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Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*

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Abstract

Somatic embryogenesis was obtained in cultures of leaves from young seedlings of *Quercus suber* L. A two-stage process, in which benzyladenine and naphthaleneacetic acid were added first at high and then at low concentrations, was required to initiate the process. Somatic embryos arose when the explants were subsequently placed on medium lacking plant growth regulators. The embryogenic lines remained productive, by means of secondary embryogenesis, on medium without growth regulators. However, this repetitive induction was influenced by the macronutrient composition of the culture medium. Both low total nitrogen content and high reduced nitrogen concentration decreased the percentage of somatic embryos that showed secondary embryogenesis. Our results suggest that alternate culture on medium that increases embryo proliferation and a low salt medium prohibiting embryo formation will partially synchronize embryo development. Chilling slightly reduced secondary embryogenesis but gave a modest increase in germination. Maturation under light followed by storage at 4 °C for at least 30 days gave the best results in switching embryos from an embryogenic pathway to a germinative one. Under these conditions 15% of embryos showed coordinated root and shoot growth and 35% formed either shoots or mostly roots. These percentages were higher than those of embryos matured in darkness. This result indicates that a specific treatment is required after maturation and before chilling to activate the switch from secondary embryo formation to germination.

Abbreviations: BA – benzyladenine, NAA – naphthaleneacetic acid, 2,4-D – 2,4-dichlorophenoxyacetic acid, IBA – indolebutyric acid, MS – Murashige & Skoog (1962) medium, SH – Schenk & Hildebrandt (1972) medium, G – Gamborg (1966, PRL-4-C) medium (macronutrients in mg l^{-1}: $NaH_2PO_4 \cdot H_2O$, 90; Na_2HPO_4, 30; KCl, 300; $(NH_4)_2SO_4$, 200; $MgSO_4 \cdot 7H_2O$, 250; KNO_3, 1000; $CaCl_2 \cdot 2H_2O$, 150), PGR – plant growth regulator

Introduction

The increasing demand for cork, a natural product from *Quercus suber* L. trees, and the low natural regeneration of the species justify intensive planting with improved material. Current trends in forest breeding strategies emphasize combined breeding and cloning (Park & Bonga, 1993). Somatic embryogenesis has the potential to be an important tool for obtaining efficient true-to-type vegetative propagation (Bonga, 1991). Somatic embryogenesis has been obtained in several species of the genus *Quercus* (Féraud-Keller & Espagnac, 1989; Gingas & Lineberger, 1989; Chalupa, 1990; Gingas, 1991). In *Q. suber* it was initiated from cotyledonary fragments (Toribio, 1986; Toribio & Celestino, 1989), from nodal segments of seedlings (El Maâtaoui & Espagnac, 1987) and from whole zygotic embryos (Bueno et al. 1992).

The two major issues in somatic embryogenesis are 1) increasing efficient multiplication of somatic embryos and 2) improving rates of conversion of embryos into plants. Papers often state how many

embryos are converted into plantlets, but usually they do not mention what happens with those that failed to germinate. However, we feel it is important that this be shown and therefore this paper describes factors affecting both secondary embryogenesis and embryo germination.

Material and methods

Induction of somatic embryogenesis

Apical leaves with petioles were removed from 4-month-old seedlings, from acorns collected in Valdelosa (Salamanca, Spain), and used as explants to induce somatic embryogenesis. They were washed for 15 min in 0.2% Benlate® (Du Pont, 50% benomyl) and 2% Tween 20. This was followed by soaking in an agitated solution of 10% NaOCl (7% active chlorine) plus a few drops of Tween 20 for 15 min, and three rinses in sterile distilled water for 15 min each. Entire leaves were placed with the abaxial surface touching the medium in plastic petri dishes (55 mm diameter, 10 ml medium, 2 leaves per dish) sealed with Parafilm®. Explants were exposed to various light-dark regimes and culture media compositions (macronutrient formulation and type and concentration of plant growth regulators). None of the assayed conditions gave somatic embryogenesis, except those described in the Results section. The successful experiments were repeated three times and in each case the results were similar.

