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GENETIC STABILITY EVALUATION OF *QUERCUS SUBER* L. SOMATIC EMBRYOGENESIS BY RAPD ANALYSIS

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Abstract

A reliable protocol for adult *Quercus suber* L. somatic embryogenesis (SE) was developed recently. To evaluate the potential use of this protocol in cork oak forest breeding programs, it is essential to guarantee somatic embryos/emblings genetic stability. Random Amplification of Polymorphic DNA (RAPD) is currently used to assess somaclonal variation providing information on genetic variability of the micropropagation process. In this work, SE was induced from adult trees by growing leaf explants on MS medium supplemented with 2,4-D and zeatin. Embling conversion took place on MS medium without growth regulators. DNA from donor tree, somatic embryos and emblings was used to assess genetic variability by RAPD fingerprinting. Fourteen primers produced 165 genetic loci with high quality and reproducibility. Despite somatic embryos originated some poor quality PCR-profiles, replicable and excellent fingerprints were obtained for both donor plant and embling. Results presented no differences among regenerated emblings and donor plant. Hence, the SE protocol used did not induce, up to moment, any genetic variability, confirming data previously obtained with other molecular/genetic techniques, supporting that this protocol may be used to provide true-to-type plants from important forestry species.

Introduction

Quercus suber L. is a very important forest species, in terms of environment and economy, mostly in the Mediterranean. However, as a consequence of the oak population aging and susceptibility to environmental factors (*e.g.* fungus-host interactions, forest fires), a large percentage of cork oak populations are declining (Anon., 1996). Moreover, classical cork oak breeding programs are mostly conditioned by vegetative propagation limitations and by low rates of seed conservation. *In vitro* propagation is a valuable complement to classical breeding strategies (Santos *et al*., 2005) and provides an opportunity to develop clones with improved productivity or resistance (Brito *et al*., 2003; Santos *et al*., 2006).

It is well known that *in vitro* culture can induce somaclonal variation for example, mutation and/or epigenetic changes (Loureiro *et al*., 2007), which may hamper the implementation of clonal forestry programs or, on the counterpart, may provide interesting mutants. From all the *in vitro* techniques, somatic embryogenesis (SE) is the most promising method for clonal mass propagation, mostly because both root and shoot meristems are present.

In *Q. suber*, SE was achieved from several sources such as from leaves of seedlings (Fernández-Guijarro *et al*., 1995), zygotic embryos (Manzanera *et al*., 1993), and from leaf explants of juvenile or adult plants (Fernandes *et al*., 2008). However, for the inclusion of a SE-protocol in breeding programs, the quality (*e.g.* genetic variability) and performance of regenerated plants must be assessed (Conde *et al*., 2004).

Using *Q. suber* somatic embryos from several embryogenic lines obtained from young material (zygotic embryos), no somaclonal variation has been detected by Random Amplified Polymorphic DNA (RAPD) analyses (Gallego, 1997). This result was later confirmed for several embryogenic lines by Amplified Fragment Length Polymorphisms (AFLP) markers (Hornero *et al*., 2001). However, when using embryogenic lines from mature explants, AFLP analyses detected somaclonal variation in one genotype. Loureiro *et al*., (2005), using the SE protocol developed by Pinto *et al*., (2002) for leaves of mature cork oaks, found no ploidy or DNA content variations among somatic embryo lines. However, Lopes *et al*., (2006) using SSR markers found one mutation in one embryogenic line derived from an adult tree, while other adult or young-derived SE lines remained stable. These data suggest that the SE process, the age of explant and/or genotype influence the genetic stability. More, despite their reliability as markers, the individual information given by each marker is restricted.

Consequently, the information concerning genetic stability of this cork oak SE protocol must be confirmed by other markers together with continuous phenotypic evaluation. RAPD analyses have been used as a reliable, quick and inexpensive method to identify clones and cultivars (Ali *et al*., 2009; Çelebi *et al*., 2009; Khatri *et al*., 2009) and to assess somaclonal variation (Valladares *et al*., 2006) . Therefore, the aim of this work was to evaluate the genetic stability of the *Q. suber* complete SE process, covering the mother plant, somatic embryos and resultant emblings, using RAPD fingerprinting.

