Induction of *Quercus ilex* L. haploid and doubled-haploid embryos from anther cultures by temperature-stress

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Abstract

This paper describes a method to obtain haploid and doubled-haploid (DH) embryos using anther cultures of holm oak (*Quercus ilex* L.). The production of haploids and DH through gametic embryogenesis provides an attractive biotechnological tool for developing homozygous lines from heterozygous parents, which is important in breeding programs, as well as in genetic studies. As a consequence, protocols to produce homozygous plants have a significant impact on forest tree improvement. Anthers were subjected to different temperature treatments for embryo induction: a cold pre-treatment (4 °C) from 3 to 7 days was carried out at the beginning, followed by a heat shock (33 °C) from 2 to 5 days. Most anthers responding to these stress treatments contained vacuolated microspores, indicating that this developmental stage is responsive to embryogenesis induction in holm-oak microspores. In all cases, embryos grew from the interior of the anthers, breaking through the degenerating anther walls. Under these conditions, embryo formation occurred in 31 anthers between 46 and 95 days after culture initiation. Embryo analysis performed with flow-cytometry and DNA-microsatellite markers showed haploid profiles and/or spontaneous doubling of the chromosomes during early regeneration stages. This is, to our knowledge, the first published report on gametic embryogenesis in holm oak.

Key words: anthers, holm oak, microsatellite DNA-markers, pollen embryogenesis, *Quercus ilex*.

Introduction

Holm oak (*Quercus ilex* L.) is the dominant tree species of most natural communities over large areas of the Western Mediterranean basin. The dominant current structure in holm oak forests and its major economic importance is the agroforestry system known as "dehesa" (wooded meadow). Holm oaks have a long life and late sexual maturation with irregular reproductive cycles, showing difficulties for seed conservation, vegetative reproduction, and for the establishment of seed orchards. All these problems make it difficult to obtain plant material focussed on forest restoration and reforestation objectives, as well as on breeding and genetic programmes.

A high genetic diversity both within and among populations has been measured in holm oak (Michaud et al., 1995) and propagation by somatic embryogenesis has been assayed in this species (Mauri and Manzanera, 2004, 2011). However, conventional breeding methods have not experienced significant improvements, which may be mainly due to the long juvenile period of this species. Thus, the use of the classical methods of repeated backcrossing in order to obtain pure lines (production of homozygous lines) is nearly impracticable, and there are no known attempts of breeding programs by the classic method of artificial selection and hybridization, traditionally performed in agricultural crops.

The use of microspore or anther culture to generate doubled-haploids (DH) is an important adjunct to tree breeding. Doubled haploid plants are useful in tree breeding programs, since completely homozygous plants may be obtained in a few months while backcrosses take many years, due to the long life cycles of trees (Germanà, 2011). The generation of stable haploids may have a great impact on future developments in forest genomics. Haploid and DH lines play an important role in genomics (Forster et al., 2007; Ferrie and Möllers, 2011), and they have been used for genetic mapping. There are two principal methods to obtain haploid plants from the male gamete: anther culture and pollen or microspore culture. Embryo induction from pollen or microspores is more easily and simply obtained through anther culture. After the initial reports of successful production of haploids from anther culture in *Datura* (Guha and Maheshwari, 1966), haploids have been obtained in more than 250 plant species (Maluszynski et al., 2003), including a wide variety of economically important crops as well as some woody species, such as *Quercus suber* L. (Bueno et al., 2004). The large number of species in which anther culture has been used successfully to produce haploids provides evidences of the benefits it offers. There has been a great increase in the research aimed at developing and optimizing protocols to obtain well developed embryos and, as a consequence, good plant regeneration rates (Pintos et al., 2010). Nevertheless, anther culture presents some disadvantages if compared to pollen (microspore) culture (diploid anther walls and smaller potential of haploid plant production). The presence of diploid tissues opens the possibility of obtaining diploid embryos from anther walls. In this

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sense, there are diverse methods to determine ploidy levels: 1) direct methods, such as chromosome counting and isozyme or DNA markers (i.e. SSRs); or 2) indirect methods such as flow cytometry, with which only the ploidy level can be assessed.

A better understanding of embryogenesis in woody plants (i.e. LIPPERT et al., 2005; SGHAIER-HAMMAMI et al., 2009), and specifically on gametic embryogenesis (GÓMEZ et al., 2009) should lead to more efficient protocols for the production of haploid and DH plants. Haploid embryogenesis has been assayed at different stages of development of the microspore or the pollen grain. In a vast majority of cases, the optimal stage is the late vacuolated microspore (PINTOS et al., 2005), and less frequently the early binucleated pollen grain (KIM et al., 2004). In those stages, stress appears to be a major signal switching development from the gametophytic to the sporophytic pathway. Therefore, gametic embryogenesis usually requires reprogramming of microspores by stress, e.g. cold, heat, and starvation, followed by embryo development under stress-free conditions (SHARIATPANAHI et al., 2006). Heat or cold stress treatments are commonly used for anther or microspore culture of many species, such as *Triticum aestivum* (GUSTAFSON et al., 1995), *Quercus suber* (BUENO et al., 1997), *Capsicum annuum* (KIM, 1999), and *Populus nigra* (DEUTSCH et al., 2004). It has been proposed that cold treatment slows the degradation process in anther tissues, thus protecting microspores from toxic compounds released in decaying anthers. As a consequence, it assures the survival of a greater portion of embryogenic pollen grains as compared to the heat treatment (DUNCAN and HEBERLE, 1976). Sucrose starvation is another stress treatment with positive results in haploid embryo induction from isolated microspore cultures, e.g. in rice (OGAWA et al., 1994) and barley (RODRIGUEZ-SERRANO et al., 2012). Although the specific mechanism remains unknown, both types of stress have been the major and probably general stimuli responsible for stopping gametophytic development and inducing the alternative sporophytic development of the microspore (BUENO et al., 1997).

