Universidade de Aveiro Departamento de Biologia
2008

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VARIABILIDADE GENÉTICA EM EMBRIOGÉNESE SOMÁTICA DE *QUERCUS SUBER* L.

GENETIC VARIABILITY IN *QUERCUS SUBER* L. SOMATIC EMBRYOGENESIS

dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia Molecular, realizada sob a orientação científica da Drª. Maria da Conceição Lopes Vieira dos Santos, Professora Associada do Departamento de Biologia da Universidade de Aveiro

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Agradecimentos

Desejo agradecer a todos os que contribuíram para este trabalho. Pela sua sabedoria, apoio, trabalho, dedicação, paciência, mas principalmente por acreditarem.

"Nunca te ofereceram um desejo sem te darem ao mesmo tempo o poder de o tornares realidade. Contudo, é possível que tenhas de lutar por ele."
(in *Ilusões*, Richard Bach).

Prof. Conceição Santos
Drª Tina Lopes
Drª Glória Pinto
Tânia Almeida
Pedro Fernandes
Joana Macedo
Engº Armando Costa

Aos meus pais, irmãos e amigos de sempre. Obrigado por estarem na minha vida.

Palavras-chave

Marcadores moleculares, PCR, Histone H3 type I element, RAPD, embriogénese somática, *Quercus suber* L.

Resumo

As culturas de tecidos celulares ou tecidos de plantas comportam-se, em muitos aspectos, de modo semelhante aos microorganismos (por exemplo, em ambos os casos são requeridas condições de meios de cultura e assépsia adequados, apresentam curvas de crescimento sigmóide, podendo também ocorrer variações genéticas). A caracterização e análise da variabilidade genética destas culturas são, actualmente, efectuadas com base em marcadores moleculares, muitos dos quais foram desenvolvidos inicialmente para estudos em microrganismos ou células animais (como por exemplo a "Polymerase Chain Reaction") e mais tarde transferidas para a análise da variabilidade genética em culturas celulares de plantas (por exemplo, como forma de assegurar a fidelidade clonal).

A fidelidade clonal é o factor de maior importância na comercialização de material micropropagado obtido por métodos de cultura de tecidos "in vitro". Este facto é da maior importância nos programas de melhoramento florestal, dado que a micropropagação de plantas superiores é um meio rápido de produção de stocks de plantas clonais para programas de reflorestação e conservação de germoplasma elite ou de elevado interesse ecológico. Contudo, devido ao longo ciclo de vida das espécies lenhosas, uma análise alargada de parâmetros genéticos e fenotípicos é essencial, especialmente quando as plantas derivam de embriogénese somática, processo no qual se consideram as células como estando sobre condições de stress (por exemplo, exposição a auxinas), bem como estados de ciclo celular repetitivos.

Atendendo a estas considerações, utilizámos embriões somáticos de *Quercus suber* L. (genótipo QsG3) obtidos a partir de explantes de folha de uma árvore adulta, os quais foram mantidos no nosso laboratório por um ano.

A variabilidade genética dos embriões somáticos e das plantas resultantes foi avaliada por dois marcadores moleculares: "histone H3 promoter type I element" e "RAPD".

Não foi encontrada variabilidade genética de acordo com estes dois marcadores durante todo o processo de embriogénese, assegurando assim a reprodutibilidade deste processo "in vitro".

Concluindo, os marcadores moleculares usados neste trabalho podem representar uma ajuda adicional como técnicas para assegurar variabilidade genética em complemento com outras técnicas.

Keywords Molecular markers, PCR, Histone H3 promoter - type I element, RAPD, somatic embryogenesis, *Quercus suber* L.

Abstract

Plant cell or tissue cultures behave, in many aspects, in a similar way to microorganisms (e.g. all require a suitable culture medium and asseptic conditions, they present a sigmoid growth curve and genetic variation may occur in those cultures). The characterization and analysis of genetic variability of these cultures is presently performed by molecular markers, many of which were first developed for microorganisms or animal cell studies (e.g. Polymerase Chain Reaction) and later transferred to analysis of genetic variability in plant cell cultures (e.g. to assess clonal fidelity).

In fact, clonal fidelity is a major concern in commercial micropropagation using in vitro tissue cultures. This is particularly important in forest breeding programs as micropropagation of tree species since it offers a rapid means of producing clonal planting stock for forestation programmes and conservation of elite and rare germplasm. But due to the long period of woody species life-cycle, a screening for genetic and phenotypical parameters of micropropagated plants is essential, in particular when plants derived from somatic embryogenesis, where cells may be considered to be under stressing conditions (e.g. auxins), as also under repetitive cell cycles.

Within this scope, we used *Quercus suber* L. somatic embryos (Gs3 genotype) achieved from leaf explants of a mature adult plant, maintained in our laboratory for one year. Genetic variability of somatic embryos and emblings was evaluated by using two molecular markers: histone H3 promoter type I element and RAPD.

We found no genetic variability according with these two markers during the whole process of embryogenesis, assessing the reliability of this in vitro regeneration process.

In conclusion, the molecular markers used in this work may represent an added value as tools of genetic variability assessment in complement with other techniques.

Index:

Abbreviations:

2,4 D – 2,4-Dichlorophenoxyacetic Acid

AFLP – Amplified Fragment Length Polymorphisms

B5 – Gamborg's Medium

ChIP – Chromatin Immunoprecipitation

DNA – Deoxyribonucleic acid

EB – Ethidium Bromide

FISH – Fluorescent in situ hybridization

KCl – Magnesium chloride

MS – Murashige and Skoog Medium

MS_{WH} – Murashige and Skoog Medium without Hormones

PCR – Polymerase Chain Reaction

RAPD – Random Amplified Polymorphic DNAs

RFLP – Restriction Fragment Length Polymorphisms

SE – Somatic Embryo

SH – Schenk and Hilderbrant Medium

SSR – Single Strand Repeats (microsatellites)

WPM – Woody Plant Medium

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CHAPTER 1 – GENERAL INTRODUCTION

1.1 – The model species: *Quercus suber* L.

Quercus suber L. (cork oak), a medium-sized evergreen oak tree, is an angiosperm dicotyledon (vascular, seed and flowering plant) that taxonomically belongs to the Order of the Fagales, Family of the Fagaceae and Genus *Quercus* (IPF, 1988; Natividade, 1990; AGRO.GES - Sociedade de Estudos e Projectos Lda, 2000).

The cork oak is native from the southwest Europe and the northwest Africa. The tree is widely cultivated in Spain, Portugal, Algeria, Morocco, France, Italy and Tunisia. It prefers climates with soft temperature changes, height atmospheric humidity and insulation (Natividade, 1990; AGRO.GES - Sociedade de Estudos e Projectos Lda, 2000). In Portugal this species is spread all over the country.

Cork oak is a monoeciuos wind-pollinated species with a protandous system to ensure cross-pollination. Its propagation is based on seeds, which unfortunately lose their germination capability very quickly (Boavida et al., 1999). It grows up to 15-20 meters high, although, in exceptional cases it can reach 25 meters. The leaves are 4-7 cm long, weakly lobed or coarsely toothed, dark green above, paler beneath, with the leaf margins often downcurved and the acorn (seed) is a 2-3 cm long (Natividade, 1990; AGRO.GES - Sociedade de Estudos e Projectos Lda, 2000). The tree trunk forms a thick, rugged and corky bark, which can be harvested every 10-20 years as cork after the firsts 30 years-old (Gil, 1998).

The European cork industries produce 340,000 tonnes of cork per year (Portugal is responsible for 50% of this production), with a value of €1.5 billion and employ 30,000 people (Gil, 1998; Oliveira and Oliveira, 2000; Medeiros, n.d.). The regular extraction of cork is very important for the sustainability of the cork oak fields, because this stable and multifunctional system is in equilibrium with is environment. In fact, the value of this tree is related, not only with the cork industry but also with other industries as civil engineering, automobile and aeronautics (Gil, 1998).

Cork oak cannot legally be cut down in Portugal, except for forest management felling of old or unproductive trees (DR, 2001, 2004). Unfortunately, in the last few years we have observed a great reduction on the natural areas of *Quercus suber* L., genetic variability and germplasm, as a consequence of forest fires, anthropogenic pressure, as well as diseases such as the ink disease caused by *Phytophthora cambivora* (Varela and Eriksson, 1995).

1.2– Plant Tissue Culture

1.2.1. General Considerations

Research work on plant tissue culture has been ongoing for decades. Unfortunately, most tree propagation in vitro has been difficult compared to other plants.

There are several factors that can limit the in vitro plant regeneration processes: the disinfection protocol used; the tissue source; response of explants, which is primarily determined by genotype and by the donor individual age (juvenile tissues are more responsive than the mature ones); the physiological state of the donor tissue, and time/season of the year when the explants are collected and cultured. The culture media composition used to establish aseptic cultures is also important (Linington, 1991; Huang et al., 1994; Marks and Simpson, 1994; Harada and Murai, 1996; Toribio et al., 1998; Pinto et al., 2002a; Giri et al., 2004; Park et al., 2006). The best media for woody tree species micropropagation depend on species/genotype, as also on the tissue source (e.g. seeds, roots, leaf-protoplasts, zygotic embryos, leaf). Media like Murashige and Skoog - MS (Murashige and Skoog, 1962), Woody Plant Medium – WPM (McCown and Lloyd, 1981), Schenk and Hilderbrandt medium – SH (1972) or Gamborg's medium - B5 (Gamborg et al., 1968) are frequently recommended to dicotyledonous woody plants (Giri et al., 2004). Also the plant growth regulators combination (e.g. mostly auxins/citokinins balance) present in the medium are considered determinants to optimize plant regeneration by micropropagation, including other external factors such as the carbon source used (e.g. sucrose, glucose), the light conditions and stress factors (e.g. pH, low and high temperature, heavy metals) (Gaj, 2004; Giri et al., 2004; Jiménez, 2005).