Materials and Methods

Somatic embryogenesis induction, maintenance and conversion: Somatic embryogenesis was induced using a modified (Pinto *et al*., 2002) protocol. Briefly, branches were collected during May and June from a 60-years-old *Q. suber* tree (QsG3) in the north of Portugal. Sprouted leaves were disinfected using ethanol (70%) and sodium hypochlorite (10%). Explants were placed on MS medium (Murashige & Skoog, 1962) supplemented with 30 $g.L^{-1}$ sucrose, 2.5 $g.L^{-1}$ Gelrite® and 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 9.0 µM zeatin. The pH was adjusted to 5.8. Explants were kept in the dark at 24±1ºC. Somatic embryos, when present, were isolated and transferred to MS medium without growth regulators (MS_{WH}). Cultures were grown under a photoperiod of 16 hours and light intensity of 98±2 $\mu mol.m^{-2}.s^{-1}$. Every four weeks, somatic embryos were subcultured on fresh MS_{WH} medium and maintained by

repetitive somatic embryogenesis. Somatic white-opaque dicotyledonary embryos were isolated and transferred to MS_{WH} for conversion and emblings then acclimatized to *ex vitro* conditions (Lopes *et al*., 2006). All chemicals used in these experiments were purchased from Duchefa (Haarlen, Netherlands).

DNA extraction and PCR amplification: For all experiments one genomic line was used (QsG3). For each sample genomic DNA was extracted from about 150 mg fresh material using DNeasy® Plant Mini Kit (QIAGEN™, Germany), according to the specifications of the supplier. DNA concentration and purity were estimated by 0.8% agarose gel electrophoresis stained with Ethidium bromide (EB) and compared with a standard molecular mass marker (lambda *Hind*III, NEB) by spectrophotometry at 260 and 280 nm (1 A_{260} Unit of dsDNA = 50µg mL^{-1} H_2O; Pure DNA: $A_{260}/A_{280} \geq 1.8$).

Amplifications were carried out in a Px2 Thermal Cycler. A total of forty decamer primers, primers 1 to 20 from kit C and 1-20 from kit S (Operon Technologies) were screened using 2 random samples each from regenerated plantlets, somatic embryos and donor tree for their effective utilization in RAPD analysis of *Quercus suber* somatic embryogenesis (Rocha *et al*., 2006). The PCR volume was 25µL and contained: 25 to 50ng of template DNA, 100µM each dNTP, 200µM primer, 3mM $MgCl_2$ and 2 U of Stoffel fragment (Applied Biosystems, USA) in 1x reaction buffer (100mM Tris-HCl, 100mM KCl, pH 8.3). The thermocycler program consisted of a preliminary step of 2 min at 94ºC; 10 cycles of 30 sec at 94ºC, ramp of 1.5ºC.s^{-1} to reach annealing temperature, 1 min at 55ºC, a ramp of 1.5ºC.s^{-1} to reach 72ºC and 4.5 min at 72ºC; 25 cycles of 30 sec at 94ºC, a ramp of 1.5ºC.s^{-1} to reach annealing temperature, 1 min at 45ºC, a ramp of 1.5 min up to 72ºC and 4.5 min at 72ºC; a final step of 1 min at 72ºC. PCR products were stored at 4ºC until resolution by electrophoresis on 1.5% (w/v) agarose gels with EB staining, in 1xTBE (Tris-Borate-EDTA, pH 8.0), at 150V and room temperature.

PCR fragments were scored using the image analysis software GeneTools (Syngene, USA) on the basis of presence (1) or absence (0) of the amplified product and assembled in a data matrix. Profiles were compared using the Simple Matching coefficient. The software package SPSS for Windows (version 15.0, SPSS Inc.) was used for all calculations.

Results

Two sets of PCRs were carried out for RAPD fingerprinting of each sample. Only bands reproducible on all runs were considered for analysis. Each primer generated a unique set of amplification products ranging from 226 bp in OPC8 to 1910 bp in OPC9 in size. The number of bands for each primer varied from 2 in OPC18 to 10 for OPS12 (Table 1).

Table 1. Primers used in RAPD studies and number of reproducible scoreable bands for each primer.

Primer	Scoreable Bands
OPC1	7
OPC2	12
OPC3	9
OPC5	9
OPC8	15
OPC9	11
OPC14	10
OPC18	6
OPC19	13
OPS12	11
OPS14	12
OPS16	13
OPS17	11
OPS18	10
OPS19	16
Average	11

Fifteen primers used in this analysis yielded 165 scoreable bands, 92 for the OPC primers plus 73 fragments for the OPS primers (Table 1), with an average of 11 bands per primer. These 165 markers were monomorphic across all the samples. An example of this is shown in Fig. 1. The total number of reproducible scoreable bands was 990. No significant genetic variability among tested samples was detected with all 990 markers. Simple matching coefficient tests showed reproducible similarities above 95% (Table 2).

There are, in this species, some low intensity PCR products for somatic embryos. Despite these products seemed, at a first glance inexistent, the use of GeneTools for spectra analysis showed they were present (Fig. 2). These faded bands are restricted to somatic embryos and are mainly due to poor quality of PCR-products for this type of material.

