Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA)
Available online at www.inia.es/forestsystems
doi: http://dx.doi.org/10.5424/fs/2014232-05829

Forest Systems 2014 23(2): 191-198
ISSN: 2171-5068
eISSN: 2171-9845

Embryogenesis in Oak species. A review

Aranzazu Gomez-Garay*[1], Jose A. Manzanera[2] and Beatriz Pintos[1]

[1] Departamento de Biología Vegetal I: Fisiología Vegetal. Facultad de Biología. UCM. C/ José Antonio Novais, nº 2. 28040 Madrid, Spain. [2] ETSI Montes. Universidad Politécnica de Madrid. 28040 Madrid, Spain

Abstract

Aim of study: A review on the propagation methods of four *Quercus* species, namely *Q. suber, Q. robur, Q. ilex* and *Q. canariensis*, through somatic embryogenesis and anther embryogenesis are presented.

Area of study: The study comprises both Mediterranean and Atlantic oak species located in Spain.

Material and methods: Somatic embryogenesis was induced on immature zygotic embryos of diverse oak species, permitting the multiplication of half-sib families. Induction of haploid embryos and doubled haploids was assayed in both *Q. suber* and *Q. ilex* by temperature stress treatments of anthers containing late vacuolated microspores. The haploid origin of the anther embryos has been evaluated by quantitative nuclear DNA analysis through flow cytometry and by DNA microsatellite markers. Genetic transformation of cork oak has also been performed by means of *Agrobacterium tumefaciens* vectors. Proteomic analysis has been conducted to screen the diverse protein profiles followed by *in vitro* derived embryos during their development.

Research highlights: Successful plant regeneration from both somatic and haploid embryos has been achieved. In the particular case of cork oak, doubled-haploid plants were obtained. Plantlets regenerated from selected parent trees through somatic embryogenesis were acclimated in the greenhouse and in the nursery, and were planted in an experimental plot in the field. Preliminary evaluation of the cork quality of the plants showed a good heritability correlation with the parent trees. This article revises the work of and is dedicated to Dr. M. A. Bueno, who devoted much of her professional life to the research on Biotechnology and Genetics of forest species, leading the Laboratory of Forest Biotechnology at the Spanish Institute of Agronomic Research (INIA).

Key words: anther embryogenesis; microspore; pollen; *Quercus canariensis; Quercus ilex; Quercus robur, Quercus suber*; somatic embryogenesis.

Somatic embryogenesis in cork oak

Cork oak (*Quercus suber* L.) is a forest species playing a major role in many Mediterranean ecosystems. Besides, this tree produces cork of economic value for the champagne and wine stopper industry and many other manufactures, contributing to the rural development in its natural area. However, cork oak is a long life cycle tree with irregular fructification seasonality and difficulty of seed conservation. Furthermore, vegetative propagation of mature individuals is not viable by classic methods. Thus, all these difficulties have impeded the development of genetic improvement programs for this species. Among the alternatives to overcome the vegetative propagation problem, somatic embryogenesis has been the most successful. The traditional breeding methods of self-pollination and backcrossing can be advantageously substituted by pollen or haploid embryogenesis, also permitting the obtainment of heterosis by hybridization.

First attempts to regenerate cork oak (*Quercus suber*) through somatic embryogenesis were conducted at the INIA Tissue Culture Laboratory by exploring the regeneration capacity of zygotic embryos, endosperm and ovules (Bueno and Manzanera, 1992). Samples were collected every two weeks along the acorn development period, from June to September. The culture medium was composed of Sommer *et al*. (1975) macronutrients and Murashige and Skoog (MS) micronutrients (1962), plus 88mM sucrose and 3.4 mM glutamine (Gln). By far, zygotic embryos were the most responsive explant type, giving rise to somatic embryos. Although a small amount of embryos were obtained from ovules, it could not be elucidated if those embryos from ovular origin were somatic or zygotic

* Corresponding author: magom02@bio.ucm.es
Received: 27-02-14. Accepted: 30-04-14.

Figure 1. Cork-oak somatic embryo originated from zygotic embryo.

embryos protruding from inside the ovule (Bueno and Manzanera, 1992).