Production of DH comprises three main steps: induction, regeneration and duplication of the haploid genome. Such duplication is sometimes indirectly induced by the treatments used to promote androgenic development. Thus, low temperature also increases the frequency of endo-reduplication, which entails an increase in spontaneous DH plants (AMSSA et al., 1980). However, an additional step of direct chromosome doubling must usually be included in the protocol (PINTOS et al., 2007b, 2010).

The totipotency of plant cells would permit the regeneration from either haploid cells of the anther locule (microspores) or from diploid cells of the anther wall. For this purpose, flow-cytometry can be used to analyze the ploidy level of embryos (BUENO et al., 2003). Furthermore, to elucidate if diploid embryos were originally haploids which spontaneously duplicated their genome, or alternatively those embryos were regenerated from the diploid tissue of the anther wall, a genetic test was designed by means of microsatellite markers (GÓMEZ et al., 2001).

First attempts of inducing haploid embryogenesis in oaks were assayed on *Quercus petraea* anthers from two trees (JÖRGENSEN, 1988). Anthers cultured on modified Woody Plant Medium (LLOYD and MCCOWN, 1981) with plant growth regulators benzyl-adenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) produced callus at a low frequency (2 to 6%). This callus was induced to embryogenesis with 2.5 µM BA, but only three haploid clones were obtained (JÖRGENSEN, 1993). More reliable results of haploid and DH embryos and plants were achieved in the related species *Q. suber*, where anther cultures produced direct haploid embryogenesis from multiple microspores. This could be confirmed by microscopic observation of embryogenic microspores inside the anther, by chromosome counting and by isozyme and microsatellite molecular markers (BUENO et al., 2000; PINTOS et al., 2007a). In this paper, we report an efficient and reproducible procedure of anther-culture for haploid and DH embryo induction in the tree *Quercus ilex* L. This is, to our knowledge, the first published report for gametic embryogenesis in this species, as well as one of the few papers that has been reported on successful anther culture in forest trees.

Materials and Methods

Plant material

Branches with catkins were collected during the flowering period from two ornamental holm oak trees located in a park near INIA (Madrid) in April 2009 and 2010.

Characterization of the Developmental Stages of Catkins, Anthers and Microspores

Catkins were collected at three different phenological stages (less than 1 cm, between 1 and 1.5 cm, and between 1.5 and 2.5 cm). Anthers were green-yellowish of about 1–1.5 mm size. Anthers from those stages were dissected in a Nikon magnifying glass and the respective microspores were stained with 4'-6-diamidino-2-phenylindole (DAPI) for the determination of their developmental stage. The anthers were placed on a glass slide with a few drops of 1 mg/l DAPI in Phosphate buffered saline (PBS) solution plus 1% Triton X-100, and tapped softly through the coverglass. Microspores were examined under a Nikon fluorescence microscope and photographed under ultraviolet light ($\lambda = 360$ nm) with a digital Coolpix 4500 Nikon camera.

Cold Pre-treatment

Branches bearing catkins were transported to the laboratory, preserved in darkness with moist cotton wrapped at the base and enveloped in aluminium foil. Then they were stored at 4 °C for 3 to 7 days.

Surface sterilization procedure

Catkins were surface-sterilized by immersion in 70% ethanol for 30 s and then placed in 2% sodium hypochlorite solution with a few drops of Tween 20 for 20 min, followed by three rinses in distilled sterile water.

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Anthers were isolated from the catkins in sterile conditions with a Nikon magnifying glass in a laminar flow cabinet.

Induction medium

Anthers were plated in Petri dishes (12 cm diameter, ca 40 anthers per plate) in a basal induction medium containing macronutrients (SOMMER et al., 1975) and microminerals and cofactors (MURASHIGE and SKOOG, 1962), supplemented with 30 g/l sucrose and 10 g/l activated charcoal at pH 5.6, and solidified with 8 g/l agar. The medium was previously autoclaved at 0.1 MPa (121°C) for 20 min.

Heat Shock Stress Treatment

A temperature shock was applied to the isolated anthers containing vacuolated microspores to induce embryogenesis. Anthers isolated from the same catkin were cultured in darkness from 2 to 5 days at 33 °C and then they were transferred to 25 °C.

Two factorial experiments were designed. In the first experiment, anthers were subjected to a cold pretreatment for 3 or 7 days at 4 °C, followed by a heat shock treatment at 33 °C for 3, 4 or 5 days. In the second experiment, anthers pretreated at 4 °C for 5, 6 or 7 days, followed by a heat shock treatment of 33 °C for 2 or 5 days.