Embryogenic lines were maintained by secondary embryogenesis. Subcultures were made monthly on proliferation medium with macronutrients of SH and micronutrients, Fe-EDTA, vitamins, inositol and sucrose of MS medium. This medium lacked PGRs, and agar (Sigma, type E) was at 6 g l^{-1}. The pH was adjusted to 5.7 before autoclaving at 114 °C and 50.66 kPa for 23 min. 40 sec. Cultures in closed Magenta^{TM} vessels (Sigma, 95 x 67 mm, height x width, 60 ml medium), were placed in a growth chamber at 25 ± 1 °C and a 16-h photoperiod (mixed cool-white and grolux fluorescent lamps, 50 μmolm^{-2}s^{-1}).

Somatic embryo development

Isolated immature somatic embryos were used in different experiments. These immature embryos were early dicotyledonary, had an average length of 3 mm, and were soft and translucent. At the stage that we call maturity, the embryos had large (about 9 mm long) swollen cotyledons, a firm surface and an opaque white yellowish colour. The following criteria were used to record growth and development: NR = no response, i.e. most embryos become mature but do not grow further; AE = secondary (adventive) embryogenesis, i.e. after maturing the embryos show *de novo* secondary embryogenesis and some germinate precociously; R/S = either the root or the shoot of the embryo elongates; CG = coordinated germination, i.e. the embryos become plantlets.

To study the influence of macronutrient formulation on morphogenic responses, isolated embryos were cultured for 40 days on media with different macronutrients. Micronutrients, Fe-EDTA, vitamins and inositol were from MS for all media. Sucrose was at 10 g l^{-1} and agar at 6 g l^{-1}. The macronutrient formulations assayed were from half-strength MS (30.0 mM total nitrogen; 10.3 mM ammonium), full SH (27.3 mM total nitrogen; 2.6 mM ammonium) or half-strength, and full G (13.0 mM total nitrogen; 3.1 mM ammonium) or half-strength. Some of the media instead of MS vitamins included the following cofactors (C): 10 μM ascorbic acid, 2.5 μM calcium pantothenate, 10 μM nicotinic acid, 5 μM thiamine-HCl, 5 μM pyridoxine-HCl, 40 μM arginine and 20 μM glutamine. Some treatments included 1 μM BA, but most of them lacked PGRs. Growth conditions were as described above (9 embryos per Magenta vessel).

To study the influence of culture on a proliferation-inducing medium (P) first and then on a growth-retarding medium (R), embryos were subcultured monthly, alternatively on medium with SH and half-strength G macronutrients with the rest of the components from MS, except agar that was at 6 g l^{-1}. Both media lacked growth regulators. Immature embryos were placed singly in test-tubes (160 × 25 mm, 30 ml medium per tube) with sero-caps sealed with Parafilm.

The next sets of experiments were conducted following a sequential order: 20 days for maturation (which equals the mean time required to achieve maturation *in situ*), cold treatment (storage at 3 ± 1 °C in darkness for 15 or 30 days) and 30 days for germination. Unless otherwise stated, maturation and germination were carried out in the growth chamber under the conditions described above (9 embryos per Magenta vessel). Further details are given in the Tables.

Results were analyzed using the Chi-square test of independence and F-test at 5% significance level.

Number of embryos per treatment are indicated in the Tables.

Results and discussion

Induction of somatic embryogenesis

In preliminary experiments IBA was ineffective in inducing callus, and 2,4-D induced non-embryogenic calluses. The use of MS and $\frac{1}{2}$MS media resulted in necrotic calluses after 3 months of culture, which is similar to what happened in foliar tissue of *Quercus rubra* (Gingas & Lineberger, 1989). Embryogenic lines were obtained on medium with macronutrients from G and the other components (micronutrients, Fe-EDTA, vitamins, inositol and sucrose) from MS (agar 6 g l^{-1}) if the following sequence was used: first 30 days on medium with 10 μM BA plus 10 μM NAA in darkness; second, 30 days on medium with 0.5 μM BA plus 0.5 μM NAA under a 16-h photoperiod. After that explants were subcultured on medium lacking PGRs. After about 90 days from the beginning, embryonic structures were separated from small amounts of soft white-yellowish callus (Fig. 1A) and transferred to proliferation medium. Sometimes somatic embryos developed directly from the swollen surface of the explant (Fig. 1B).