Immature zygotic embryos were subjected to different treatments with 2,4-dichlorophenoxyacetic acid (2,4-D) for 30 days. Callus was formed mainly from the hypocotyl, although cotyledons also produced callus. Then the explants were transferred to growth regulator-free medium and globular structures were visible after two or three weeks (Bueno *et al.,* 1992). Somatic embryos were formed either directly (Fig. 1) on the zygotic embryos or indirectly in the callus (Bueno *et al*., 2000b). The effect of culture on agar (8 g/l) solid medium was compared to liquid medium agitated on an orbital shaker at 100 rpm. Although a slightly higher rate of somatic embryogenesis was observed in liquid medium with 2.3 µM 2,4-D than in agar medium with higher 2,4-D concentrations, no statistically significant differences were recorded. Somatic embryo maturation was stimulated with a cold storage (5°C) treatment prior to germination. Other factors, such as spermine, sorbitol or air-drying desiccation were not effective (Bueno *et al.,* 1992). The beneficial effect of cold storage on later germination of the somatic embryos was corroborated by testing two and ten week-long cold treatments at 2 and 4°C. Significant differences were obtained for ten weeks at 4°C and two weeks at 2°C as compared to the control, but not for two or four weeks at 4°C (Manzanera *et al.,* 1993). The problem of epicotyl dormancy was overcome by placing the somatic embryos on paper bridges, in test tubes containing 10 ml medium supplemented with 0.4 µM benzyl adenine (BA). Plantlets with normally developed shoots were transferred to soil and acclimated in the greenhouse (Bueno *et al.,* 2000b). Microscopic studies revealed a well-developed histological structure of the cork oak somatic embryos, with a prominent apical meristem between cotyledons, a root meristem and the calyptra. Vascular bundles were already visible in both the embryo axis and the cotyledons (Bueno *et al.,* 2000b).

Cork oak somatic embryos have proven to be a good starting material for genetic transformation experiments. This species is threatened by several pests including members of *Lepidoptera* larvae and *Coleoptera*, which dig galleries in the bark, damaging the cork. These threats may be obviated by genetic manipulation to pest-resistance, employing any of several methods and vectors. Initial experiments were conducted with pro-embryo masses induced on immature zygotic embryos with 2.3 µM 2,4-D. As selection agents, two antibiotics were compared, kanamycin and hygromycin (Sanchez *et al*., 2005). While the first was not effective at concentrations as high as 850 µM, the latter was successful as selection agent. Cork oak pro-embryo masses were inoculated with the *Agrobacterium tumefaciens* LBA4404/p35S GUS INT/pCAMBIA 1301 strain, which contains genes *hpt*II, conferring hygromycin resistance, and *gus*A gene coding for β-glucuronidase (GUS). Transformants were selected on hygromycin 94 µM-supplemented medium. Fifty two viable embryos survived out of 400 selected on hygromycin during four months (13%). The transformation of 5.8% embryos selected on hygromycin was confirmed by expression of β-glucuronidase four months after co-cultivation (Fig. 2) and by the presence of nopaline synthase terminator, amplified by polymerase chain reaction. This method is one of the few reports on the genetic transformation of cork oak somatic embryos (Sanchez *et al.,* 2005).

The final step of plant regeneration from somatic embryos is the production of synthetic seeds. In cork oak, somatic embryos were coated with alginate and their storability for commercialization was investigated (Pintos *et al.,* 2008). The encapsulation medium consisted of Sommer *et al*. (1975) macronutrients without calcium, plus MS micronutrients and supplemented with 5% (w/v) sodium alginate. Then the embryos were immersed in 50 mM $CaCl_2$ for 20 min for complexing. The addition of 30 g/l sucrose to the capsule helped later germination, reaching a 73% rate (Pintos *et al*., 2008).