Several other limitations such as low shoot proliferation in forest trees, excessive phenolic exudation, pronounced basal callusing, vitrification and shoot tip necrosis, or rooting recalcitrance contribute to a negative vision of the micropropagation of woody forest tree species in vitro (Wilhelm, 2000; Gaj, 2004; Giri et al., 2004; Jiménez, 2005); Pinto, 2007).

The most used strategies of in vitro plant regeneration are organogenesis, embryogenesis and axillary proliferation, and all of them share some of these characteristics and limitations.

From them, the somatic embryogenesis is regarded as the best system for propagation of superior genotypes (Zimmerman, 1993; Sutton, 2002; Celestino et al., 2007), mostly because both root and shoot meristems are present simultaneously (Kim, 2000), being applied on different genus as the case of *Pinus* (Park et al., 2006), *Quercus* (Bueno et al., 1992; Kim et al., 1997; Toribio et al., 1998; Kim, 2000; Pinto et al., 2002b; Hernández et al., 2003a; Mauri and Manzanera, 2003; Toribio et al., 2004; Valladares et al., 2006), *Picea* (Fourré et al., 1997; Arnold et al., 2005) and *Eucalyptus* (Pinto et al., 2002a).

1.2.2. Somatic Embryogenesis

Cork oak populations are traditionally propagated by acorns but evident phenotypic heterogeneity due to free hybridisation and localised problems leading to deforestation make it urgent to implement regeneration improvement programs (Boavida et al., 1999). Also, considering that, in *Quercus*, seeds are recalcitrant for storage, verification that the juvenile-adult correlation is low and the delay to reach sexual maturity and produce seeds (good seeds only occur once every 3-5 years), the need to find a good in vitro culture protocol for this specie is a priority (Wilhelm, 2000).

Vegetative propagation (by macropropagation) of oak is, in general, almost impossible for mature trees, because rooting percentages are negligible. Due to phase change phenomena or ontogenetic aging, mature trees are also often recalcitrant for in vitro propagation via organogenesis (Wilhelm, 2000). Some of these problems may be overcome by the use of other micropropagation techniques. Despite a lot of studies still have to be performed in the field, recent works showed that micropropagation by stem cuttings and, mostly by somatic embryogenesis, presents a huge potential to be used in oak species breeding programs in a similar way as the case of eastern Canadian breeding programs (*e.g.* Park, 2002).

Regarding *Q. suber,* somatic embryogenesis was achieved from leaves and seedlings (Fernández-Guijarro et al., 1995), nodal segments (El Maataoui and Espangnac, 1987) and zygotic embryos (Bueno et al., 1992; Manzanera et al., 1993). The inability to initiate embryogenic cultures from mature trees was one of the major limitations of this process until some years ago. Recently, somatic embryos were obtained from leaf

explants of mature plants, with a frequency induction of up to 20% (Toribio et al., 1998; Hernández et al., 1999; Hornero et al., 2001a; Hernández et al., 2003b; Hernández et al., 2003a), and Pinto et al. (2001) reported somatic embryogenesis in calluses from leaves of 3 years old cork oak plants and from leaves of a 60-years-old cork oak tree, including plant conversion (Pinto et al., 2002b) that, despite apparent morphological abnormalities, showed no significant genetic or ploidy changes (Loureiro et al., 2005; Lopes et al., 2006).

The occurrence of phenotipically anomalous embryos was reported for this species by for example Pinto et al. (2002b), Lopes et al., 2006, see Fig 1. Phenotypic variations, analysed with morphologic or protein markers, can be the result of a modification of the genome sequence itself or simply a change in the expression of genes. Genotypic variations can be genomic, chromosomic or genic. Genomic mutations affect the number of chromosomes (the ploidy) and can be detected by, for example, flow cytometry or chromosome counting. Chromosomic mutations like inversion, deletion or translocation and genic mutations could be detected by genetic molecular markers like Restriction Fragment Length Polymorphisms (RFLP) and Random Amplified Polymorphic DNA (RAPD) that can detect DNA sequence modifications. It is also important to remember that mutations can occur on nuclear as well as on mitochondrial or chloroplast DNA (Fourré et al., 1997).

Changes on DNA-ploidy in somatic embryogenic cell lines of various oak species has been monitored by flow cytometry and molecular markers (e.g. (Gallego, 1997; Loureiro et al., 2005; Wilhelm et al., 2005; Valladares et al., 2006). No somaclonal variation was detected applying RAPD (Gallego, 1997) or AFLP markers (Hornero et al., 2001b). Recently our laboratory showed the successful utilization of microsatellite markers for the assessment of genetic stability of somatic embryogenesis clonal materials in this particular specie (Lopes et al., 2006; Santos et al., 2007). The absence of genetic variability in clones derived from the protocol used may open perspectives to its use in industrial propagation of *Q. suber* (Santos et al., 2007). Once the process of somatic embryogenesis has been initiated, the multiplication cycle proceeds via repetitive embryogenesis, which can be maintained indefinitely, a useful condition to the application of this process to mass propagation and bioreactors (*e.g.* Wilhelm, 2000).

As stated above, somatic embryogenesis presents huge potential in forest breeding programs, and a standard protocol (Fig 1) was already established for several cork oak genotypes. However, some steps still require optimization: in particular,

maturation and low germination frequencies are the main bottlenecks for a broader use of this technique (Fernandes, 2007).

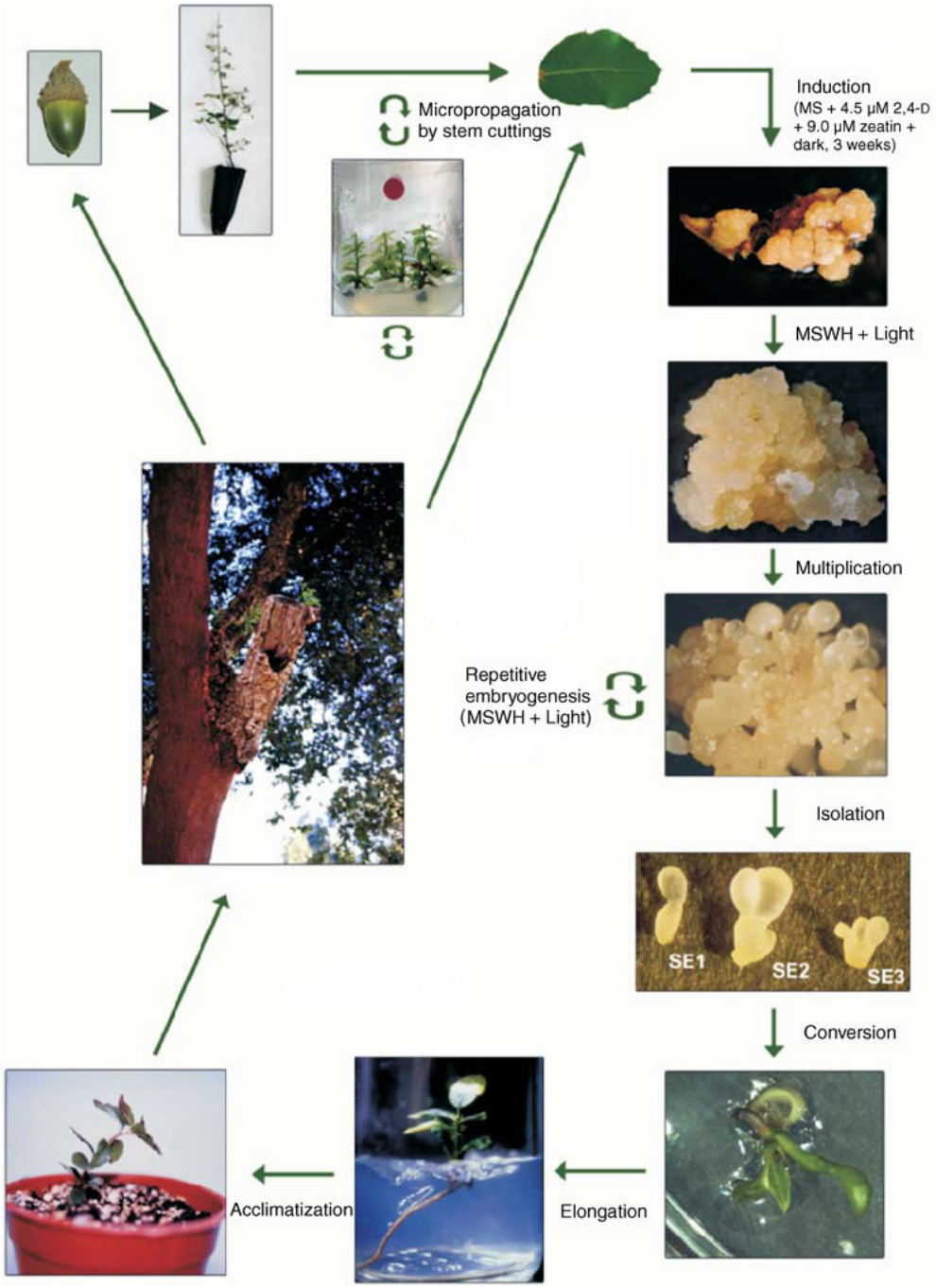

Fig 1 – Schematic diagram illustrating the embryogenic process used in our laboratory to obtain somatic embryos (SE) in *Quercus suber* (from Lopes et al., 2006). Three somatic embryo morphotypes are shown: an abnormal SE with 1 cotyledon (SE1), a normal dicotyledonary embryo (SE2) and an abnormal SE with more than two cotyledons (SE3).

The somatic embryogenesis process presents intense cell divisions and cells suffer severe functional and physiological changes (Pinto, 2007). Besides, as a highly organized and complex process (Giri et al., 2004), somatic embryogenesis is, therefore under a complex regulation, where histones may play an important role, as already describe for alfalfa (Kapros et al., 1992).