Proliferation medium

Globular embryos emerged from anthers were transferred to individual plates containing macronutrients (SOMMER et al., 1975) and microminerals and cofactors (MURASHIGE and SKOOG, 1962), supplemented with 500 mg/l glutamine, 30 g/l sucrose and 8 g/l agar and pH 5.6, and at a temperature of 25 ± 1°C in the dark. Every thirty days, these embryos were subcultured on fresh medium of the same composition. These subcultures were maintained along the year, providing embryos for DNA analysis.

Flow cytometry

For nucleus release, approximately 0.5 cm^2-size samples were chopped with a sharp razor blade in a 55 mm plastic Petri dish containing 500 µl extraction buffer (Partec Cystain UV precise P Kit), and then filtered through a Partec 30 µm celltrics disposable filter. Small globular embryos, the proliferating tissue of the embryo base and the embryo axis without cotyledons provided a good material for the analysis of the ploidy level. The suspension of released nuclei was stained with 1500 µl staining solution (Partec Cystain UV precise P Kit) for 60 seconds.

The relative fluorescence of total DNA from isolated nuclei was analysed with a PA Ploidy Analyzer, Partec. The sample size was at least 10,000 nuclei. Leaf tissue of the male donor trees was used to determine the standard peak of diploid cells (2C DNA).

Microsatellite DNA-markers analysis

Genetic marker analysis allowed a non-tissue-specific search of genotypes in different structures, such as leaves and embryos. These genetic markers may efficiently be used in the characterization of *in vitro* material. In our study, two Single Sequence Repeat (SSR) markers have been used for DNA analysis in *Q. ilex* anther-culture embryos.

DNA Extraction: Leaves of the parent tree were first vacuum-dried for 45 minutes in order to avoid moisture and then they were ground. DNA samples from leaves and embryos regenerated from different anther cultures

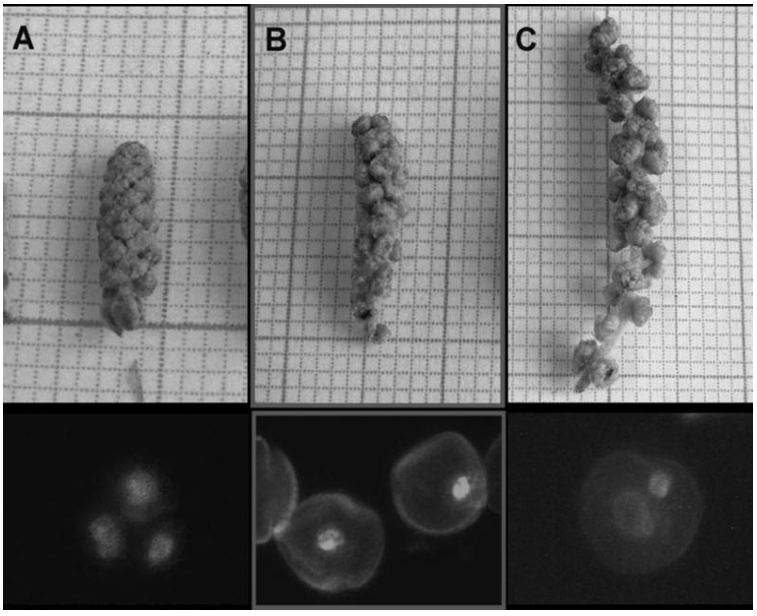

Figure 1. – Series of the parallel development among catkins and microspores stages before pollen release. (A) Tetrad. (B) Uni-nucleated microspore. (C) Early bi-nucleated pollen.

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were isolated following the UltraClean™ Plant DNA Isolation protocol (Mo Bio) after grinding.

Microsatellite-markers: two microsatellite loci were amplified by PCR. DNA was amplified with a Perkin-Elmer 9600 thermocycler. The amplification was prepared following the protocol described by the kit 289006-STR illustra Hot Start Mix RTG (0.2 ml tubes 5x96 rxn) (GE Healthcare). For both microsatellites, the amplification was prepared for a final volume of 25 µl (12 µl milli-Q H2O + 3 µl DNA + 5 µl, 1µM primer F, Direct + 5 µl, 1 µM primer R, Reverse). Primers, SSrQpZAG 36 and SSrQrZAG 20, were designed by STEINKELLNER et al. (1997) for *Quercus petraea* L. and they have been widely used in oak population genetic studies. DNA amplifications were obtained using the following PCR cycling profile: 95 °C for 6 min, 35 cycles of 92 °C for 1 min, 50 °C (SsrQpZAG36) or 52 °C (SsrQrZAG20) for 30 s and 72 °C for 1 min, and then 8 min extension at 72 °C.

Fluorescently labelled amplification products were separated and analysed in an automated DNA sequencer (3730 DNA Analyzer Applied Biosystem) by the Peak Scanner software V1.0 Applied Biosystem. Homologous fragments of the same satellite amplification differing in size – i.e., at least one base pair – were considered "alleles" of the same "gene" or locus.