The percentage of initial induction was low: 4% of the cultured leaves were embryogenic. When somatic embryos were generated, all the natural developmental forms (globular, torpedo, heart, dicotyledonary) were seen. 2,4-D, the PGR widely used for inducing embryogenesis, was not effective in our study. This result seems to be typical of *Quercus* species: 2,4-D induces somatic embryogenesis in zygotic embryos (Gingas & Lineberger, 1989; Bueno et al. 1992), with some exceptions (Chalupa, 1990), but to initiate primary embryogenesis in non-embryonic explants other auxins, often in combination with BA, are needed (El Maâtaoui & Espagnac, 1987; Féraud-Keller & Espagnac, 1989). In *Quercus ilex* a multi-stage induction treatment, similar to ours, was required (Féraud-Keller & Espagnac, 1989).

It has been claimed that genotype plays a major role in morphogenic responses, but only a few studies have dealt with it in depth (Park et al. 1993). Also important is the developmental stage of the explants (Gingas & Lineberger, 1989; Sotak et al. 1991). Since our material was randomly collected from several trees, both variation in the developmental stage of the explants and genotype may have affected the low induction percentages that we have obtained.

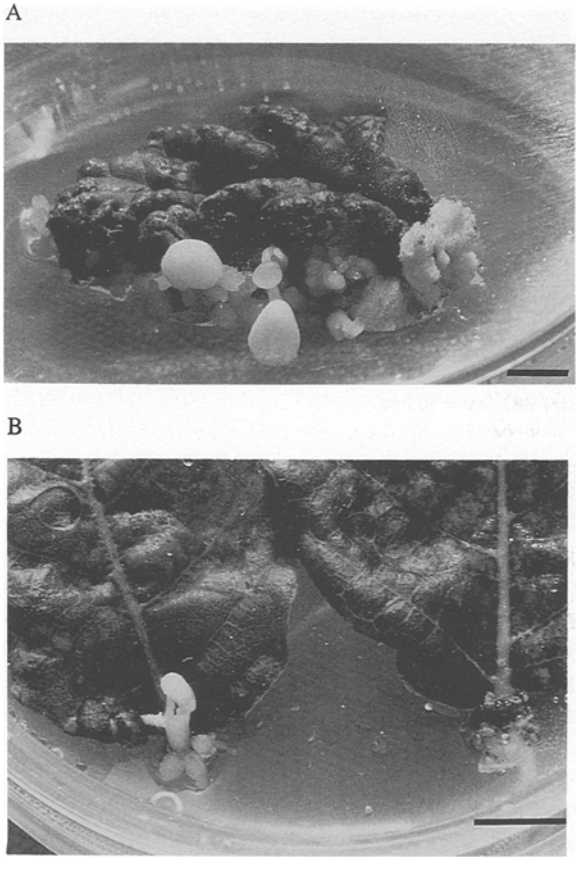

Fig. 1. Somatic embryogenesis on leaves of *Q. suber*. (A) Somatic embryos arising from the abaxial side with some callus formation. (B) Putative direct embryogenesis on the cut surface on the petiole. The somatic embryo shows secondary embryogenesis. Scale bar = 50 mm.

Maintaining and controlling repetitive embryogenesis

The embryogenic lines generated from leaves of cork oak have been maintained for more than 2 years by secondary embryogenesis, simply by subculturing the somatic embryos on media lacking PGRs. This fact agrees with what has been reported for *Quercus rubra* (Gingas & Lineberger, 1989), for *Quercus suber* (El Maâtaoui et al. 1990), for a *Juglans nigra x regia* hybrid (Deng & Cornu, 1992) and for *Aesculus hippocastanum* (Kiss et al. 1992). Gingas & Lineberger (1989) also stated that for the induction of somatic embryogenesis on zygotic embryos in the proper stage of development, auxin was not required. Obtaining

Fig. 2. Isolated somatic embryos showing secondary embryogenesis. Scale bar = 50 mm.

somatic embryogenesis without the use of PGRs has the advantage of reducing the risk of somaclonal variation (Berlyn et al. 1986).