Therefore a better knowledge of mechanisms involved in cell cycle (e.g. histone regulation) will provide useful tools to control the process reducing the empirical approaches. Recent studies showed different cell cycle dynamics during cell/tissue cultures (Loureiro, pers. comm.), and some studies demonstrated that histones (e.g. H3) may be involved in the regulation of the different G0/G1, S, G2 stages (Kapros et al., 1992). In particular one H3 variant seems to be associated to S phase (Kapros et al., 1992), but studies on its gene expression variation during somatic embryogenesis process and the impact of putative mutations in its H3 gene sequence or in its promoter remain completely unknown and challenging.

As other in vitro micropropagation techniques, somatic embryogenesis may lead to genetic instability of the embryogenic cell lines. As spontaneous mutations rarely offer valuable traits for breeding programs, mutation is often in large scale production programs regarded as a negative aspect of the process and therefore the regenerated plantlets should be monitored both at the chromossomal and molecular level, preferably in combination with the plant phenotypic performance in the field (e.g. Pinto, 2007).

1.3– Somaclonal Variation

As referred earlier, many aspects in the somatic embryogenesis process are responsible for occurrence of somaclonal variation (Larkin and Scowcroft, 1981), which is often heritable and involves changes in both nuclear and cytoplasmic genomes, and their character can be of genetic and/or epigenetic nature (Henry, 1998; Gaj, 2004).

Genetic changes include polyploidy, aneuploidy, (point) mutations, and new insertions of (retro)transposons. Genetic changes behave as Mendelian traits in crosses. Epigenetic changes do not involve changes of the primary DNA sequence, but are the result of alterations in DNA methylation, of changes in histone modifications, or a combination of these epigenetic mechanisms that modify gene expression. They are in theory temporary (plants ‘revert’ to normal phenotype), but are sometimes nevertheless

taken over into the progeny as well (de Klerk, 1990; Smulders, 2005). Typical for epigenetic changes due to adventitious regeneration, is that the same change often occurs at a higher frequency (whereas genetic changes occur, in principle, at random) (Smulders, 2005).

The incidence of somaclonal variation in somatic embryogenesis is influenced by genotype, by ploidy level (polyploids giving rise to greater variation), tissue source, culture age and procedure (Larkin, 1987; Karp, 1989; Bednarek et al., 2007), as also by the fact that cells suffer intense and multiple cellular divisions that are prone of replication errors. Additionally, stress conditions and the exogenous plant growth regulators supplied can influence directly the gene expression profile (Chugh and Khurana, 2002; Che et al., 2006), as a consequence, for example, of modification in DNA methylation (promote activation of transposons and retrotransposons, which can activate or silencing genes), chromosome changes, point mutations (Kaeppler et al., 2000; Smulders, 2005).

Several studies have shown that somaclonal variation can be assessed by analysis of phenotype, chromosome number and structure, proteins or direct DNA evaluation of plants (de Klerk, 1990). The types of variation that are frequently observed may differ from species to species, and it is often difficult to determine the genetic nature of the observed variation (Saunders et al., 1992).

1.4– Molecular Markers

Compared to the other techniques, molecular markers had been reported as less subject to the influences of environmental factors and developmental stage, at the same time that they reveal immense number of characters for comparison (Patterson, 1988; FAO, 1994; Karp et al., 1996). Nevertheless, as stated by Smulders (2005) the use of each molecular marker, alone, to assess genetic variability, gives limited information, and always the largest combination of tools (e.g. RAPD, microsatelittes, ploidy) must be used together with phenotypic parameters.

We can distinguish between two classes of molecular markers - molecular genetic markers (those derived from direct analysis of polymorphism in DNA sequences), and biochemical markers (those derived from study of the chemical products of gene expression). The major types of molecular markers are: Isozymes, Restriction Fragment Length Polymorphisms (RFLP), and those based on the polymerase chain reaction (PCR)

such as Randomly Amplified Polymorphic DNAs (RAPD), Microsatellites (SSRs) and Amplified Fragment Length Polymorphisms (AFLP) (FAO, 1994).

1.4.1. Isozymes

In isozyme analyses, the particular enzyme is extracted from the plant tissue, and the different forms separated by gel electrophoresis, on the basis of molecular size, shape and electrical charge (by *e.g.* polyacrylamide gels and isoelectric focusing). A number of enzyme systems can be examined in this way. The two alleles at an allozyme locus in a heterozygous individual can be detected. Allozymes are thus co-dominant markers. They are also multiallelic, fast and inexpensive to analyse. The number of markers is limited by the number of enzymes available for analysis (Weeden, 1989; FAO, 1994).

In somaclonal studies the isoenzymes were used for example in the detection of this variations in tissue culture-derived date palm plants (Saker et al., 2000), in *Quercus robur* (Racchi et al., 2001) and in *Quercus suber* L. (Bueno et al., 2000), showing in this case that all the embryos resulting from the same anther had the same isoenzyme allele.

1.4.2. Restriction Fragment Length Polymorphisms (RFLP)

Used widely in research programmes since the early 1980s, RFLP have been well described in a number of reviews (*e.g.* (Landry and Michelmore, 1987; Nance and Nelson, 1989; Tanksley et al., 1989; Neale and Williams, 1991). The basic technique involves the extraction and then the digestion of DNA with a restriction enzyme, which cuts the DNA at occurrences of a particular recognition sequence (usually 4 to 8 bases in length) throughout the strand. The number and lengths of resulting fragments depends then on the number and distribution of recognition sites. Following digestion, the fragments that have been generated are separated by gel electrophoresis. The generally large genomes of higher plants and animals produce too many fragments for clear resolution, and a Southern blotting is therefore applied to the fragment array (Nance and Nelson, 1989).

Despite of being codominant and multiallelic markers, RFLP analyses posses several technical limitations: a good supply of probes is needed and, if heterologous probes are unavailable, cDNA or genomic DNA must be developed; the blotting and hybridization steps are time-consuming and difficult to automate; sufficient quantities (10µg per digestion) of good quality DNA are required; much more expensive; require the use of radioactive material; and RFLP are, thus, not applicable where very limited amounts of source material or preserved tissue are available (Karp et al., 1996).

RFLP is most suited to studies at the intraspecific level or among closely related taxa. In plant genetic analysis, RFLP markers were initially used for estimating genetic distance and fingerprinting in wheat, but some studies in somaclonal variation as also reported (Tanksley et al., 1989; Devarumath et al., 2002).

1.4.3. Randomly Amplified Polymorphic DNAs (RAPD)

The Polymerase Chain Reaction (PCR) came to overcome some of the limitations of the RFLP. All those techniques involve the use of a single arbitrary primer and result in the amplification of several discrete DNA products. The most common version is Randomly Amplified Polymorphic DNAs (RAPD) (Karp et al., 1996).

RAPD were first described only in 1990 (Williams et al., 1990). Briefly, an oligonucleotide will prime amplification from a genomic template if binding sites on opposite strands of the template exist within a distance which can be traversed by the DNA polymerase (up to several thousand nucleotides).

Genomic polymorphisms at one or both priming sites result in the non-amplification of a band. RAPDs are thus dominant markers, resulting in an inability to distinguish homozygotes from heterozygotes. All other alleles at the priming site will be represented by absence of the band. A primer usually amplifies several bands, each originating from a different genomic location (Williams et al., 1990; Rafalski et al., 1991; Rafalski et al., 1993).

The nature of the fragments amplified is influenced dramatically by the sequences of both primer and template. Fragments are separated on agarose gels and stained with ethidium bromide. Primers most commonly used are 10 nucleotides in length with at least

50% Guanine-Cytosine content. Each different primer used will result in a different banding pattern.

RAPD analyses can be conducted much more quickly (and with fewer laboratory restrictions) than those involving RFLP. At the same time, there is no requirement for DNA probes or sequence information for the design of specific primers; since the procedures involve no blotting or hybridization steps, it is quickly, simple and automatable; very small amounts of DNA (10 ng per reaction) are required (Karp et al., 1996). However is absolutely critical to maintain strictly constant PCR reaction conditions in order to achieve reproducible profiles.

Use of RAPDS markers may permit mapping in areas of the genome not accessible to RFLP analysis due to the presence of repetitive DNA sequences.

RAPD markers are one of the most used techniques for the determination of somaclonal variation in plant cell cultures. In *Quercus* this technique was been described for the analysis of somaclonal variation in micropropagation processes of *Q. robur*, *Q. suber*, *Q. affinis*, *Q. laurina* (Barrett et al., 1997; Gallego, 1997; Concepcion Sanchez et al., 2003; Sanchez et al., 2003; Gonzalez-Rodriguez et al., 2004; Valladares et al., 2006). Other conifers micropropagation processes, specially somatic embryogenesis, were also been study as the case of *Picea abies* (Heinze and Schmidt, 1955; Fourré et al., 1997), *Picea mariana* (Isabel et al., 1993), *Betula pendula* (Ryynänen and Aronen, 2005), *Pinus thunbergii* Parl. (Goto et al., 1998), *Picea glauca* (DeVerno et al., 1999).

1.4.4. Microsatellites

Microsatellites are DNA sequences composed of a tandem repetition of a simple short sequence, occurring in the genome of many higher organisms (Rafalski et al., 1993). The most common are dinucleotide repeats. They are very common, and very polymorphic (there are many variants) (Morgante and Olivieri, 1993).

Providing the sequence of the DNA surrounding a microsatellite is known and suitable PCR primers can be designed, the segment of DNA incorporating the microsatellite can be amplified and its length determined by electrophoresis (Jones et al., 1997). Multiple allelic length variants can be identified at most microsatellite loci.

Advantages of microsatellites are their abundance, high degree of polymorphism, multi-allelic and co-dominant nature, and that they give highly reproducible profiles (Karp et al., 1996; Koreth et al., 1996).