Also, a new method for the automatic monitoring of somatic embryo growth with a digital system of image

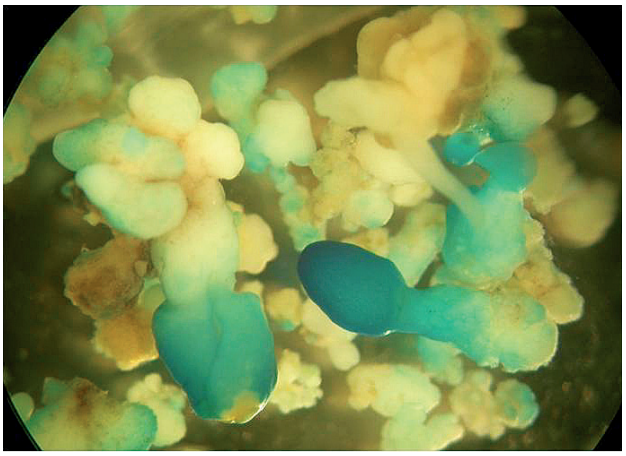

Figure 2. Cork-oak somatic embryo showing GUS positive reaction.

capture was tested (Pintos *et al.,* 2008). For this purpose, the projected area of each embryo was measured with an image analyzer and regression models fitted between the projected area and fresh weight. The best fit was obtained with the power regression (Adjusted R-squared = 0.96, $p < 0.0001$). This methodology permits growth monitoring without contamination risk and will be a helpful tool for the automated control of culture growth for the up scaling of plant production (Pintos *et al.,* 2008).

After optimizing the production of cork oak somatic embryos and synthetic seeds in the laboratory, they are ready for transfer to soil and the establishment of plantations. Somatic embryos germinated into plantlets, producing more than 900 individuals which were transferred to 100 ml nursery pots filled with peat: perlite: vermiculite (1:1:1). These plantlets were acclimated in successive steps in the greenhouse and in the nursery. The high survival rate obtained, 78% (Pintos *et al.,* 2010), proved that this methodology is applicable for large scale plantlet production. In the second year of nursery, the plantlets were transferred to 2 l pots with peat: vermiculite (3:1). Around 500 plantlets were established in a plot in their natural area, as a progeny test at 3.5 m × 3.5 m spacing (Pintos *et al.,* 2009).

Phenotypic traits of parent trees and half-sib plantlets derived from these parent trees by somatic embryogenesis were evaluated following standard histology techniques. The percentage of virgin cork thickness of the main shoot of the somatic embryo-derived plantlets showed a moderately strong association with the cork thickness in twigs of their parent trees. This progeny test permitted the estimation of a high heritability of this trait from parent phenotypes to their progenies (Pintos *et al.,* 2009). Therefore, cork oak plantlets were successfully regenerated and planted in the field and showed a cork quality trait related to that of their parent trees. These results open a new perspective for the large-scale propagation of cork oak plants, ready for future extraction of high quality cork. The transfer of this technology will be interesting for this forest industry sector.

Somatic embryogenesis in *Quercus robur*

Pedunculate oak (*Q. robur*) is widespread in Europe, from the Atlantic coast to the Ural and Caucasus Mountains, and from the Mediterranean to Southern Scandinavia. This wide distribution leads to a great intraspecific variability. This tree can be several centuries old and reach 40 m height and 1 m diameter at breast height. The wood is durable, dense and of fine grain. This species is also appreciated as ornamental. The acorn is difficult to store and production is irregular. Traditional vegetative propagation is also very problematic. To solve these difficulties, somatic embryogenesis has also been assayed in this oak. First results in this species were obtained by Chalupa (1990), who reported successful plantlet regeneration from immature embryos cultured on MS or Woody Plant Medium (WPM; Lloyd and McCown, 1981), supplemented with BA 4.4 µM, alone or combined with 2.9 µM gibberellic acid (GA_3) or indole-butyric acid (IBA) 0.5 to 5 µM. Similar results were obtained by Hubner *et al.* (1995) on medium supplemented with 1 µM BA + 1 µM 2,4-D.

Zygotic embryos of pedunculate oak (*Quercus robur* L.), extracted from acorns collected at different stages of development from June to November, were established at the INIA Tissue Culture Laboratory on WPM supplemented with Gln. The best explants form somatic embryogenesis induction were the immature embryos collected in June and July, while later stages were not responsive. Two weeks after culture onset, the first embryos were visible (Fig. 3). Other types of explants, such as excised cotyledons from mature embryos, shoot internodes and leaf fragments also were cultured but only gave rise to callus or roots without further response (Manzanera *et al.,* 1996).