We have not observed secondary embryogenesis on heart- or torpedo-stage embryos, nor on immature dicotyledonary ones. This agrees with what has been reported for zygotic embryo explants: only relatively mature dicotyledonary embryos produced high percentages of embryogenic lines (Gingas & Lineberger, 1989; Wetzstein et al. 1989; Bueno et al. 1992; Kiss et al. 1992). Similar results have been reported for some coniferous species (Tautorus et al. 1991; Thompson & von Aderkas, 1992). Therefore, it seems that for many species a short period of embryo explant development is required before induction of somatic embryogenesis happens. It appears that if the naturally ocurring shift from embryo enlargement to arrested growth (Kermode et al. 1986) is prevented, secondary embryogenesis will occur. Usual in vitro culture conditions may impede that switch. In fact, the main problem to obtain proper germination of isolated somatic embryos is to stop their embryogenic potential (Fig. 2).

Secondary embryos appeared chiefly on the root pole and secondarily on the main axis and cotyledons. This has previously been reported for cork oak (El Maâtaoui et al. 1990) and also for other species (Merkle & Wiecko, 1989; Gingas, 1991; Kiss et al. 1992) (Fig. 3).

Isolated immature embryos of cork oak had longer and more swollen cotyledons when grown on media with a high nitrogen content than when grown on a medium with low nitrogen. In addition the percentage of embryos giving secondary embryogenesis was significantly higher when 27.3 mM total nitrogen (SH) instead of 6.5 mM ($\frac{1}{2}$G) was used. In the later case,

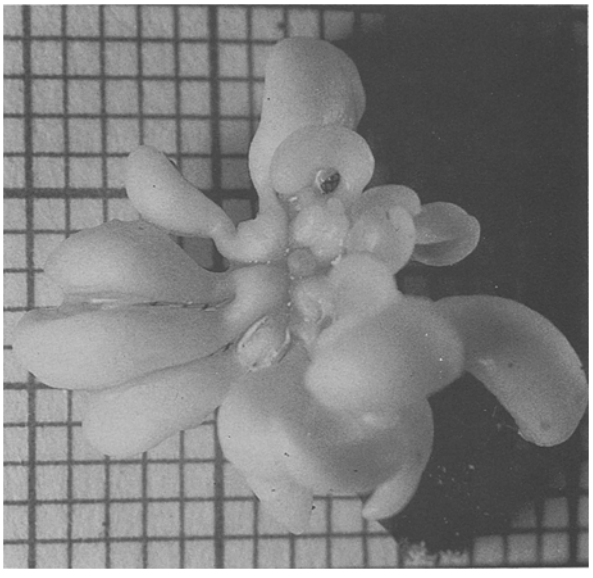

Fig. 3. Secondary embryogenesis on the root pole of an isolated somatic embryo. Initial embryo is upside down showing a darkgreen cotyledon. Graph paper, squares = 1 sq mm.

close to 60% of the embryos remained arrested (Table 1). It has been stated that adequate total nitrogen levels must be supplied to support growth (McCown & Sellmer, 1987). In our case they also influenced the induction of secondary embryogenesis.

The ratio of reduced nitrogen to nitrate may also have had an effect. Higher concentrations of reduced nitrogen, e.g. in media with cofactors C (presence of glutamine and arginine), decreased the percentage of explants that were polyembryonic (Table 1). Morphogenesis is determined by the form of nitrogen (Kirby et al. 1987). Reduced nitrogen is generally claimed to promote somatic embryogenesis (Ammirato, 1983), but there are some cases in which the addition of ammonium or amino acids decreases the yield (von Arnold, 1987). *Quercus suber* seems to belong to the latter group. The germination of isolated embryos cultured without any specific treatment was negligible. The addition of 1 μM BA did not reduce secondary embryogenesis and improved germination significantly.

We observed that continuous subculture of embryo aggregates on proliferative medium led to asynchrony in embryo development and hence to a decreasing ability to produce new detachable embryos without malformations. Alternating culture on proliferation and growth retarding medium almost doubled the number of new embryos at a similar stage of development at a

Table 1. Effect of macronutrients, presence of cofactors C and 1 μM BA on secondary embryogenesis. Data were recorded after 40 days of culture.

