATION OF CORK-OAK (*QUERCUS SUBER* L.) CULTURES

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Abstract

Cork-oak cultures were stored *in vitro* without an intervening subculture, at 5°C in the dark for 5 months without loosing their capacity to regenerate when subcultured under standard conditions. The viability, proliferation rate and elongation of the explants were evaluated during 5 months of storage. Proliferation rate of stored cultures was observed during the following 3 proliferation cycles and compared to unstored controls of the same age. Shoot cultures survived with 100% viability and the proliferation rate was not statistically different from the controls. The results here described indicate the feasibility of establishing an *in vitro* active gene bank for *Quercus suber*.

K.W.: cold storage, cork-oak, germplasm, medium-term storage, micropropagation, *Quercus suber* L.

Resumen

Cultivos de alcornoque han sido almacenados a 5 ° en oscuridad durante 5 meses sin ninguna intervención de subcultivo. Los cultivos no han perdido la capacidad de regeneración cuando cultivados en las condiciones estandardizadas. La viabilidad, la tasa de multiplicación y la alongación de los explantos han sido analizados durante 5 meses. La multiplicación de los cultivos almacenados se estudió durante los siguientes 3 ciclos de multiplicación y se comparó con los controlos sin almacenaje con la misma edad. Los cultivos han presentado 100% de viabilidad y la tasa de multiplicación no ha sido estadisticamente diferente del control. Los resultados presentados demuestran la capacidad de establecer *in vitro* un banco de genes activo para el alcornoque (*Quercus suber* L.).

P.C: Almacenaje en el frio, alcornoque, germplasm, micropropagación, *Quercus suber* L.

INTRODUCTION

In recent years, techniques of *in vitro* storage have been applied with varying degrees of success to a wide range of species and culture systems (GROUT, 1991).

Whilst seed storage might appear to be the most attractive conservation strategy, low viability in storage and a relative long generation time, indicate *in vitro* storage techniques as potentially valuable in genetic conservation of *Quercus suber* L..

Plant tissue culture techniques are the basis for *in vitro* conservation. Frequent transfer of *in vitro* cultures onto fresh medium is the simplest *in vitro* preservation method. However, it is costly in terms of laboratory management and can lead to serious practical problems, such as an increased risk of contamination, equipment failure and risks of genetic variation, which increase with the culture duration in axenic conditions and can lead to the production of plants which are not true to type.

These problems can be overcome by reducing the growth rate of cultures through their maintenance under suboptimal conditions. Growth reduction is generally obtained by lowering the culture temperature, reducing or suppressing light intensity, or applying growth inhibitors (ENGELMANN, 1991). Normally, a simple reduction in the growth temperature is sufficient. However, there are specific differences in the tolerance of low growth temperatures and it is important to establish the most suitable temperature and frequency of subculturing in each case. The success of the chosen method is based on the reduction in the subculturing interval, the length of exposure to the limiting factor, good surviving and recovery after return to standard conditions.

In order to improve the planning production, a method for short term *in vitro* preservation of *Quercus suber* L. cultures was performed. The aim of this work was to develop a medium-term, low-input *in vitro* maintenance system, through the manipulation of the culture temperature, to extend the normal subculture interval.

MATERIALS AND METHODS

STORAGE OF CULTURES IN SLOW GROWTH

Cork-oak (*Quercus suber* L.) was established *in vitro* (ROMANO AND MARTINS-LOUÇÃO, 1992). Shoot cultures have been maintained with a reduced proliferation rate (ROMANO *et al*, 1992), on a proliferation medium consisting of GD (GRESSHOFF AND DOY, 1972) basal medium, supplemented with 0.5 mg l^{-1} 6-

benzylaminopurine (BAP) and 0.1 mg l^{-1} indolacetic acid (IAA). Sucrose at 20 g l^{-1} was used as carbon source and Difco Bacto agar at 7 gl^{-1} as the gelling agent. Media were adjusted to pH 5.8. Ten shoots per flask were cultured on 30 ml of medium in 200 ml flasks and closed by transparent polycarbonate caps. Media were previously autoclaved at 121° for 20 min. Cultures were incubated at 25° (day) and 22° (night) under a 16h photoperiod at 60 µmol m^{-2} s^{-1} produced from cool-white fluorescent lights. The explants were sub-cultured onto fresh multiplication medium every four weeks. These conditions are termed standard and they were considered the controls in experiments described below.