Statistical analysis

For statistical analysis, the software IBM® SPSS® Statistics Release 20.0.0 (2011) was used. A log-linear model was fitted to all experiments using a Chi-square test. Asymptotic standard errors of the parameter estimates were computed by the Delta method (LEE, 1977). The ratio of the log-linear parameter estimate to its standard error was used to obtain the frequency significance level to compare frequencies of positive response to the treatments.

Results

Characterization of the Developmental Stages of Catkins, Anthers and Microspores

The induction of gametic embryogenesis in *Quercus ilex* was obtained from anther cultures. The phenological stage of holm oak catkins evolved in parallel with anther size and colour, and the microspore stage observed (*Fig. 1*). Immature catkins up to 1 cm size showing green anthers corresponded well with the tetrad stage (*Fig. 1a*). Catkins containing potentially androgenic cells (i.e., either late vacuolated microspores or early bicellular pollen grains) were intermediate in size, between 1 and 1.5 cm, with anthers of green-yellow colour (*Fig. 1b*). At this stage of the microspore, the nucleus is moved towards a pole due to the presence of a big central vacuole. The mature phenological stage, with catkins bigger than 2.5 cm size corresponds with yellowish anthers, late binucleated pollen stage and mature pollen (*Fig. 1c*).

Embryogenesis induction on anther cultures

Isolated anthers containing vacuolated microspores were cultured on induction medium, and subjected to different temperature regimes and in darkness to induce embryogenesis. In the first experiment, both the previous cold treatment at 4 °C and the heat shock treatment at 33 °C stimulated the induction of anther embryogenesis. The log-linear analysis proved that both factors

Table 1. – Number of anthers cultured in the first experiment of embryogenesis induction and percentage of embryogenic anthers. Cold pretreatments at 4 °C lasted 3or 7 days. Heat shock treatments lasted 3, 4, and 5 days at 33 °C.

	Cold Pretreatmet			
	3 days		7 days	
Heat shock (33 °C)	No. of anthers	% embryogenesis	No. of anthers	% embryogenesis
3 days	280	2.5% *	600	0
4 days	480	1.5% *	640	0
5 days	400	0	800	0

*: statistically significant at the 0.05 level.

Table 2. – Number of anthers cultured in the second experiment of embryogenesis induction and percentage of embryogenic holm oak anthers subjected to previous cold treatments of 5, 6 and 7 days at 4 °C and then subjected to a heat shock treatment for 2 or 5 days at 33 °C after culture initiation on induction medium.

	Heat shock (33 °C)			
	2 days		5 days	
Cold Pretreatmet (4° C)	No. of anthers	% embryogenesis	No. of anthers	% embryogenesis
5 days	960	1% *	1160	0.3%
6 days	1400	0.1%	320	0
7 days	600	0.2%	800	0

*: statistically significant at the 0.05 level.

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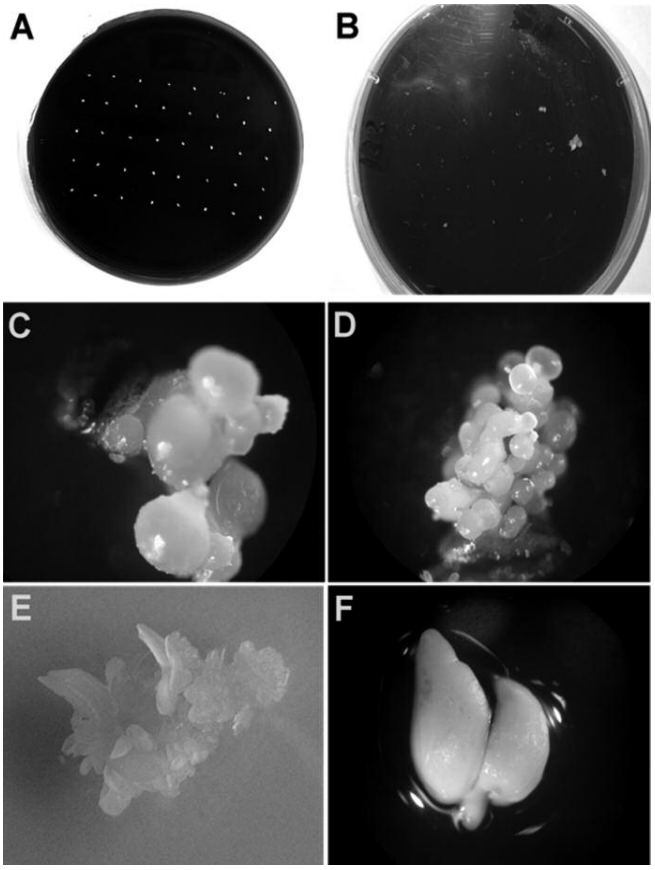

Figure 2. – (A) Holm oak anther cultures on induction medium supplemented with activated charcoal. (B) Microspore embryogenesis from some holm oak anthers. (C, D) Detail of small globular anther embryos emerging from inside the anther. (E) Proliferation of holm oak haploid embryos. (F) Mature embryo of *Quercus ilex* L.

were statistically significant. The frequency of embryogenic anthers was highest for short stimulation periods of 3 days at 4 °C ($p = 0.000$). Heat shock duration was also statistically significant ($p = 0.002$), with highest rates of embryogenesis induction for 3 and 4 days at 33 °C, in combination with a previous cold treatment of 3 days at 4 °C (*Table 1*). A total of 14 embryogenic anthers were obtained in this experiment.