Medium[1,2]	N[3]	Response[4] (%)			
		NR	AE	CG	R/S
$\frac{1}{2}$MS ^{ab}	27	30	62	4	4
SH ^{a}	19	21	79	0	0
$\frac{1}{2}$SH ^{ab}	23	38	62	0	0
G ^{ab}	18	39	61	0	0
$\frac{1}{2}$G ^{b}	23	61	39	0	0
$\frac{1}{2}$MS+C^{ns}	18	66	34	0	0
$\frac{1}{2}$SH+C*	18	77	23	0	0
$\frac{1}{2}$G+C*	18	100	0	0	0
$\frac{1}{2}$MS+BA^{ns}	28	28	61	4	7
SH+BA^{ns}	90	23	57	4	16

[1]Media with the macronutrients indicated. Micronutrients, Fe-EDTA,vitamins and inositol from MS medium. Sucrose 10 g l^{-1}. All media lacked PGRs, except those quoted BA.
[2]Cofactors C (they were included instead of vitamins from MS): 10 μM ascorbic acid, 2.5 μM calcium pantothenate, 10 μM nicotinic acid, 5 μM thiamine-HCl, 5 μM pyridoxine-HCl, 40 μM arginine and 20 μM glutamine.
[3]N: number of isolated embryos tested per treatment. They were grown in the growth chamber.
[4]NR = no response, AE = secondary embryogenesis, CG = coordinated germination, R/S = either the root or the shoot of the embryo elongates.
^{a,b}Treatments followed by the same letter are not significantly different for the overall response. ^{*,ns} Indicate significant or non-significant differences between equivalent media including either cofactors C or MS vitamins, and between equivalent media with or without BA (Chi-square test, p = 0.05).

given time (Table 2). Starvation stress induced in the growth-retarding medium may have been the cause for this improved response. Culture of somatic embryos of *Populus* (Michler & Bauer, 1991) on solid medium arrested their development at the torpedo stage and, since this effect was not observed in liquid medium, the authors claimed that nutrient availability was a possible reason for that result. Starvation also improved the embryogenic potential of birch cells in suspension culture (Nuutila & Kauppinen, 1992).

Embryos that have matured in the light germinated properly after cold treatment (Table 3). There was a significant difference between non-cold-treated embryos and those chilled for 30 days, not only in germinative response (CG plus R/S) but also in reduced adventitious embryogenesis. As long as the embryos are programmed for further embryo initiation, normal completion of development is prevented. This program has to be interrupted if we want to achieve normal germination. Chilling is the most usual treatment applied to stop growth, to break dormancy and to hasten germination. Cold treatment of maturing somatic embryos to induce their germination has sometimes been unsuccessful (Gingas & Lineberger, 1989), but usually favourable (Tulecke & McGranahan, 1985; Deng & Cornu, 1992). Present results confirm the latter trend. The length of the cold storage was important for both improving the percentage of germination and obtaining better coordinated germination. In the case of walnut 4 weeks were not enough and 8 to 10 weeks were needed (Tulecke & McGranahan, 1985). Cork oak does not require more than 5 weeks of chilling because 30 days of treatment

Table 2. Effect of alternate culture on proliferation (P) and growth retarding (R) media on morphogenic response and on the production of detachable secondary embryos. Data were recorded after 90 days of culture.

Culture condition	N[1]	Response[2] (%)				DE[3]	T[4]
		NR	AE	CG	R/S		
Alternate R-P-R^{ns}	29	0	65	7	28	63	3.3^{a}
Continuous P-P-P^{ns}	30	7	73	3	17	38	1.7^{b}

[1]N: number of isolated embryos tested per treatment. They were grown in the growth chamber. The cultures were subcultured every 30 days.
[2]NR = no response, AE = secondary embryogenesis, CG = coordinated germination, R/S = either the root or the shoot of the embryo elongates.
[3]DE: number of detachable embryos obtained per treatment.
[4]T: mean number of detachable embryos obtained per embryo with secondary embryogenesis.
^{a,b}Treatments followed by the same letter are not significantly different for column T (Multiple Range Test, p = 0.05). ^{ns} Indicate non-significant differences between treatments for the overall response (Chi-square test, p = 0.05).