The highest percentages of direct somatic embryogenesis were obtained with the addition of BA 4.4 µM

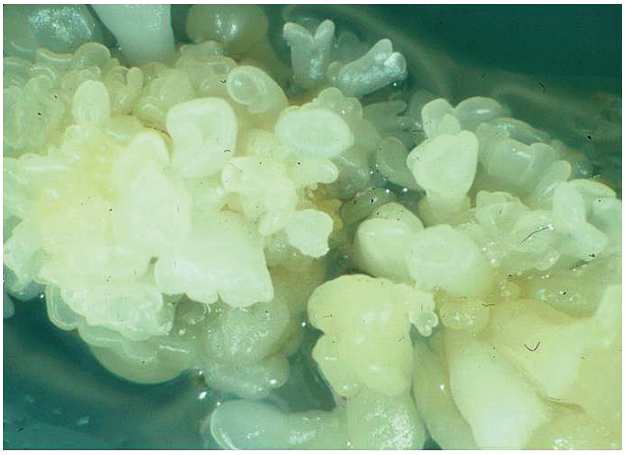

Figure 3. *Quercus robur* somatic embryo originated from zygotic embryo.

alone or BA 0.4 µM combined with 1-naphthaleneacetic acid (NAA) 0.05 µM, while higher concentrations (BA 44.4 µM with NAA 5.7 µM) were ineffective. In zygotic embryos subjected to 2,4-D 4.5 µM, a friable callus was observed. Then this callus was transferred to liquid medium. After two weeks, 66% showed indirect somatic embryogenesis and a few weeks later the first somatic embryos were visible. Somatic embryos were transferred to WPM supplemented with lower BA concentrations (0.9 to 2.7 µM) in the light for further development (Manzanera *et al.,* 1996).

Somatic embryogenesis in *Quercus canariensis*

Quercus canariensis is an oak endemic of the Western Mediterranean, thriving in the Toledo Mountains, south west and north east of Spain, and also in southern Portugal, Tunisia, Algeria and Morocco. Very often, this oak appears sharing mixed stands with other oaks, such as *Q. suber, Q liex, Q. pyrenaica* and *Q. faginea*. This oak species plays an important ecological role as the acorn nourishes many species of the ecosystem. The conservation of this interesting species has been first approached by somatic embryogenesis at the INIA Tissue Culture Laboratory. Somatic embryos were induced on immature zygotic embryos collected in July. The explants were cultured in Petri dishes in WPM macronutrients with MS micronutrients, 88 mM sucrose, 8 g/l agar and Gln. Globular structures appeared three weeks after induction onset. After two months, more than 30% explants subjected to 4.5 µM

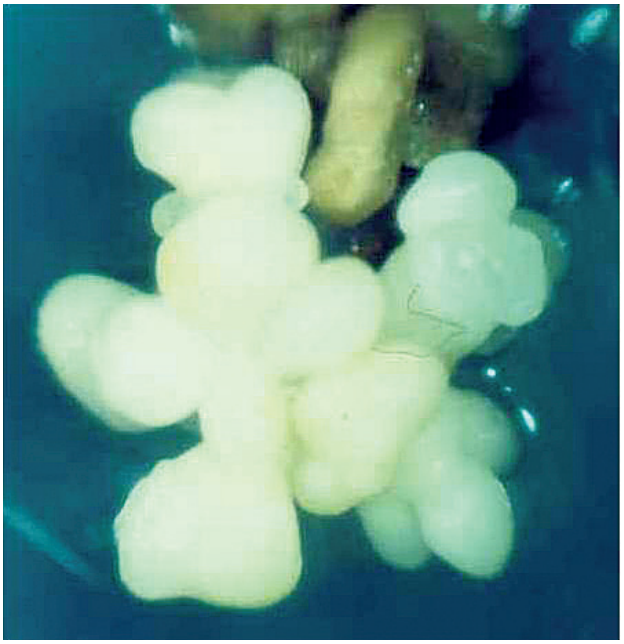

Figure 4. *Quercus canariensis* somatic embryo originated from inmature somatic embryo.