In the second experiment, the highest rate of embryogenesis induction was obtained in anthers subjected to a previous cold treatment for 5 days at 4 °C, followed by a heat shock at 33 °C for 2 days ($p = 0.000$; *Table 2*). A total of 17 embryogenic anthers were obtained in this experiment. Therefore, we obtained a total of 31 embryogenic anthers after 46 to 95 days on induction medium (*Fig. 2a-b*). Several embryos grew from the interior of each of the embryogenic anthers, breaking through the degenerating anther walls. When no visible embryos or calluses were formed, the not-responsive anthers eventually degenerated and died. Initial translucent globular structures, observed growing from the anther (*Fig. 2c*), developed later into heart-shaped and torpedo-shaped embryos, until formation of well-developed cotyledons could be seen (*Fig. 2d*).

Embryo Proliferation

All the embryos obtained from the anther cultures were independently transferred to agar plates with an embryogenic proliferation medium in darkness, where the embryos were clonally propagated. Spontaneous secondary embryogenesis occurred, and subsequent embryos proliferated on the surface of the original embryo (*Fig. 2e*). The embryos changed in appearance from translucent to opaque during the maturation process (*Fig. 2f*). The clones have been maintained along the year under these conditions, and subculture onto fresh plates was carried out once a month.

Determination of Ploidy Level by Flow Cytometry

The ploidy level was analysed by flow cytometry in a sample of 18 anther-derived embryos. Results were displayed in histograms showing the number of nuclei according to relative fluorescence intensity, which is proportional to DNA content (*Fig. 3*). Analysis of the tissues by flow cytometry produced clear peaks of DNA-content at channel 34 showing a clear haploid profile (DNA amount 1C) corresponding to the anther embryos, and at channel 68 using leaf tissue of the male donor tree as diploid control (DNA amount 2C; *Fig. 3a*). Peaks of haploid tissues and the diploid control were clearly resolved, demonstrating that the diploid control contained exactly twice as much DNA as the sample. A high percentage of anther embryos (83.4%) were, in fact, haploids (*Fig. 3b*), fact that confirms their origin from microspores or pollen grains, and not from parental tissue of the anther wall. Nevertheless, some exceptions were found revealing other ploidy levels: 11.1% showed a mixoploid (haplo-diploid) pattern, while 5.5% anther-derived cultures showed a diploid one (DNA amount 2C; *Fig. 3c*).

SSR analysis

The parent trees were heterozygotic for both microsatellite *loci* (*Table 3*). All alleles of the parent trees were also found in the progeny, but only one allele per locus was amplified in each of the 17 haploid embryos tested. All the anther-derived mixoploid embryos had a single set of alleles, a part of which must be doubled. Also, all anther diploid embryos provided evidence of the presence of a single allele per *locus*, demonstrating that those individuals were homozygous for each locus. These results prove that the non-haploid anther-derived embryos of *Q. ilex* have not a somatic but a gametic origin, and these embryos subsequently experienced spontaneous duplication of their haploid genome. Actually, these embryos can be considered doubled-haploids. The pattern of allelic heredity was ana-

Table 3. – Proportion of anther induced embryos bearing microsatellite alleles detected in holm oak parent trees.

Loci	Parent tree allele (bp)	% anther-derived embryos
SSrQp ZAG 36	207	60%*
	217	40%*
SSrQr ZAG 20	162	20%
	168	80%

*: 1:1 Segregation at significance level = 0.05 (Chi-square test).