Disadvantages are the requirement for cloning and sequencing of microsatellite loci (when specific primers are not readily available), the need for high resolution gels, and the difficulty of plus/minus assays (Rafalski et al., 1993).

Studies related to the analysis of somaclonal variation in cell culture process involving conifers had been reported in different genus in a similar way as also reported for the others molecular markers (Barrett et al., 1997; Hornero et al., 2001a; Helmersson et al., 2004; Wilhelm et al., 2005; Lopes et al., 2006; Burg et al., 2007)

1.4.5. Amplified Fragment Length Polymorphisms (AFLP)

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA. The technique involves three main steps: (i) restriction of the DNA and ligation of oligonucleotide adapters, (ii) selective amplification of sets of restriction fragments, and (iii) gel analysis of the amplified fragments. Using this method, sets of restriction fragments may be visualized by PCR without knowledge of nucleotide sequence (Vos et al., 1995; Jones et al., 1997).

The method allows the specific co-amplification of high numbers of restriction fragments. However the results analyses are dependent on the resolution of the detection system. Typically 50-100 restriction fragments are amplified and detected on denaturing polyacrylamide gels (Karp et al., 1996).

The AFLP technique provides a novel and very powerful DNA fingerprinting technique for DNAs of any origin or complexity, but unfortunately, compared with the other techniques referred earlier this is the most expensive one (Mueller and Wolfenbarger, 1999). They also share the same limitations, with respect to bands homologies and identities as the case of RAPD. Fortunately, this type of technique seems to have the same reproducible than RFLP, but requires more DNA (1µg per reaction) than RAPD (Karp et al., 1996).

Although high costs involved on this technique, some studies to evaluate the somaclonal variation in somatic embryogenesis had been recently made (Cervera et al., 2000; Hornero et al., 2001b; Jain, 2006).

1.4.6. DNA methylation and post- transcriptional changes

Somaclonal variation is a very complex problem that needs several approaches to be correctly appreciated. Obviously, the only use of molecular markers like RAPD, RFLP, AFLP, Microsatellite or Isozymes to assess the genetic stability of an in vitro production system is insufficient, and the morphological and cytogenetical approach appears to be a valuable complementary tool (Fourré et al., 1997).

For this reason, the study of alterations in DNA methylation, of changes in histone modifications, or gene expression is also important (Zhang and Jacobsen, 2006; Boyko and Kovalchuk, 2008).

DNA methylation consists on the methylation of cytosine residues in DNA by action of methyltransferases. In plants, methylcytosine can occur at any cytosine residue CpG, CpNpG and CpNpN sequence context (where N = A, C, or T) (Takeda and Paszkowski, 2006). In plants, as in mammals, DNA methylation has dual roles in defence against invading DNA and transposable elements and in gene regulation. Although originally reported as having no phenotypic consequence, reduced DNA methylation disrupts normal plant development (Finnegan et al., 1998; Vaillant and Paszkowski, 2007). Relative to histone post-translational modifications, such as acetylation, methylation, phosphorylation, ubiquitination, glycosylation, ADP ribosylation, carbonylation, and biotinylation, they occur at the amino-terminal tails. This tails are responsible for interaction with DNA and thereby facilitate the chromatin assembly (Chen and Tian, 2007).

Studies show that these processes (specially between the DNA methylation and the histone H3-K9 methylation) are related between them, function as signals for each other assessing propagation of the silence state (regulation of heterochromatin condensation) during the cell cycle (Reichheld et al., 1995; Reichheld et al., 1998; Johnson et al., 2002; Vaillant and Paszkowski, 2007).

1.4.7. Brief reference to other techniques for detecting genetic and epigenetic changes in cell cultures

Technical variations from these main techniques described above are presently available (for review see Karp et al., 1996; Smulders, 2005; Bednarek et al., 2007). Besides, other genetic changes such as chromosomal changes can be detected by flow cytometry, chromosome counting or by other techniques such as FISH analysis.

On the other hand, epigenetic changes as alterations on the DNA methylation profile can be detected by protocols that permits converting the unmethylatd cytosine residues in uracil, as the one presented by Frommer et al., 1992, whilst the histone modifications are analysed by chromatin immunoprecipitation (ChIP) (Johnson et al., 2002; Haring et al., 2007).

1.5– Aim of this Work

The aim of this work is to analyse genetic changes in somatic embryos and plants obtained according to Pinto et al. (2002b) with respect to both RAPD markers and the histone H3 promoter type I element.

RAPD analyses will permit the evaluation of the genetic variability in the DNA sequence, evaluating randomly very parts of all the genome.

The information obtained with the histone H3 promoter type I element will permit an evaluation of the nucleotide sequence stability of the promoter regulatory region of these genes. The presence of this cell cycle-specific promoter element in the H3-1 alfalfa gene supports the idea of cell cycle-related control for this histone H3 variant (Kapros et al., 1992). So the determination of changes in the histone H3 promoter type I element could permit the identification of mutants that will be valuable tools to study the role of H3 in somatic embryogenesis evolution.

CHAPTER 2 - GENETIC VARIABILITY EVALUATION OF *QUERCUS SUBER* L. EMBRYOGENESIS BY HISTONE H3 PROMOTER AND RAPD

This chapter includes a manuscript to be submitted to an international journal:

Cristina Rocha, Pedro Fernandes, Glória Pinto, Conceição Santos (2008) Genetic variability evaluation of *Quercus suber* L. embryogenesis by Histone H3 promoter and RAPD. Submitted to Scientia Horticulturae

Genetic variability evaluation of *Quercus suber* L. embryogenesis by Histone H3 promoter and RAPD

2.1 – Abstract

A reliable protocol for *Quercus suber* L. somatic embryos production has been developed in the last years. To evaluate the potential of this protocol within cork oak breeding programs it is essential to guarantee somatic embryos/emblings genetic stability. RAPD are currently used to assess somaclonal variation and provide (as other molecular markers) large information on genetic variability of the micropropagation process. Besides, assessing the stability of genomic and promoter sequences of genes of interest may also give important information on genetic fidelity of the micropropagated material with respect to that specific gene. In particular, studying histone gene sequences, proteins involved in cell cycle, may contribute to a better understanding of the role of cell cycle in somatic embryogenesis process. In this work, somatic embryogenesis was induced from leaves of field trees on MS medium supplemented with 2,4-D and zeatin. Embling convertion took place on MS medium without growth regulators. DNA from donor tree, somatic embryos and emblings was used to assess genetic variability by RAPD (a total of fifteen primers were used) and by Histone H3 promoter – type I element. The results obtained from the RAPD and the Histone H3 promoter – type I element data analyses, demonstrate that the somatic embryogenesis protocol used did not induce, up to moment, any genetic variability, confirming data obtained with other molecular and genetic techniques, supporting that this standard protocol may be used to provide true to type plants.

Key words: Histone H3 promoter - type I element, Molecular markers, PCR, *Quercus suber* L., RAPD, somatic embryogenesis.

2.2 – Introduction

Quercus suber L. is a very important species, in terms of environment and economy, mostly in the Mediterranean. According the last governmental information available, in Portugal this forestry species occupies approximately 800,000 ha (Direcção Geral de Florestas, 2001). However, as a consequence of the oak population aging and susceptibility to environmental factors (*e.g.* fungus-host interactions, soil water-nutritional imbalances and forest fires) a large percentage of the native cork oak populations are now declining. In order to satisfy the environmental and economic demands, a compromise between both situations includes the in vitro propagation of the best trees for both cases.

It is well known that in vitro culture can induce somaclonal variation (by e.g mutation and/or epigenetic changes) (e.g. Kaeppler et al., 2000). The frequency of these changes depends on several factors, such as genotype, growth regulators (Larkin and Scowcroft, 1981; Berlyn et al., 1986), which may hamper the implementation of clonal foresty programs or, on the counterpart, may provide mutants with useful commercial or scientific value. From all the in vitro techniques used, somatic embryogenesis is the most promising method for clonal mass propagation of forest species (Merkle, 1995), mostly because both root and shoot meristems are presented simultaneously (Kim, 2000).

In *Q. suber*, somatic embryogenesis was achieved from leaves of seedlings (Fernández-Guijarro et al., 1995), nodal segments (Maataoui and Espagnac 1987), zygotic embryos (Bueno et al., 1992; Manzanera et al., 1993), and from leaf explants of juveniles (Toribio et al., 1998; Hernández et al., 1999; Hornero et al., 2001a) and adult plants (Pinto et al., 2001; Pinto et al., 2002b). But for the inclusion, within a near future, of this somatic embryogenesis-protocol in cork oak breeding programs, the plant quality (e.g. genetic variability) and performance must be assessed.

Using *Q. suber* somatic embryos from several embryogenic lines obtained from 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 of this species by Amplified Fragment Length Polymorphisms (AFLP) markers (Hornero et al., 2001b). However, when using embryogenic lines from mature explants, AFLP analyses detected somaclonal variation in one genotype. These data suggest an influence in the process, by the age of explant and/or of the genotype on

genetic stability (Hornero et al., 2001b). Also using somatic embryos and emblings derived from mature plants, Loureiro et al. (2005) found no significant ploidy or DNA content variations among somatic embryos, by using flow cytometry. Later, using SSR markers Lopes et al. (2006), found no variability in somatic embryogenic lines resulting from young trees but one mutation was found when the line was obtained from an old one, supporting the putative importance of genotype and/or of the plant donor age.

Despite their reliability as markers, the individual information given by each marker is restricted (e.g. Smulders, 2005) and must be combined with other markers such as RAPD and genetic variability in highly conserved genome sequences (together with phenotypic analyses). RAPD analyses have been used as a reliable, quick, and inexpensive method to identify clones and cultivars (Williams et al., 1990; Lin et al., 1994; Barrett et al., 1997; Li and Nelson, 2001) and to assess somaclonal variation (Isabel et al., 1993; Heinze and Schmidt, 1995; Gallego, 1997; DeVerno et al., 1999; Saker et al., 2000; Raimondi et al., 2001; Martins et al., 2004; Valladares et al., 2006).