2,4-D produced embryos (Fig. 4). These somatic embryos grew and developed in basal medium supplemented with Gln. Six months later, the embryos were chilled for two weeks at 4°C in darkness before they were transferred to germination medium. This has been the single attempt to date to regenerate this species by somatic embryogenesis or tissue culture in general (Bueno *et al.,* 1996). Also, the stability in ploidy level was assessed by flow cytometry in somatic embryos clonally propagated for more than one year in culture. All somatic embryos were diploid, with a DNA content of 2.2 ± 0.1 pg per nucleus, slightly greater than the 1.9 pg nuclear content measured in cork oak (Bueno *et al.,* 1996). So far, this has been the first and single published work on the regeneration of this species by somatic embryogenesis. More research is needed to improve and upscale this technology.

Pollen embryogenesis in cork oak

Direct embryogenesis from pollen is a process by which germ cells shift from their normal gametophytic development to a sporophytic development. Therefore, instead of mature pollen grains, haploid embryos are obtained. Microspore embryogenesis in cork oak is one of the first examples in woody plants. Specifically, the INIA Tissue Culture Laboratory was the first to initiate

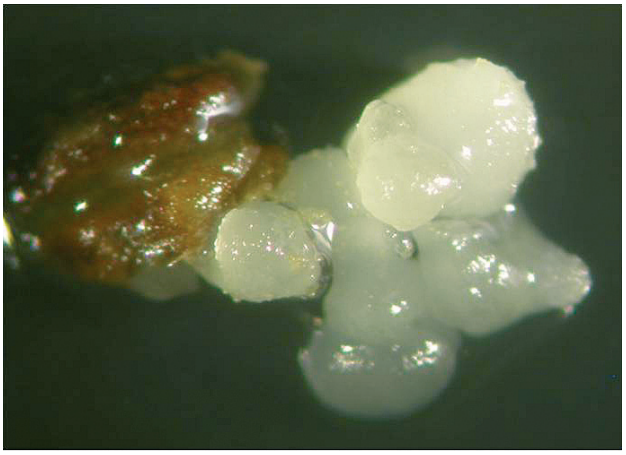

Figure 5. Small globular embryos of cork-oak emerging from inside the anther.

this work on cork oak. Anthers were subjected to different types of stress, such as thermic stress or starvation. In cork oak, microspore embryogenesis was successfully achieved by culturing isolated anthers on basic agar-solidified medium composed of macro- and micronutrients at33°C for five days. It is hypothesized that microspores suffer sucrose and nutrient starvation inside the anther thanks to the barrier effect of the anther walls. The induction medium is also supplemented with activated charcoal, which absorbs inhibitory substances, *e.g.* polyphenols and their oxidation derivatives, the quinones. After the induction under thermic stress, cultures are maintained at 25°C until embryos burst out of the anther (Fig, 5). Once the anther wall is broken, the embryos feed on the culture medium and grow normally (Bueno *et al.,* 1997).

The process of embryogenesis induction only takes place when the microspores are in a narrow developmental stage. The optimal period for the induction of the sporophytic pathway in cork oak is the late uninucleated microspore, which is characterized by the presence of a big central vacuole and the nucleus in lateral position. Although the maturation of cork oak pollen is asynchronous, a close relationship has been established between the phenology of catkins and anthers and the developmental stage of microspores. This has facilitated the adequate application of the thermic stress to the anthers (Pintos *et al.,* 2005).

The verification of anther embryo origin is a problem associated to anther culture. In cork oak, the induction medium does not contain plant growth regulators which would favor the induction of somatic embryos from the anther tissues, but it is necessary to assess the ploidy level of anther embryos by flow cytometry or chromosome counting to warrant the haploid origin of those embryos (Bueno *et al.,* 1997). Furthermore, isozyme (Bueno *et al.,* 2000a) and microsatellite DNA molecular markers have been used to verify the haploid origin of anther embryos by analyzing their genotype for loci that show heterozygosity in the parent tree (Gomez *et al.,* 2001).