Discussion

Molecular markers (*e.g.* AFLP, microsatellites, RAPD, RFLP) had been used as reliable, powerful and quick tools in the analyses of somaclonal variation in somatic embryogenesis of both conifers and angiosperms (Leal *et al*., 2006; Burg *et al*., 2007; Lopes *et al*., 2009) and particularly in the *Quercus* genus (Hornero *et al*., 2001; Wilhelm *et al*., 2005; Valladares *et al*., 2006; Lopes *et al*., 2006; Fernandes *et al*., 2008). Sanchez *et al*., (2003), using 32 RAPD primers, found no intraclonal or interclonal polymorphism between embryogenic lines originated from the same seedling of *Q. robur*, concluding that these somatic embryos were genetically uniform. In the same study, no differences in DNA sequences were found between somatic embryos and the later converted emblings. Similarly, by RAPD analyses, Valladares *et al*., (2006) found no evidence of genetic variation within, or between, the embryogenic lines from three trees, or between these lines and the field tree.

Table 2. Similarity matrix for all generated fragments using 15 primers.

	Donor plant	Somatic embryo	Converted plant
Donor Plant	1,000	0,953	0,994
Somatic Embryo	-	1,000	0,959
Converted Plant	-	-	1,000

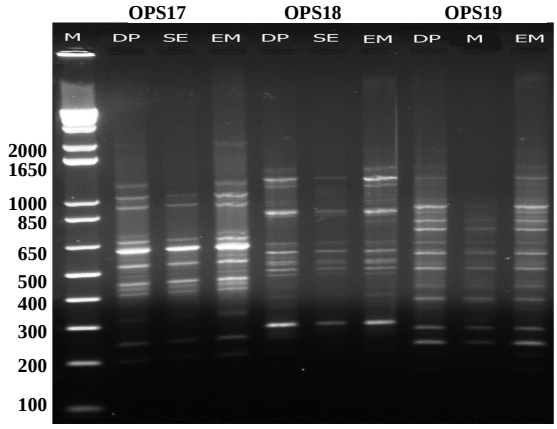

Fig. 1. DNA profiles generated by the RAPD primers OPS 17, 18 and 19, in the three different stages of the somatic embryogenesis process: donor plant (DP), somatic embryo (SE) and embling (EM). M, size marker (1Kb Plus DNA Ladder).

In the specific case of somatic embryogenic lines of *Q. suber*, until the moment, either RAPD (Gallego, 1997), AFLP (Hornero *et al*., 2001; Fernandes *et al*., 2008) or microsatellites (Lopes *et al*., 2006) were used to evaluate somaclonal variation among somatic embryos within some embryogenic lines, and/or even among the explant leaves, embryos and emblings.

Studies on somatic embryogenesis genetic stability such as those of Loureiro *et al*., (2005) and of Lopes *et al*., (2006) are, in this paper, complemented with RAPD analyses to further validate this cork oak SE protocol. The choice of RAPD presents several advantages as it is an easy-of-use and development technique with a high quantity of information output. Also, the polymorphism level of RAPD analyses for different cork oak genotypes is referred by Gallego (1997) as high as 31.9%.