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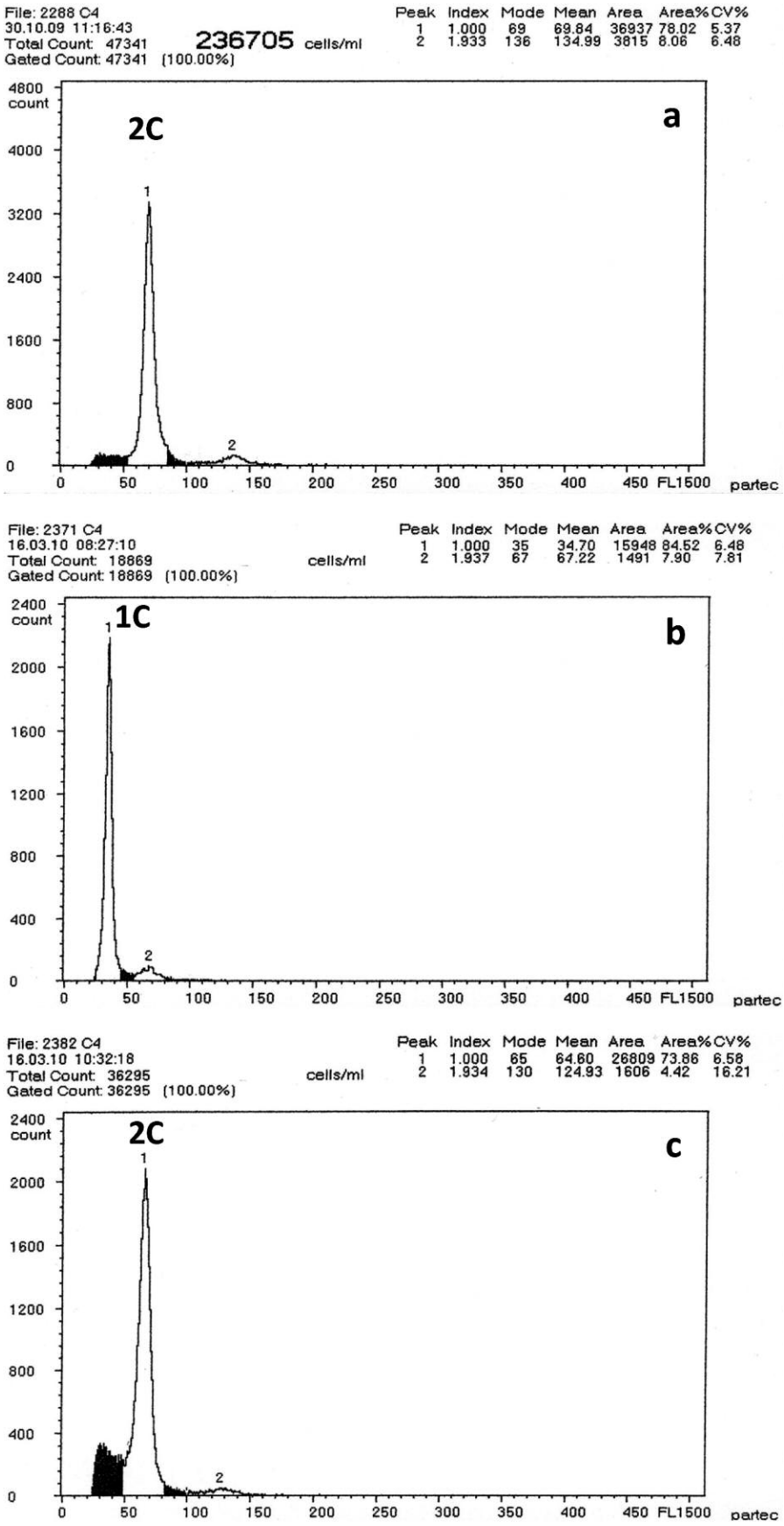

Figure 3. – Flow cytometry histograms of relative DNA content of nuclei released from anther derived holm oak embryos stained with DAPI: (a) Diploid standard from leaf tissue of one of the parental trees (DNA amount 2C); (b) Sample from an anther embryo showing haploid DNA amount (1C); (c) Sample from an anther embryo showing 2C amount of DNA.

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lyzed by a chi-squared test, obtaining a 1:1 segregation for locus SSrQp ZAG 36 only ($p = 0.05$). A total of four genotypes/haplotypes were distinguished.

Discussion

We present the first report demonstrating the successful induction of gametic embryogenesis in *Quercus ilex* L. Previously only somatic embryogenesis, mostly from diploid immature zygotic embryos, had been reported in holm oak (Mauri and Manzanera, 2004, 2011) and other related oaks (e.g., Garcia-Martin et al., 2001, 2005; Gonzalez-Benito et al., 2002; Pintos et al., 2008). Only two cases of haploid embryo induction on anther cultures from related oak species have been published in *Quercus petraea* (Jörgensen, 1988) and *Q. suber* (Bueno et al., 1997). In *Q. petraea*, the induction medium was supplemented with plant growth regulators (BA and 2,4-D), which produced callus at a low frequency (2 to 6%) and indirect embryogenesis on the callus was stimulated with 2.5 µM BA, obtaining three haploid clones (Jörgensen, 1993). In the case of holm oak, we did not add plant growth regulators to the medium and the induction protocol was based on temperature stress treatments, which efficiently switched on the sporophytic pathway of embryogenesis in microspores. Our results are similar to those obtained in *Q. suber* (Bueno et al., 1997; Pintos et al., 2005) in which haploid embryos were obtained from anthers previously preserved at 4 °C for one week, then established in culture and subjected to a heat shock treatment of 33 °C for 5 days, while other treatments at 4, 25, 35 and 37 °C were less effective. Furthermore, we observed in holm oak that responsive-type anthers contained microspores in the optimal stage for the induction of gametic embryogenesis, in a similar pattern to that observed in the related cork oak (Pintos et al., 2005). Anther embryo induction by plant growth regulator treatments is less reliable, as these agents may stimulate callus formation from the parental anther tissues, thus producing a diploid callus and hindering the identification of haploid material. Therefore, we preferred the application of a stress treatment without the use of plant growth regulators for the induction of haploid embryogenesis, as it has proven to be successful in many plant species, e.g. in *Brassica sp.* (Chuong and Beversdorf, 1985), barley (Hoekstra et al., 1992) and rice (Ogawa et al., 1994). It should be taken into account that microspores suffer starvation within the excised anther as the wall represents a barrier for nutrient diffusion from the medium. Also, the levels of carbohydrates stored in the anther decrease rapidly after excision (Prakash and Giles, 1987). Thus, this culture method is in itself a starvation treatment for the microspores. Only after the anther wall is broken down by the protruding embryos, nutrients become available.