Many genes that are involved in somatic embryogenesis process were already identified (for review see Chugh and Khuruna, 2002). However it is also important to find how those genes (i.e. their products) interact with each other and their role in the process (e.g. cyclins, histones, hormone regulators, heat shock proteins).

Some phases of the somatic embryogenesis process are rich in high cell division rates. Therefore, when studying this process it is important to focus on the cell cycle intervenients. In particular, histones, according to its function in nucleosome, are classified into core (H2A, H2B, H3, and H4) and linker (H1) proteins. Despite differences in their functions and regulation roles, core and linker histone, genes are coordinately expressed in S phase during the cell cycle (Kapros et al., 1992). In most eukaryotic cells, transcription of certain histone genes, including histone H3, begins at the onset of S phase (Kapros et al., 1992; Reichheld et al., 1995; Reichheld et al., 1998). Correlou et al. (2001), in *Fucus*, used histone H3 transcription as a S phase–specific marker using RNA gel blot analysis with a fragment of the coding region from a *Fucus* histone H3 gene. Kapros et al. (1992) found differential expression of the H3 histone gene variants during the cell cycle and during embryo development. This means that any point mutation or other type of alteration on the sequence of the type I element may be reflected in the first instance on the genetic expression of those histone genes, and then probably of other genes, as a consequence of their direct influence on the heterochromatin condensation (Johnson et al., 2002; Chen and Tian, 2007). Therefore, searching for mutations in regions of interest

(e.g. coding/promoter sequences of histones, important regulators of the cell cycle), may allow the detection of scientifically valuable mutants.

Furthermore, regions of the histone H3 promoter were recently reported to be polymorphic for *Q. suber* (Brás, 2001; Rocha et al., 2006). These studies were based on the fact that the promoter regions of the plant histone genes harbour one or more types of highly conserved, specific sequences (motifs) which could be used as molecular markers (Brignon and Chaubet, 1993). One of them, the type I element (CCACGTCANCGATCCGCG), that is a well conserved regulatory element found in the proximal promoter region of a certain class of plant histone genes, being composed of two independent cis-acting elements of the hexamer (ACGTCA) and the reverse oriented octamer (GATCCGCG) motifs (Terada et al., 1995; Minami et al., 2000).

The aim of this work is to study of the genetic variability of the somatic embryos from leaf explants of adult plants of *Q. suber,* as also from emblings obtained according the Pinto et al. (2002b) protocol by RAPD and the molecular marker histone H3 promoter type I element.

2.3 – Material and Methods

2.3.1. Induction of somatic embryogenesis

Cuttings were collected during May and June from a 60-years-old *Q. suber* tree (QsG3) in the north of Portugal and were treated as described by Pinto et al. (2002). Leaves were disinfected with commercial bleach and embryogenic calli were induced in accordance with the protocol established by Pinto et al. (2002). Briefly, explants were placed on Murashige and Skoog (1962) medium (MS) with 30 $g.L^{-1}$ sucrose, 3 $g.L^{-1}$ Gelrite®, pH adjusted to 5.8 and supplemented with 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 9.0 µM zeatin, in the dark at 24±1ºC to induce somatic embryogenesis. After 3 weeks, cultures were transferred to a photoperiod of 16 hours and exposed to a light intensity of 98±2 $\mu mol.m^{-2}$ s^{-1} for three months. After this period, somatic embryos

when present, were isolated and transferred to fresh MS medium without growth regulators (MS_{WH}). Every four weeks, somatic embryos were subcultured on fresh MS_{WH} medium and were maintained by repetitive somatic embryogenesis. The embryogenic line used in this study was maintained by repetitive somatic embryogenesis for 1 year. When somatic embryos reached the cotyledonary stage, normal somatic embryos were isolated for embryo germination and/or conversion in MS_{WH}. When plants were obtained they were acclimatized.

All chemicals used in these experiments, except the genomic DNA extraction, were purchased from Duchefa (Haarlen, Netherlands).

2.3.2. Genomic DNA extraction

For both experiments, leaves from the donnor tree (QsG3), individual dicotyledonary somatic embryos and leaves from emblings were colleted for DNA extraction. Total genomic DNA was extracted with the DNeasy® Plant Mini Kit (QIAGEN, Germany), according to the specifications of the supplier. Following extraction, DNA concentration and purity were estimated by 0.8% agarose gel electrophoresis with ethidium bromide (EB) staining, and comparison with a standard molecular mass marker (lambda *Hind*III, NEB), and also by spectrophotometry at 260 and 280 nm (1 A_{260} Unit of dsDNA = 50µg/mL H_2O; Pure DNA: $A_{260}/A_{280} \geq 1.8$).

2.3.3. RAPD

Amplifications were carried out in a Px2 Thermal Cycler. Forty 10-mer primers (from Kits C and S from Operon Technologies) were tested in this study to select the most polymorphic for this genotype. 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/sec to reach annealing temperature, 1 min at 55ºC, a ramp of 1.5ºC/sec to reach 72ºC and 4.5 min at 72ºC; 25 cycles of 30 sec at 94ºC, a ramp of 1.5ºC/sec to reach annealing temperature, 1 min at 45ºC, a ramp of 1.5 min to reach 72ºC and 4.5 min at 72ºC; a final step of 1 min at 72ºC. PCR reactions were

stored at 4ºC until their resolution by electrophoresis on 1.5% (w/v) agarose gels with EB staining, in 1XTBE (Tris-Borate-EDTA, pH 8.0), at 150V and at room temperature. After electrophoresis, the gel was photographed using the imaging system G:BOX HR (Syngene, USA). The molecular dimensions of the PCR products were deduced by extrapolation of their electrophoretic motilities on a calibration curve defined for each gel, using for this the computer program GeneTools (Syngene, USA).

2.3.4. Histone H3 promoter type I element

PCR amplifications were done by using the *Taq* PCR Master Mix Kit (QIAGEN, Germany) under instructions specified by the supplier, the type I element CCACGTCACCGATCCGCG as primer (synthesised by MWG Biotech. Germany), and carried out in a Px2 Thermal Cycler following the PCR profile described by Brás et al. (2001). For each reaction (total volume of 25 µL), 12.5µL of *Taq* PCR Master Mix, 1.75µL of 50mM MgCl2, 1.5µL of 10µM primer solution and 50ng of DNA template were mixed in that order and immediately submitted to PCR amplification. PCR products were simultaneously resolved with a 1Kb Plus DNA Ladder (Invitrogen, USA) by electrophoresis on 1.2% (w/v) agarose gels with EB staining, in 1XTBE (Tris-Borate-EDTA, pH 8.0), at 2.8 V/cm and at room temperature. After electrophoresis, the gel was photographed using the imaging system G:BOX HR (Syngene, USA). The molecular dimensions of the PCR products were deduced by extrapolation of their electrophoretic motilities on a calibration curve defined for each gel, using for this the computer program GeneTools (Syngene, USA).

2.3.5. Data Analysis

For each RAPD primers, the molecular weight of each PCR fragment was estimated. Assuming that the polymorphic bands are segregated in a Mendelian way and the alleles do not co-migrated to the same position in the gel. Data were scored for subsequent analysis on the basis of presence (1) or absence (0) of the amplified product and assembled in a data matrix. Data were analysed with a 95% confidence interval according to the descriptive variables. The association between the non-parametric variables just like for example, the state of presence or absence for the primers OPC (1, 2, 3, 5, 8, 9, 14, 18 and 19), OPS (12, 14, 16, 17, 18 and 19), and others, were estimated

by the Correlation Coefficient of Spearman (ρ) and the qui-square test (χ^2) being considered significant when P≤0.05 (Zar, 1999). Genetic distances were estimated from the data matrix using both the DICE and the Jaccard indices of similarity from the SPSS program (version 11.5, SPSS Inc., USA).

Concerning Histone H3 promoter type I element, the technique was first optimized for seven genotypes, in a total of 36 samples (Rocha et al 2006). For variability assays, two independent experiments were perfomed to assess the profile of PCR-products using the matching comparison function of the computer program GeneTools (Syngene, USA).

2.4 – Results

Di-cotyledonary somatic embryos used in this study had large, compact and when mature, green cotyledons. Emblings derived from these somatic embryos looked morphologically normal with one dominant shoot apex, well developed stem and green leaves.

2.4.1. RAPD analysis

From the screening primers experiments, primers 1, 2, 3, 5, 8, 9, 14, 18 and 19, from the Kit C (Operon Technologies) and primers 12, 14, 16, 17, 18 and 19 from Kit S (Operon Technologies) were selected. All these primers showed a reproducible profile under the conditions selected. In this study, a total of 96 fragments were obtained for the OPC primers (Fig.2 e 3) and a total of 73 fragments for the OPS primers (Fig.3 e 4).