COLD STORAGE OF CULTURES

For the storage experiment, shoot explants 1 cm long from 2-years old cultures without developed leaves, were stored at 5° in the dark. At the end of the storage period (1, 2, 3, 4, 5 months) the shoots were subcultured to fresh medium with the same composition and placed under standard conditions, described above.

After the storage periods, observations were made on the extension of the growth of the new shoots, the presence of necrotic leaves and the number of surviving shoots. The effect of the storage period on proliferation and elongation rate, and on the percentage of surviving explants was assessed after being cultured for 4 weeks under standard conditions. These observations were made during the following 3 subcultures.

RESULTS AND DISCUSSION

Cok-oak cultures under optimal conditions normally produce 4-5 axillary shoots every 4 weeks. Moreover, under the standard storage conditions here described, cultures could be maintained without reducing proliferation rate for 2 years (ROMANO *et al*, 1992).

At the end of storage periods most of the leaves were necrotic and presented no visible growth. After 4 weeks under standard conditions they recovered their normal development and the new shoots were vigorous. This occurred independently of the duration of the storage period.

Shoot cultures survived with 100% viability. Proliferation rate, after transfer to standard conditions, was not significantly different between stored and unstored cultures, for all stored periods tested. We also observed an increase in the elongation of the shoots with the duration of the storage period (Table 1). Statistical differences

were not observed in the variation of proliferation and elongation rates during 3 following proliferation cycles under standard conditions (Table 2). Similar results were observed by SHARMA AND THORPE (1990) working with mulberry cultures, which could be stored at 4° in darkness for 6 months without any loss of multiplication rate. However for longer periods they observed high mortality. On the contrary, for apple a so effective response was not observed because the increase of proliferation rate was dependent on the rootstocks used (ORLIKOWSKA, 1991).

The results here presented show that cold storage of cultures for 5 months without an intervening subculture is feasible. The storage of cultures at 5° appears to offer considerable promise as a technique for maintenance and medium-term conservation of cork-oak germplasm. This method, very simple and effective, is the first approach to the possibility of establishing an *in vitro* active gene bank for *Quercus suber*.

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Table 1 - Effect of cold storage duration at 5° in the dark on shoot proliferation and elongation rates. Values represent means ± s.e. of 20 replicates. Values in a column sharing the same letter are not significantly different at 5% level.

Duration of storage (months)	*N*	*N control*	*L(mm)*	*L control(mm)*
1	1.95 ± 0.11 ab	2.25± 0.18 ab	16.20 ± 1.01 a	16.60 ± 1.18 ab
2	2.00 ± 0.14 ab	2.45 ± 0.17 ab	17.30 ± 1.10 ab	14.65 ± 0.83 a
3	1.90 ± 0.12ab	2.25 ± 0.20 ab	18.05 ± 1.05 ab	18.75 ± 1.45 b
4	1.84 ± 0.16 a	1.95 ± 0.15 a	18.63 ± 1.43 ab	14.35 ± 0.96 a
5	2.32 ± 0.17 b	2.71 ± 0.26 b	19.53 ± 1.31 b	13.71 ± 0.63 a

Values for L and L control are significantly different for 4 and 5 months of storage.

Table 2 - Effect of cold storage for 1 month at 5° in the dark on the variation of the shoot proliferation and elongation rates along the first 3 subcultures under standard conditions. Values represent means ± s.e. of 20 replicates. Values in a column sharing the same letter are not significantly different at 5% level.

Subculture	*N*	*N control*	*L(mm)*	*L control(mm)*
1 st	1.95 ± 0.11 a	2.25 ± 0.18 ab	16.20 ± 1.01 a	16.60 ± 1.18 b
2 nd	2.20 ± 0.14 a	2.45 ± 0.17 b	16.05 ± 0.87 a	17.20 ± 1.23 b
3 th	2.24 ± 0.22 a	1.17 ± 0.18 a	17.23 ± 1.11 a	12.80 ± 1.01 a