A high proportion of the holm oak embryos regenerated from anther culture were haploid (83.4%), indicating that the probable origin were the microspores enclosed in the locules. The haploid amount of DNA of these embryos was measured by DAPI staining of the nuclei and flow cytometry, using a similar technique to that employed in the ploidy level assessment of cork oak anther embryos (Pintos et al., 2007a). On the other hand, the haploid composition of *Quercus petraea* anther embryos was assessed by chromosome count (Jörgensen, 1993), which only permits the analysis of a reduced sample, as compared to the flow cytometry methodology. Nevertheless, we found a small amount of anther embryos containing either a diploid (2C) DNA amount (5.5%) or mixoploid (11.1%). In order to prove the hypothesis of a spontaneous diploidization of the haploid anther embryos, SSR markers have been used. Deutsch et al. (2004) investigated in *Populus* for the ploidy level by flow cytometry and microsatellite markers to assess the true haploid origin of calli and embryos from immature pollen culture. Höfer et al. (2008) successfully applied SSR markers in *Malus* which revealed 100% homozygosity and confirmed that all alleles of the parent cultivars were also found in the progeny. Previously in *Quercus suber*, the use of microsatellite gene markers permitted the verification of the hypothesis that anther embryos are induced from different microspores or pollen grains, as it has been proved by the diverse genetic composition of embryos from the same anther. The donor trees were heterozygous at all *loci*, this being a prerequisite for investigating the haploid origin of the regenerants (Gómez et al., 2001).

In *Quercus ilex*, all haploid as well diploid embryos exhibited one of the paternal alleles at all SSR loci. In the diploid embryos, homozygosity and thus multiple haploidy were confirmed. One of the aims of our analysis by microsatellite markers was to prove the hypothesis of a spontaneous duplication of the haploid genome and the results obtained in this work verify this hypothesis. Diploid anther embryos had only one allele per locus, revealing a homozygotic genome for all loci tested. This result confirms the applicability of microsatellite markers as indicators of the ploidy level in embryo regeneration from anther cultures.

Conclusion

We successfully obtained haploid and doubled-haploid embryos from holm oaks. Catkins containing potentially embryogenic cells were between 1–1.5 cm size, with 2 mm size flowers and 1 to 1.5 mm size anthers of green-yellow colour. The best stress treatment consisted of a cold pre-treatment (4 °C) on the anther for 3 days, followed by a heat shock (33 °C) for 3 or 4 days, in a simple agar medium plus 1% activated charcoal and without growth regulators. Embryos were clonally propagated, and ploidy level and the origin of such embryos, either somatic or gametic, were elucidated by flow-cytometry and SSR markers.

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References

AMSSA, M., J. DE BUYSER and Y. HENRY (1980): Origin of diploid plants obtained by in vitro culture of anthers of young wheat (*Triticum aestivum* L.). Cr Acad Sci D Nat **290**: 1095–1097.

BUENO, M. A., A. GÓMEZ, M. BOSCAIU, J. A. MANZANERA and O. VICENTE (1997): Stress-induced formation of haploid plants through anther culture in cork oak (*Quercus suber*). Physiol Plantarum. **99**: 335–341.

BUENO, M. A., M. D. AGUNDEZ, A. GÓMEZ, M. J. CARRASCOSA and J. A. MANZANERA (2000): Haploid origin of cork oak anther embryos detected by enzyme and RAPD gene markers. Int J Plant Sci **161**: 363–367. DOI: 10.1086/314265

BUENO, M. A., A. GÓMEZ, F. SEPULVEDA, J. M. SEGUÍ, P. S. TESTILLANO, J. A. MANZANERA and M. C. RISUEÑO (2003): Microspore-derived from *Quercus suber* anthers mimic zygotic embryos and maintain haploidy in long-term anther culture. J Plant Physiol **160**: 953–960.

BUENO, M. A., B. PIONTOS, M. J. PRADO, A. GÓMEZ and J. A. MANZANERA (2004): Androgenesis: A tool for woody plant breeding, pp. 365–383 Vol 1. Part II in Recent Research Developments in Genetics & Breeding edited by S.G.Pandalai, Research Signpost

CHUONG, P. V. and W. D. BEVERSDORF (1985): High frequency embryogenesis through isolated microspore culture in *Brassica napus* L. and *B. carinata* Braun. Plant Sci **39**: 219–226.

DEUTSCH, F., J. KUMLEHN, B. ZIEGENHAGEN and M. FLADUNG (2004): Stable haploid poplar callus lines from immature pollen culture. Physiol Plantarum **120(4)**: 613–622.

DUNCAN, E. J. and E. HEBERLE (1976): Effect of temperature shock on nuclear phenomena in microspores of *Nicotiana tabacum* and consequently on plantlet production. Protoplasma **90**: 173–177.

FERRIE, A. M. R. and C. MÖLLERS (2011): Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. Plant Cell Tiss Organ Cult **104**: 375–386.