A great number of microspores shift to the sporophytic pathway and a great diversity of haplotypes is obtained, providing useful material for future studies. Embryogenic lines can be characterized and traced by means of different molecular markers, such as Random Amplified Polymorphic DNA (RAPD) and microsatellites (Bueno *et al*., 2000a; Gomez *et al*., 2001). These embryogenic lines are subcultured for recurrent embryogenesis, and the secondary embryos maintain the original ploidy level and anatomic traits, warranting the quality of this system of pollen embryo production (Bueno *et al.,* 2003).

Flow cytometry analysis of pollen embryo samples in cork oak has shown a low rate of diploid embryos (7.78%). The haploid origin and later spontaneous diploidization of those embryos has been assessed from their homozygous genotype, as revealed by molecular marker tests (Bueno *et al.,* 2000a, 2003; Gomez *et al.,* 2001). However, pure-bred, also called true breeding organisms are interesting for research and for practical breeding. Cork oak pure-bred has been induced by means of antimitotic agents that stimulate chromosomic doubling. Among the antimitotics tested, colchicine, amiprophos-methyl and oryzalin, the latter has been more efficient. Anther embryos treated with 0.01 mM oryzalin for 48 h showed a 50% chromosome doubling rate (Pintos *et al.,* 2007).

Haploid and doubled-haploid plants open a wide range of future applications for genetic improvement, hybridization to obtain heterosis, shortening of breeding cycles, genome sequencing, generation of completely homozygous lines, genetic transformation, somatic hybridization, etc.

Pollen embryogenesis in *Quercus ilex*

Holm oak (*Quercus ilex* L.) is the predominant tree species in many natural communities of the West Mediterranean. The dominant structure in the natural areas of this species is as wooded meadows, and its

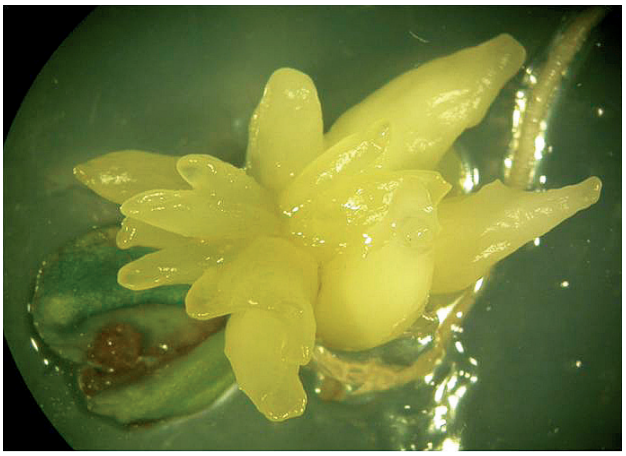

Figure 6. Small globular embryos of *Quercus ilex* emerging from inside the anther.

main economic importance is the agroforestry system. This structure is well known in Mediterranean countries and receives specific names, such as "dehesa" in Spanish, "montado" in Portuguese, etc. As Holm oak has a prolonged life and a late sexual maturation with irregular reproductive cycles, difficulties are found for seed conservation, vegetative reproduction, and for the establishment of seed orchards. All these problems hamper the production of plant material for forest restoration, reforestation and for breeding and genetic programs. The breeding process has been shortened through the induction of haploid embryos and doubled-haploids (Pintos *et al.,* 2013). No previous results on gametic embryogenesis of Holm oak have been described.

Catkins were collected during middle flowering at different phenologic stages, finding a relationship with the anther phenology. This relationship has permitted the characterization of the optimal phase for induction of embryogenesis from microspores. The highest rate of late vacuolated microspores and early bi-cellular pollen grains was found in 1 to 1.5 cm-long, green-yellow anthers. Late microspores show a polar nucleus and a great central vacuole. At this stage, anthers were subjected to a cold stress pre-treatment of 4°C for 3 days, followed by a heat shock treatment at 33°C for 3 or 4 days. As a result, embryos were induced in the anther locule. A few days later, the embryos emerged from the interior of the embryogenic anthers, breaking through the walls (Fig. 6). Initial translucent globular embryos developed later into heart-shaped and torpedo-shaped embryos, until formation of well-developed cotyledons could be observed. Later on, the quantitative and qualitative DNA analysis performed through flow cytometry and DNA-microsatellite markers showed haploid profiles and/or spontaneous doubling of the chromosomes during early regeneration stages (Pintos *et al.,* 2013).