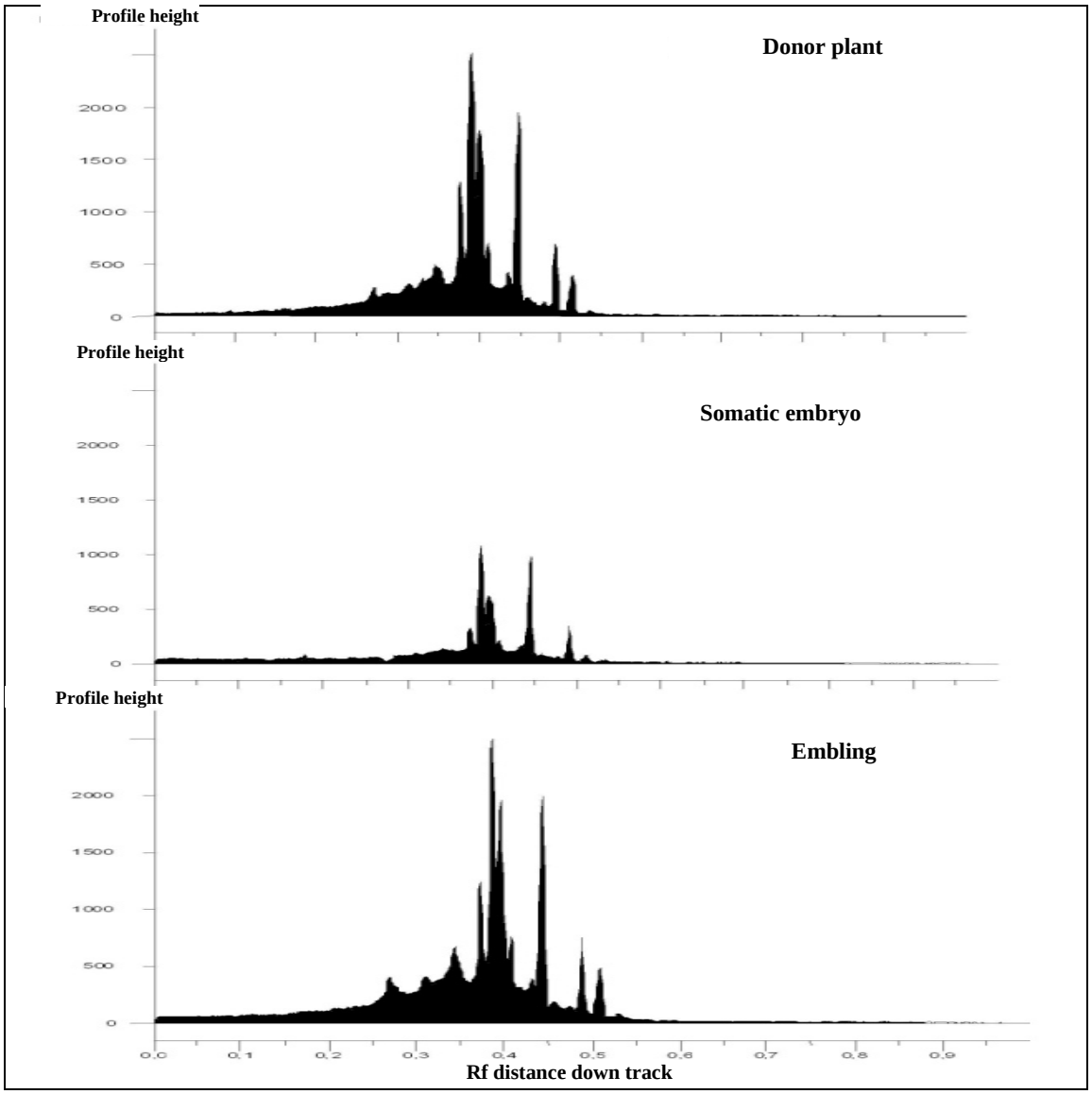

Fig. 2. DNA profiles generated by the RAPD primer OPS 12, in the three different stages of the somatic embryogenesis process: donor plant, somatic embryo and embling.

In our study, RAPD analyses do not show any somaclonal variation between the field tree, somatic embryos and the emblings obtained,using the modified somatic embryogenic protocol developed by Pinto *et al*., (2002). The total of 165 PCR products analyzed proved a perfect similarity between all the samples from all the three different somatic embryogenesis stages. Despite some reports of emblings regenerated from embryogenic callus cultures of e.g., date palm (Saker *et al*., 2000) have been found to exhibit somaclonal variation by RAPD analysis. Our study presents exactly the opposite. Emblings presented no differences compared to donor plant. However, somatic embryos showed lower band intensity (Fig. 2). This fact could be due to the type of material and its high water content (>80%), which difficult amplification and further evaluation and scoring of PCR products. Nevertheless, despite the inherent importance of the process, since no variability was found at the converted plants, if there is any at the somatic embryo stage, it was, so far, not relevant. Also, Wilhelm *et al*., (2005), using microsatellites, found variation in *Q. robur* embryogenic lines but not in the regenerated plantlets. Since embryogenic cultures are exposed to plant growth regulators and suffer high cellular division rates, some somaclonal variation may occur. However, none of this putative variation was found at the emblings. This supports that the somatic embryogenic process did not induce changes in gene structure, which could significantly affect regenerated plantlets. Similar findings have been reported for *Q. serrata* (Thakur *et al*., 1999).

Our studies using SSR, FCM and now RAPD analysis show that our method is in general genetically stable and reliable. Yet, one must however not exclude the putative occurrence of other genetic changes (alteration in the DNA methylation, activation/inactivation of transposons and retrotransposons, activation/silencing genes, changing gene expression) (Gaj, 2004).

In conclusion, this work shows no genetic variability in plants obtained according to the modified available protocol, with respect to RAPD fingerprinting. Together with these results, those obtained by flow cytometry (Loureiro *et al*., 2005), microsatellites (Lopes *et al*., 2006) and morphological characterization of somatic embryos in this species (*e.g.* Pinto *et al*., 2002) support that our protocol used to somatic embryogenesis may be performed to provide true to type plants.

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