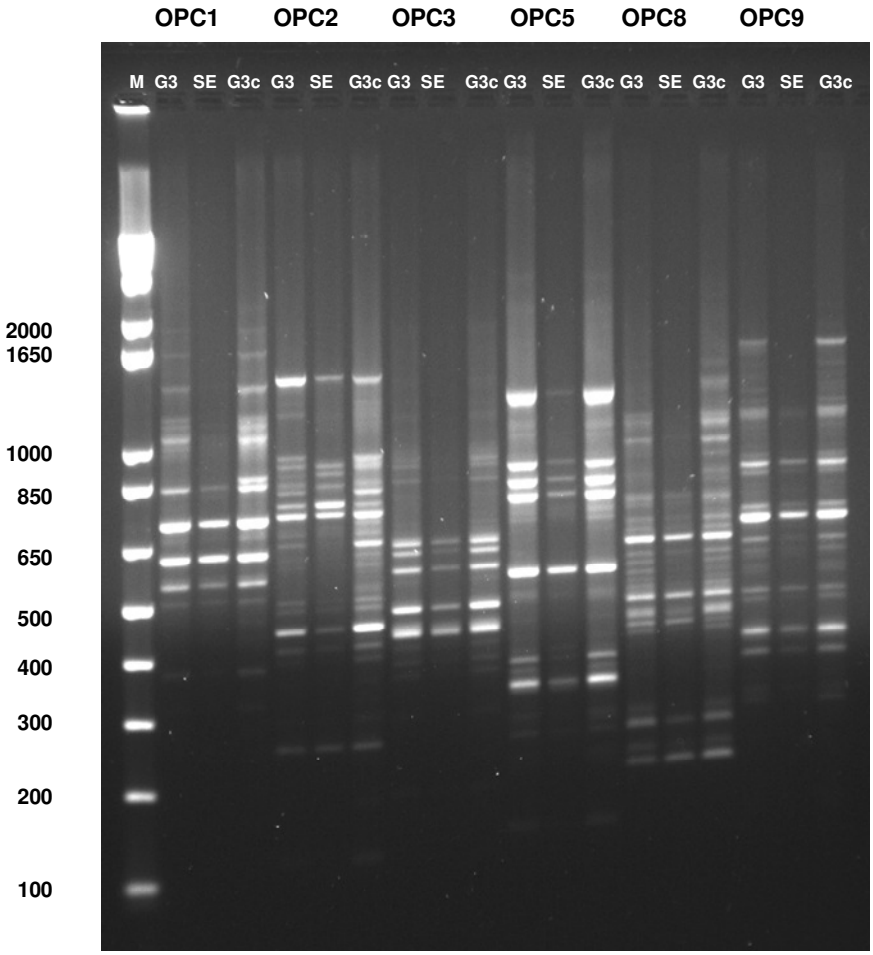

Fig. 2: DNA profiles generated by primers OpC 1, OpC2, OpC3, OpC5, OpC8 and OpC9 in the three different stages of the somatic embryogenesis process: mother tree, somatic embryo and embling. M, size marker (1Kb Plus DNA Ladder)

The OPC primers individually generated from 6 (OPC18) to 15 (OPC8) bands, with molecular weight ranging between 226 and 1899 bp, whilst OPS primers generated individually, RAPD profiles composed of 10 (OPS18) to 16 (OPS19) bands, with molecular weight between 230 and 1417 bp.

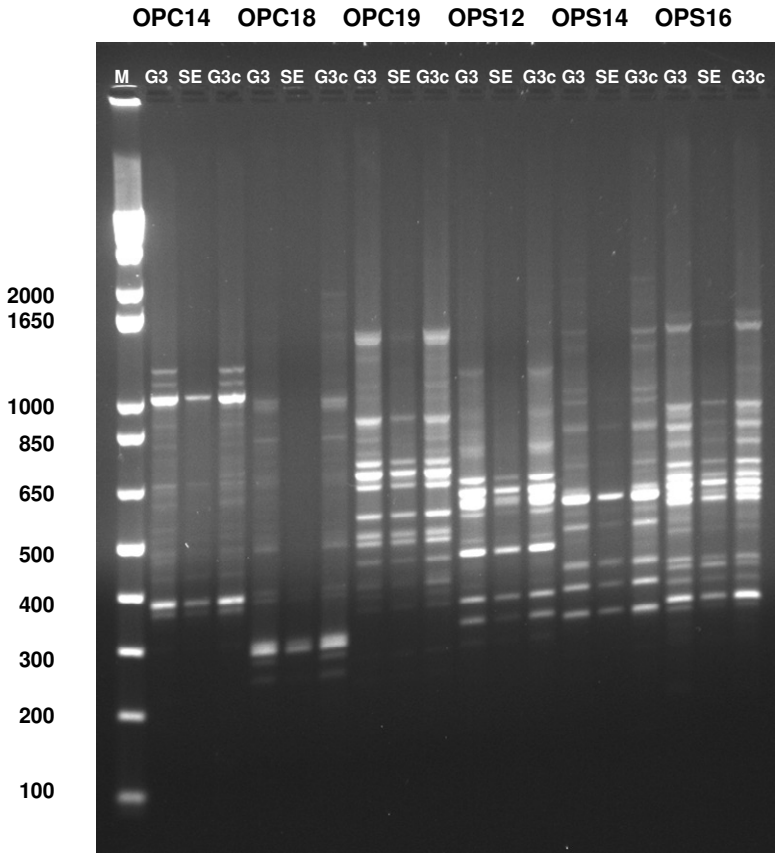

Fig. 3: DNA profiles generated by primers OpC 14, OpC18, OpC19, OpS12, OpS14 and OpS16 in the three different stages of the somatic embryogenesis process: mother tree, somatic embryo and embling. M, size marker (1Kb Plus DNA Ladder)

For profile analyses only clearly amplified fragments were considered and scores of 1 (present) or 0 (absent) were used to form a matrix.

Before being analysed for genetic variability, data were evaluated in terms of significance as non parametric variables by tests of correlation with coefficient of Spearman and by qui-square (Table 1). These results show that all data belong to the interval of the 95% establish for non-parametric variables, being the differences not significant between them ($P>0.05$). By the use of the coefficients DICE and JACCARD with the SPSS program, the genetic variability values ranged from 0.863 to 0.953 for coefficient DICE, and from 0.759 to 0.910 for coefficient JACCARD (Table 2).

According to the significance values determined (Table 1), the results for both kits of primers do not show variability between the three stages of the somatic embryogenic process.

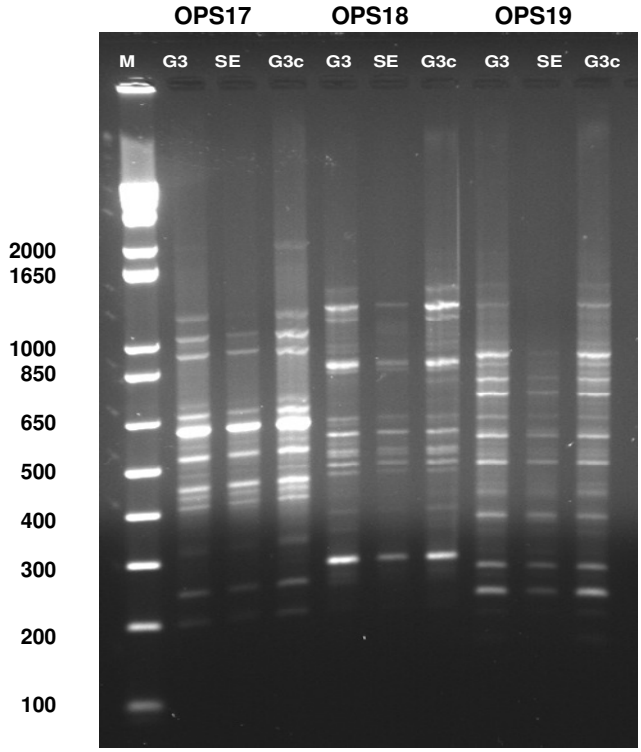

Fig. 4: DNA profiles generated by primers OpS 17, OpS18 and OpS19 in the three different stages of the somatic embryogenesis process: mother tree, somatic embryo and embling. M, size marker (1Kb Plus DNA Ladder)

The RAPD analyses (Table 2) show that the genetic similarity between the mother tree and the embling had more proximal coefficient values than those referred to the somatic embryos.

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Table 1: Values of non-parametric correlation coefficient of Spearman (ρ) and the Qui-square (χ^2) tests for the PCR products obtained with the primers OPC and OPS selected being significant when P≤0.05.

Primers		OPC			OPS				OPC			OPS		
		G3	SE	G3C	G3	SE	G3C		G3	SE	G3C	G3	SE	G3C
	ρ	1.000	-0.100	-0.049	1,000	-0.122	-0.050							
G3	p	.	0.332	0.636	.	0.302	0.675							
	n	96	96	96	73	73	73							
	ρ	-	1.000	-0.113	-	1.000	-0.105	χ^2	9.183	6.658	13.630	5.481	2.718	8.051
SE	p	-	.	0.275	-	.	0.375							
	n	-	96	96	-	73	73	p	0.327	0.574	0.092	0.360	0.743	0.153
	ρ	-	-	1.000	-	-	1.000							
G3C	p	-	-	.	-	-	.							
	n	-	-	96	-	-	73							

Table 2: Similarity relations (Dice and Jaccard coefficients/index) obtained with the Kits S and C of the Operon Technology among the different stages of the somatic embryogenesis process by the SPSS software program.

Coefficients	DICE (Czekanowski or Sorenson) OPC			DICE (Czekanowski or Sorenson) OPS			JACCARD OPC			JACCARD OPS		
Samples	G3	SE	G3c	G3	SE	G3c	G3	SE	G3c	G3	SE	G3c
G3	1.000	0.870	0.953	1.000	0.863	0.949	1.000	0.770	0.910	1.000	0.759	0.904
SE	-	1.000	0.864	-	1.000	0.878	-	1.000	0.760	-	1.000	0.783
G3c	-	-	1.000	-	-	1,.000	-	-	1.000	-	-	1.000

2.4.2. Histone H3 promoter type I element

Following the optimisation of the technique, analyses of different *Quercus suber* L. genotypes by this primer showed its reproducibility as a polymorphic marker; these genotypes show band profiles (composed of five to eight PCR products) specific to all of them (see Annex).

After ensuring polymorphism within genotypes, the Histone H3 promoter type I element was used as a molecular maker to assess genetic variability during the somatic embryogenesis. Our data show no variability along the somatic embryogenesis process (Fig. 5).