Proteomic study of the process of development in *Quercus suber*

Proteomics has permitted the determination of gene expression changes during the development of somatic embryos of cork oak, from early mass proliferation stages to the mature stage, when it is ready for germination (Gomez *et al.,* 2013). Relevant changes are observed in proteins involved in the detoxification of Reactive Oxygen Species (ROS) and ROS-related stress (38.6% differential proteins), cell division (31.8%), accumulation of storage substances (mainly starch and reserve proteins, 15.8%), glycolysis (15.8%) and in a lesser extent, ethylene and polyamine synthesis (8%).

In the proliferation stage, fermentation is the main energy source for the cells. Among the proteins involved in this process, some of them are specifically induced during somatic embryogenesis, for instance serine hydroxyl-methyl-transferase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triose-phosphate isomerase. This phase is characterized by a high cell division rate, which demands a great amount of energy. Thus, many proteins involved in this process are identified, *e.g.,* RNA-binding protein, Phosphate binding-protein, 14-3-3 proteins and alpha-tubulin proteins. During embryo development, an increment in ROS detoxification-related proteins has been observed, *i.e.* in superoxide dismutase (SOD), catalase and ascorbate peroxidase. This implies a certain level of oxidative stress during proliferation, which is controlled to permit further development to the formation of cotyledons. In this stage, proteins involved in carbohydrate accumulation (starch phosphorylase and granule bound starch) and reserves (legumin) are observed. Embryo maturation seems to be regulated by ethylene and polyamine synthesis, in association with increasing levels of methionine-related proteins (methionine synthetase and 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase).

The comparative proteomic analysis of somatic and doubled-haploid embryos has shown significant differences between both types (Gomez *et al.*, 2009). Hete-

rozygous somatic embryos and homozygous doubled-haploid embryos differed in the stress regulation, mainly in the quantity of SOD and peroxidase. Other differences have been observed in the actin content, involved in pollen development, which is more abundant in embryos induced from late microspores than in somatic embryos. The relative content of proteins involved in tannin and phenylpropanoid metabolism is also greater in pollen embryos. These components represent two of the major synthetic pathways of cork chemistry, and they could be related to the homozygosis of pollen embryos. The early detection of elevated contents of these proteins could be used as marker tests of cork quality.

Acknowledgements

We are grateful to Dr. María Angeles Bueno, who devoted much of her professional life to the research on Biotechnology and Genetics of forest species, leading the Laboratory of Forest Biotechnology at the Spanish Institute of Agronomic Research (INIA). We also thank Nieves Sanchez, Elena Garcia, Dolores Salvador, Carmen Garcia and to all our colleagues of the Laboratory who helped Dr. Bueno.

References

Bueno MA, Astorga R, Manzanera JA, 1992. Plant regeneration through somatic embryogenesis in *Quercus suber*. Physiol Plant 85: 30-34.

Bueno MA, Manzanera JA; 1992. Primeros ensayos de inducción de embrioides somáticos de *Quercus suber* L. Scientia Gerundensis 18: 29-37.

Bueno MA, Gómez A, Vicente O, Manzanera JA, 1996. Stability in ploidy level during somatic embryogenesis in *Quercus canariensis*. In: MR Ahuja, W Boerjan & DB Neale (eds): Somatic Cell Genetics and Molecular Genetics of Trees. Kluwer Academic Publishers, pp: 23-28.

Bueno MA, Gómez, A, Boscaiu M, Manzanera JA, Vicente, O, 1997. Stress-induced formation of haploid plants through anther culture in cork oak (*Quercus suber*). Physiol Plant 99: 335-341.

Bueno MA, Agundez MD, Gómez A, Carrascosa MJ, Manzanera JA, 2000a. Haploid origin of cork oak anther embryos detected by enzyme and RAPD gene markers. Int J Plant Sci 161: 363-367.