FORSTER, B. P., E. HEBERLE-BORS, J. KASHA K. and A. TOURAEV (2007): The resurgence of haploids in higher plants. Trends Plant Sci **Vol. 12 No. 8**: 368-375.

GARCÍA-MARTÍN, G., M. E. GONZÁLEZ-BENITO and J. A. MANZANERA (2001): *Quercus suber* L. somatic embryo germination and plant conversion: pretreatments and germination conditions. In Vitro Cellular & Developmental Biology- Plant. **37(2)**: 190–198.

GARCÍA-MARTÍN, G., J. A. MANZANERA,and M. E. GONZÁLEZ-BENITO (2005): Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos. Plant Cell Tissue Organ Cult. **80(2)**: 171–177.

GERMANÀ, M. A. (2011): Gametic embryogenesis and haploid technology as valuable support to plant breeding. Plant Cell Rep **30**: 839–857.

GÓMEZ, A., B. PINTOS, E. AGUIRIANO, J. A. MANZANERA and M. A. BUENO (2001): SSR markers for *Quercus suber* tree identification and embryo analysis. J Hered **92**: 292–295.

GÓMEZ, A., J. A. LÓPEZ, B. PINTOS, E. CAMAFEITA and M. A. BUENO (2009): Proteomic analysis from haploid and diploid embryos of *Quercus suber* L. identifies qualitative and quantitative differential expression patterns. Proteomics **9**: 4355–4367.

GONZÁLEZ-BENITO, M. E., G. GARCÍA-MARTÍN and J. A. MANZANERA (2002): Shoot development in *Quercus suber L.* somatic embryos. In Vitro Cellular and Developmental Biology-Plant **38**: 477–480.

GUHA, S. and S. C. MAHESHWARI (1966): Cell division and differentiation of embryos in the pollen grains of *Datura in vitro*. Nature **212**: 97–98.

GUSTAFSON, V. D., P. S. BAENZIGER, M. S. WRIGHT and W. W. STROUP (1995): Isolated wheat microspore culture. Plant Cell Tissue Organ Cult **42**: 207–213.

HOEKSTRA, S., M. H. VAN ZIJDERVELD, J. D. LOUWERSE, F. HEIDEKAMP and F. VAN DER MARK (1992): Anther and microspore culture of *Hordeum vulgare* L. cv. Igri. Plant Sci **86**: 89–96.

HÖFER, M., C. H. GRAFE, A. BOUDICHEVSKAJA, A. LÓPEZ, M. A. BUENO and D. ROEN (2008): Characterization of plant material obtained by *in vitro* androgenesis and in situ parthenogenesis in apple. Sci Hortic-Amsterdam **117**: 203–211.

JÖRGENSEN, J. (1988): Embryogenesis in *Quercus petraea* and Fagus sylvatica. Journal of Plant Physiology **132(5)**: 638–640. http://dx.doi.org/10.1016/S0176-1617(88)80269-4.

JÖRGENSEN, J. (1993): Embryogenesis in *Quercus petraea*. Ann For Sci 50 Suppl 1, 344s-350s.

KIM, M. (1999): The influence of temperature pretreatment on the production of microspore embryos in anther culture of *Capsicum annuum* L. Kor J Plant Tissue Cult **26**: 71–76.

KIM, M., J. KIM, M. YOON, D. I. CHOI and K. M. LEE (2004): Origin of multicellular pollen and pollen embryos in cultured anthers of pepper (*Capsicum Annuum*) Plant Cell, Tissue and Organ Culture **77(1)**: 63–72. DOI: 10.1023/B:TICU.0000016506.02796.6a.

LEE, S. K. (1977): On the asymptotic variances of u-terms in loglinear models of multidimensional contingency tables. J Amer Statist Assoc **72**: 412–419.

LIPPERT, D., J. ZHUANG, S. RALPH, M. GILBERT, R. OLAFSON, K. RITLAND, B. ELLIS, C. DOUGLAS and J. BOHLMANN (2005): Proteome analysis of early somatic embryogenesis in *Picea glauca*. Proteomics **5**: 461–473.

LLOYD, G., and B. H. MCCOWN (1981): Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. Proc. Int Plant Propag Soc. **30**: 421–427.

MALUSZYNSKI, M., J. K. KASHA K. and SZAREJKO (2003): Published doubled haploid protocols in plant species, in Doubled Haploid Production in Crop Plants, edited by M. MALUSZYNSKI et al. IAEA, Netherlands.

MAURI, P. V. and J. A. MANZANERA (2004): Effect of Abscisic Acid and stratification on somatic embryo maturation and germination of holm oak (*Quercus ilex* L.). In Vitro Cell. Dev. Biol. Plant **40**: 495–498.

MAURI, P. V. and J. A. MANZANERA (2011): Somatic embryogenesis of holm oak (*Quercus ilex* L.): ethylene production and polyamine content. Acta Physiologiae Plantarum **33**: 717–723. DOI: 10.1007/s11738-010-0596-5.

MICHAUD, H., L. TOUMI, R. LUMARET, T. X. LI, F. ROMANE and F. DI GIUSTO (1995): Effect of geographical discontinuity on genetic variation in *Quercus ilex* L. (holm oak). Evidence from enzyme polymorphism. Heredity **74**: 590–606.

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