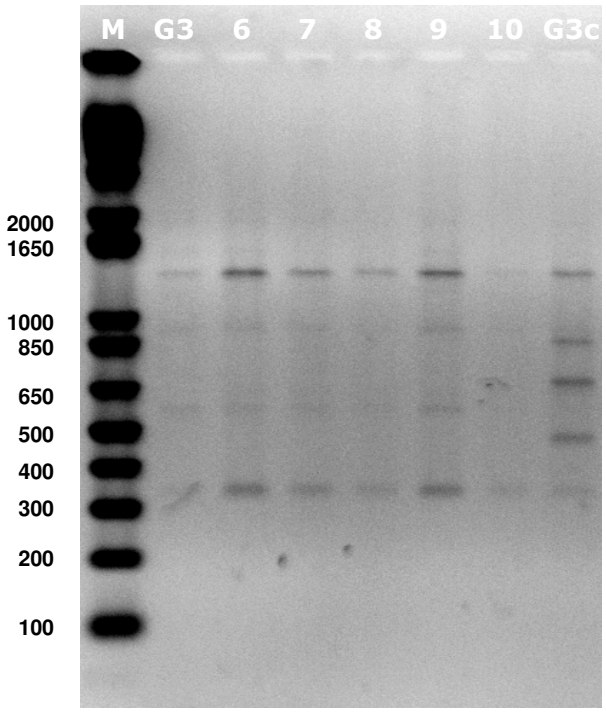

Fig. 5: Histone H3 promoter type I element PCR products from the mother tree (G3), somatic embryos (6 to 10) and embling (G3c) simultaneously fraccionated with 1Kb Plus DNA Ladder (M - Invitrogen).

The QsG3 genotype demonstrates to generate a profile of 6 bands, with molecular weights between the 1663 bp and the 305 bp. The analysis of Fig. 5 shows that the visualization (naked eye) of some bands may be sometimes difficult, and specific softwares must be used. This difficulty is, at least partially, due to the large amounts of template DNA that are in general required for this histone analyses, a demand that may hamper the analyses of individual somatic embryos due to both their small size and high water content (app. 80%). However, due to the objective of the work it was decided do not use pools of somatic embryos, in way to maintained the individuality of the samples.

Table 3 shows molecular weights for the PCR products obtained in each sample; values presented in the table were the result of alignments based on the matching comparison function of the computer program GeneTools, showing no variability among the whole embryogenic process. Therefore, as no intraclonal variation was found, molecular weights presented in the SE column of Table 3 refer to all the embryos analysed.

Table 3: Diagram representing the molecular weights obtained by Histone H3 promoter type I element for the somatic embryogenesis of *Q. suber*, considering the mother plant, dycotiledonary somatic embryos and emblings

	Mother tree	**Somatic embryos**	**Embling**
PCR products (bp)	1663	1662	1620
	1425	1425	1437
	961	961	961
	555	555	550
	422	426	443
	313	310	305

2.5 – Discussion and Conclusions

Molecular markers (molecular genetic markers: RFLP, microsatellites, RAPD, AFLP) had been used as reliable, powerful and quickly tools in the analyses of somaclonal variation in somatic embryogenesis of conifers (Isabel et al., 1993; Heinze and Schmidt, 1995; Fourré et al., 1997; Helmersson et al., 2004; Burg et al., 2007), specially in *Quercus* genus (Hornero et al., 2001b; Sanchez et al., 2003; Wilhelm et al., 2005; Lopes et al., 2006; Valladares et al., 2006).

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 either within or between the embryogenic lines from three trees, or between these lines and the mother tree. Contrarily, Wilhelm et al. (2005), using microsatellites found variation in embryogenic lines of *Q. robur* but not in the regenerated plantlets, concluding that only the non modified genomes are capable of regeneration.

In the case of somatic embryogenic lines of *Q. suber*, until the moment using RAPD (Gallego, 1997), AFLP (Hornero et al., 2001b) or microsatellites (Lopes et al., 2006), no somaclonal variation was observed among somatic embryos within these lines, or even between the explant leaves and the embryos.

One must consider that these techniques have some limitations, some of them related with collection of molecular data. In general, the characters of methods using presence vs.absence of bands are not independent and there is a pronounced asymmetry in the probability of loosing/gaining bands (Karp et al 1996). Based on this fact, it is important to minimize the putative occurrence of errors associated with molecular techniques, ensuring the reproducibility of the technque and the quality of the data (Karp et al., 1996). Therefore, and as highlighted by Smulders (2005) it is advisable to perform genetic analyses with different molecular markers to achieve reliable results. Our laboratory has combined several genetic and molecular analyses of the somatic embryogenesis process in Quercus suber: for example, based on the protocol for somatic embryogenesis in Q. suber designed by Pinto et al. (2002b), Loureiro et al (2005) found

no nDNA content nor ploidy changes in *Q. suber*, while Lopes et al (2006) found only one SSR mutation in a long-term embryogenic culture.

Those genetic studies are, in the present paper, complemented with RAPD analyses to further validate the somatic embryogenesis protocol. The choice of RAPD presents several advantages as it is a cheap, quick and easy technique. Furthermore, the polymorphism value of RAPD analyses was already proved by Gallego et al (1997) who found a polymorphism level as high as 31.9% for different cork oak genotypes. Additionally, Gallego et al (1997) also used RAPD to asses somaclonal variation in *Q. suber* but restricted the study to somatic embryos, not using emblings.

In our study RAPD analyses do not show somaclonal variation in both somatic embryos and emblings by using the somatic embryogenic protocol developed by Pinto el al (2002b). A total of 169 PCR products, obtained with fifteen primers, were analyzed independently by two coefficient of similarity: DICE and JACCARD. Both statistical tests comproved a great similarity between the mother tree and the embling, showing values of 0.953 (OPC) and 0.949 (OPS).

As stated above these RAPD data support other techniques (SSR, FCM) that point out the somatic embryognesis protocol used for cork oak as a potentially true to type propagation system. 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).

As refereed earlier in introduction, the analyses of mutations in gene/promoter sequences may be helpful to understand the role of that particular gene in the micropropagation process. So, in complement to RAPD analyses, it was decided to analyse the histone H3 promoter type I element, an important component of cell cycle. The use of histone H3 promoter type I element was based on the fact that the promoter regions of the plant histone genes harbour one or more types of highly conserved, specific sequences (motifs) which could be used as molecular markers (Brignon and Chaubet, 1993; Terada et al., 1995) and the regions of the histone H3 promoter was reported to be polymorphic for *Q. suber* (Brás, 2001; Rocha et al., 2006), which was also confirmed for other cork oak genotypes (Annex, Rocha et 2006).

Furthermore, and considering that the somatic embryogenesis process presents intense cell divisions, putative mutation in this promoter would provide mutants of high interest to study the embryogenic process *per se.* Other approaches already tried to

evaluate the expression of Histone H3 during somatic embryogenic such as in alfalfa (Kapros et al., 1992) or in other in vitro culture systems (Reichheld et al 1995; 1998). Later, Minami et al (2000) identified that the highly preserved sequences in histone H3 promoter type I element play a crucial role in the S-phase specific regulation. So, a better knowledge of genetic stability of histones, proteins already documented as being involved in cell cycle (Kapros et al., 1992; Reichheld et al., 1995; Reichheld et al., 1998; Minami et al., 2000), may contribute to a better understanding of the somatic embryogenesis process as also from the regulation of the different stages process.

With respect to our results on histone H3 promoter type I element analyses, we defined one profile according to Karp et al. (1996), who suggested that analyses based on variations in band intensity are unclear, being preferable to use data based on more restrict information (e.g. molecular weight). According on this, histone H3 promoter type I element produces, for the QsG3 genotype, a profile composed of 6 PCR products, which is maintained during all the somatic embryogenesis process. Therefore, these results show no modifications in the genomic sequence of the histone H3 promoters during this process.

In conclusion, this work show that no genetic variability in somatic embryos and plants obtained according to Pinto et al. (2002) with respect to both RAPD markers and the histone H3 promoter type I element. Together with the results obtained in these embryos by flow cytometry (Loureiro et al., 2005), by microsatellites (Lopes et al., 2006), as also by the morphological characterization of somatic embryos in this species (e.g. Pinto, 2002; Fernandes, 2006), is supported that the protocol used to somatic embryogenesis may be used to provide true to type plants.

CHAPTER 3 – FUTURE PERSPECTIVES

We demonstrate here that the standard protocol for somatic embryogenesis may provide true to type plants. Other aspects related to some steps of the protocol still require, however, optimization: in particular, maturation and low germination frequencies have to be considered.

We used in this work two molecular markers that showed no genetic variability for the used somatic embryogenesis protocol. In particular, considering the advantages and the demonstrated reproducibility of the RAPD-primers used in this work, they can be used as routine screening of micropropagatd plants in implementation forestry cork oak breeding programs.

The genetic stability obtained here by RAPDS and histone H3 promoter type I element confirm other data for the same protocol, using other genetic analyses. However, it should be advisable to increase the battery of techniques (e.g. AFLP) in these kind of assessments for true–to-type propagation systems.

Finally, we confirmed that the histone H3 promoter type 1 element may be used as a molecular marker in cork oak, and that no mutations in the analysed specific sequences were found. This, however, leads to more interesting questions related with histone H3 gene expression, and its/their influence in the cell cycle during the somatic embryogenesis process. Within this scope, other analyses (e.g. DNA methylation, Real Time PCR, Imunocytochemistry, In situ hybridization) may also be highly interesting to assess epigenetic induced changes during the regeneration process.

CHAPTER 4 – LITERATURE CITED

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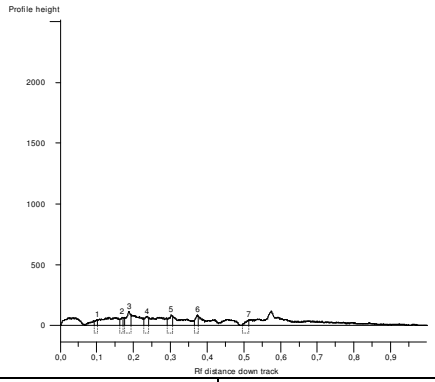
CHAPTER 5 – ANEXES:

A – Table showing the Histone H3 promoter type I element profile for the seven genotypes analysed.