Bueno MA, Gómez A, Manzanera JA, 2000b. Somatic and gametic embryogenesis in *Quercus suber* L. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic Embryogenesis in Woody Plants. Vol. 6 Kluwer Academic Publishers, pp 479-508.

Bueno MA, Gomez A, Sepulveda F, Segui JM, Testillano PS, Manzanera JA, Risueño MC, 2003. Microspore-derived embryos from *Quercus suber* anthers mimic zygotic embryos and maintain haploidy in long term anther culture. Journal of Plant Physiology 160: 953-960.

Chalupa V, 1990. Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). Plant Cell Reports 9: 398-401.

Gómez A, Pintos B, Aguiriano E, Manzanera JA, Bueno MA. 2001. SSR markers for *Quercus suber* tree identification and embryo analysis. Journal of Heredity 92(3): 292-295.

Gomez A, López JA, Pintos B, Camafeita E, Bueno MA, 2009. Proteomic analysis from haploid and diploid embryos of *Quercus suber* L. identifies qualitative and quantitative differential expression patterns. Proteomics 9: 4355-4367.

Gomez A, López JA, Bueno MA, Camafeita E, Pintos B, 2013. Proteomic perspective of *Quercus suber* somatic embryogenesis, J Prot.

Hubner E, Muller K, Heyder J, Schmitt HP, 1995. Somatic embryogenesis and shoot development as function of 2,4-D and BAP-concentrations in zygotic embryos of late flushing pedunculate oak (*Quercus robur* L). Silvae Genetica 44(5-6): 225-229.

Lloyd G, McCown BH, 1981.Commercially-feasible micropropagation of Mountain laurel, *Kalmia latifolia* by use of shoot tip culture. Int Plant Prop Soc Proc 30: 421-427.

Manzanera JA, Astorga R, Bueno MA, 1993. Somatic embryo induction and germination in *Quercus suber* L. Silvae Genetica 42 (2-3): 90-93.

Manzanera JA, Bueno MA, Pardos JA, 1996. *Quercus robur*. In: Bajaj YPS (ed): Biotechnology in Agriculture and Forestry. Vol 35. Trees IV. Springer. pp: 321-341.

Murashige T, Skoog F, 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497.

Pintos B, Manzanera JA, Bueno MA, 2005. Cytological analysis of early microspore divisions leading to gametic embryo formation in *Quercus suber* L. anther cultures. Acta Physiologiae Plantarum 27: 703-708.

Pintos B, Manzanera JA, Bueno MA, 2007. Antimitotic agents increase the production of doubled-haploid embryos from cork-oak anther culture. Journal of Plant Physiology 164(12): 1595-1604.

Pintos B, Bueno MA, Cuenca B, Manzanera JA, 2008. Synthetic seed production from encapsulated somatic embryos of cork oak (*Quercus suber* L.) and automated growth monitoring. Plant Cell, Tissue and Organ Culture 95(2): 217-225.

Pintos B, Manzanera JA, Bueno MA, Cremades A, González-Adrados JR, 2009. Acclimation and establishment of cork oak (*Quercus suber*) somatic embryo-derived plantlets and post-acclimation cork quality test. Acta Hort (ISHS) 812: 431-436.

Pintos B, Manzanera JA, Bueno MA, 2010. Oak somatic and gametic embryos maturation is affected by charcoal and specific aminoacids mixture. Annals of Forest Science 67: 205.

Pintos B, Sánchez N, Bueno MA, Navarro RM, Jorrín J, Manzanera JA, Gómez-Garay A, 2013. Induction of *Quercus ilex* L. haploid and doubled-haploid embryos from anther cultures by temperature-stress. Silvae Genetica 62: 210-218.

Sánchez N, Manzanera JA, Pintos B, Bueno MA; 2005. *Agrobacterium*-mediated transformation of cork oak (*Quercus suber*L.) somatic embryos. New Forests 29: 169-176.

Sommer HE, Brown CL, Kormanik PP, 1975. Differentiation of plantlets in longleaf pine (*Pinus palustris* Mill.) tissue cultured in vitro. Bot Gaz 136: 196-200.