Table 3. Effect of chilling and BA on secondary embryogenesis and germination. Data were recorded after 30 days in germinating conditions.

Cold storage period	N[1]	Response[2] (%)			
		NR	AE	CG	R/S
Control ^{a}	30	33	57	0	10
C15 ^{ab}	35	40	34	3	23
C30 ^{b}	40	20	30	15	35
C15+BA^{ns}	18	55	17	11	17
C30+BA^{ns}	40	40	33	17	10

[1]N: number of isolated embryos tested per treatment. Immature embryos were matured in the growth chamber, then underwent 15 (C15) or 30 (C30) days of cold storage, and were placed in the growth chamber for germination. All subcultures were on media with SH macronutrients and the rest of the components from MS. Some treatments included 0.5 μM BA in the germination media. The control did not undergo cold treatment.
[2]NR = no response, AE = secondary embryogenesis, CG = coordinated germination, R/S = either the root or the shoot of the embryo elongates.
^{a,b}Treatments followed by the same letter are not significantly different for the overall response. ^{ns} Indicates non-significant difference between equivalent media with or without BA (Chi-square test, p = 0.05).

promoted germination as much as 10 weeks of cold storage (Bueno et al. 1992). The addition of BA to the germination medium after chilling did not improve germination, in contrast to what has been observed in experiments with other species of *Quercus* (Chalupa, 1990).

The macronutrient formulation had little influence on the germination of embryos that had matured in darkness and had undergone cold treatment (Table 4). However, SH macronutrients induced the highest percentage of secondary embryogenesis while $\frac{1}{2}$MS macronutrients, having almost the same total nitrogen content but about five times more ammonium, gave

Table 4. Effect of macronutrients on secondary embryogenesis and germination. Data were recorded after 30 days in germinating conditions.

Medium[1]	N[2]	Response[3] (%)			
		NR	AE	CG	R/S
$\frac{1}{2}$MS ^{a}	34	65	9	3	23
SH ^{b}	33	27	52	3	18
G ^{ab}	33	49	27	0	24
$\frac{1}{2}$G ^{ab}	44	48	27	3	22
SH+BA ^{ns}	22	41	23	13	23

[1]Media with the macronutrients indicated and the rest of the components from MS.
[2]N: number of isolated embryos tested per treatment. Immature embryos were matured in darkness at 25 ± 1 °C. Then they underwent a 30 days cold storage and were placed in the growth chamber for germination. All subcultures were made on the same macronutrients formulation media. One treatment included BA (1μM) in the germination medium.
[3]NR = no response, AE = secondary embryogenesis, CG = coordinated germination, R/S = either the root or the shoot of the embryo elongates.
^{a,b}Treatments followed by the same letter are not significantly different for the overall response. ^{ns} Indicate non-significant difference between equivalent media with or without BA (Chi-square test, p = 0.05).

the lowest. It is interesting that treatments in which the only difference is that in one maturation occurred in the light (C30, Table 3) and in the other in the dark (SH, Table 4), there was a significant difference in response (Chi-square test). Maturation under light inhibited polyembryony and improved subsequent germination. This indicates that factors during or after maturation, but prior to chilling, were as important as cold treatment to improve embryo conversion into plants.

Conclusions

Obtaining somatic embryos from zygotic embryos, nodal segments and leaves shows that *Quercus suber* tissues have a high capacity for somatic embryogenesis. Likewise, the reported ability of leaf tissues from mature trees of a related species, *Quercus ilex* (Féraud-Keller & Espagnac, 1989), to produce somatic embryos suggests that it will soon be possible to clone selected cork oak trees. Moreover, our technique of multiplication by secondary embryogenesis reduces the risk of somaclonal variation associated with callus growth.

Synchronization of embryo development still requires improvement. Sieving techniques have proven effective (Nadel et al. 1990; Merkle, 1991) and, as our results indicate, depletion of nutrients may also help to synchronize embryo production.

The control of the switch, prior to chilling, from the embryogenic to the germination program is essential to direct embryos from repetitive embryogenesis to the conversion into plantlets. The results suggest that starvation during maturation could be effective to improve germination.

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