	GM5	GM4	GM1	GM2	G0	G3	G5
Number PCR Products	8	5	7	8	7	6	8
Molecular weights	1408	1338	1465	1404	1424	1663	1400
	1215	1081	1299	1234	1261	1425	1218
	930	963	1078	938	1068	961	1041
	799	435	961	835	787	555	921
	685	323	752	697	527	422	601
	536		526	540	332	313	522
	387		323	390	304		347
	315			331			284

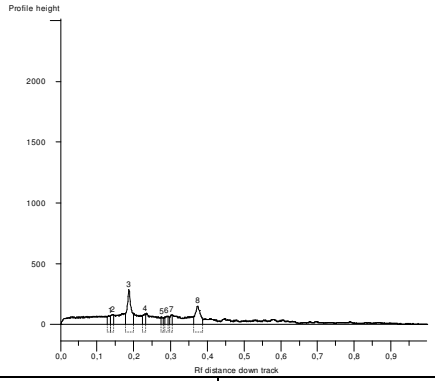
B – Examples of the graphics obtained with the SynGene Tools Program for the PCR with the Histone H3 type I element.

Track 2



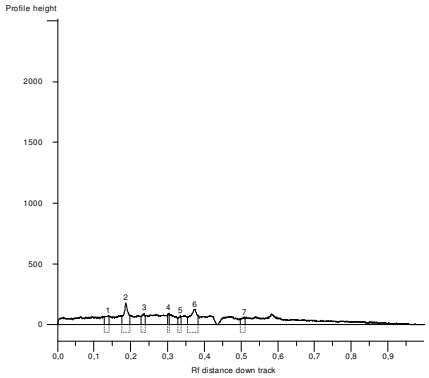
Amostra G3 mãe			
Number	**Mol. Weight**	**Height**	**Raw vol.**
1	2901,49	48,869	16979,44
2	1662,89	66,990	21952,83
3	1425,33	114,840	55611,36
4	961,23	76,273	30238,44
5	555,63	86,867	39015,74
6	313,03	86,179	29917,69

Track 3



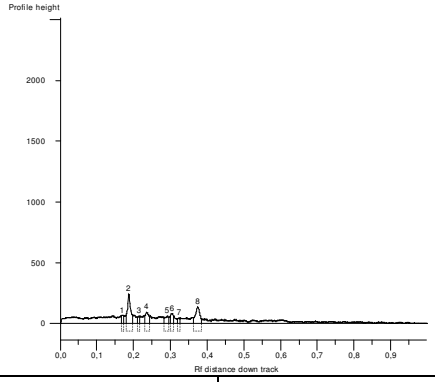
Amostra 6			
Number	**Mol. weight**	**Height**	**Raw vol.**
1	2187,17	75,139	22921,46
2	2077,62	83,306	25708,35
3	1437,59	290,179	130658,98
4	1003,28	88,081	29760,37
5	694,21	61,700	16318,93
6	626,41	71,286	18859,80
7	560,41	80,169	19978,02
8	310,36	152,226	95394,16

Track 4



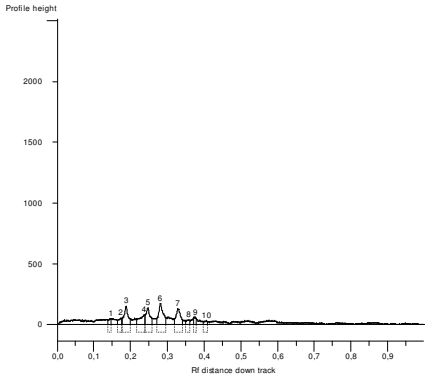
Amostra 7			
Number	**Mol. weight**	**Height**	**Raw vol.**
1	2131,70	74,850	34413,63
2	1437,59	180,914	89240,99
3	961,23	91,387	32892,73
4(m)	555,63	91,388	18082,47
5	426,08	72,739	23263,23
6	310,36	126,931	101502,09
7	102,82	63,786	28810,62

Track 5



Amostra 9			
Number	**Mol. weight**	**Height**	**Raw vol.**
1	1662,89	70,850	19286,09
2	1437,59	246,341	89703,59
3	1150,62	59,793	13739,34
4	969,49	91,966	37627,95
5	610,52	62,727	26712,92
6	550,89	84,270	26715,14
7	464,17	46,395	13663,67
8	310,36	139,317	74119,27

Track 6



Amostra G3 conv.			
Number	**Mol. weight**	**Height**	**Raw vol.**
1	2007,66	51,963	16104,24
2	1620,71	53,903	16945,25
3	1425,33	153,388	68496,11
4	961,23	82,846	54093,56
5	882,33	137,172	67626,38
6	665,11	170,398	98167,04
7	448,54	133,923	71602,18
8	349,90	39,415	14717,99
9	305,09	62,982	14423,08
10	235,97	32,856	11719,58

C – RAPD Analysis Matrices

Primer	Banda	G3	SE	G3c
OPC1	1155	1	1	1
OPC1	893	1	1	1
OPC1	738	1	1	1
OPC1	618	1	1	1
OPC1	543	1	1	1
OPC1	499	1	1	1
OPC1	343	1	1	1
OPC2	1551	1	1	1
OPC2	1054	1	0	1
OPC2	1010	1	1	0
OPC2	961	1	1	0
OPC2	882	1	1	1
OPC2	825	1	1	1
OPC2	780	1	1	1
OPC2	673	1	0	1
OPC2	502	1	1	1
OPC2	433	1	1	1
OPC2	393	1	0	1
OPC2	239	1	1	1
OPC3	1041	1	0	1
OPC3	1010	1	0	1
OPC3	938	1	1	1
OPC3	866	1	0	1
OPC3	771	0	1	1
OPC3	682	1	1	1
OPC3	649	1	1	1
OPC3	592	1	1	1
OPC3	484	1	1	1
OPC3	430	1	1	1
OPC5	1423	1	1	1
OPC5	1191	1	1	1
OPC5	1010	1	1	1
OPC5	926	1	1	1
OPC5	861	1	1	1
OPC5	790	0	1	1
OPC5	585	1	1	1
OPC5	524	1	1	1
OPC5	376	1	1	1
OPC5	335	1	1	1
OPC8	1267	1	0	1
OPC8	1155	1	1	1
OPC8	973	1	1	0

Primer	Banda	G3	SE	G3c
OPS12	1293	1	0	1
OPS12	1007	1	0	1
OPS12	833	1	1	1
OPS12	698	1	1	1
OPS12	649	1	1	1
OPS12	614	1	1	1
OPS12	578	1	1	1
OPS12	499	1	1	1
OPS12	467	1	1	1
OPS12	359	1	1	1
OPS12	318	1	1	1
OPS14	1166	1	0	1
OPS14	1090	1	0	1
OPS14	942	1	1	1
OPS14	765	1	1	1
OPS14	724	1	1	1
OPS14	629	1	1	1
OPS14	581	1	0	1
OPS14	537	1	1	1
OPS14	502	1	1	1
OPS14	436	1	1	1
OPS14	384	1	1	1
OPS14	330	1	1	1
OPS16	1057	1	0	1
OPS16	953	1	1	1
OPS16	865	1	1	1
OPS16	770	1	1	1
OPS16	733	0	1	1
OPS16	716	1	0	1
OPS16	681	1	1	1
OPS16	626	1	1	1
OPS16	564	1	1	1
OPS16	531	1	1	1
OPS16	450	1	1	0
OPS16	398	1	1	1
OPS16	361	1	1	1
OPS17	1294	1	0	1
OPS17	1174	0	1	1
OPS17	1014	1	1	1
OPS17	744	1	1	1
OPS17	696	1	0	1
OPS17	631	1	1	0

OPC8	861	1	1	1
OPC8	771	1	1	1
OPC8	734	1	1	0
OPC8	699	1	1	1
OPC8	649	1	1	1
OPC8	521	1	1	1
OPC8	478	1	1	1
OPC8	455	1	1	0
OPC8	294	1	0	1
OPC8	275	1	1	1
OPC8	243	1	1	1
OPC8	226	1	1	1
OPC9	1899	1	0	1
OPC9	1330	1	1	1
OPC9	1022	1	1	1
OPC9	825	1	1	1
OPC9	775	1	1	1
OPC9	699	1	1	1
OPC9	657	1	1	1
OPC9	537	1	1	1
OPC9	514	1	0	1
OPC9	436	1	1	1
OPC9	393	1	1	1
OPC14	1309	1	1	1
OPC14	1217	1	1	1
OPC14	1104	1	1	1
OPC14	965	1	0	1
OPC14	854	1	1	1
OPC14	681	1	1	1
OPC14	611	1	0	1
OPC14	490	0	1	1
OPC14	447	1	0	1
OPC14	403	1	1	1
OPC14	355	0	1	1
OPC14	334	1	1	1
OPC18	1097	1	1	1
OPC18	886	1	0	1
OPC18	481	1	0	1
OPC18	359	1	1	1
OPC18	269	1	1	1
OPC18	227	1	1	1
OPC19	1563	1	1	1
OPC19	1400	1	0	1
OPC19	983	1	1	1
OPC19	881	1	1	1
OPC19	813	1	0	1
OPC19	770	1	1	1

OPS17	555	0	1	1
OPS17	539	1	0	1
OPS17	480	1	0	1
OPS17	297	1	1	0
OPS17	230	1	1	1
OPS18	1383	1	1	1
OPS18	1091	1	0	1
OPS18	966	1	1	1
OPS18	882	1	0	1
OPS18	687	1	1	1
OPS18	627	1	1	1
OPS18	562	1	1	1
OPS18	519	1	1	1
OPS18	489	1	1	1
OPS18	284	1	1	1
OPS19	1417	1	0	1
OPS19	1118	1	0	1
OPS19	1033	1	1	1
OPS19	978	1	1	1
OPS19	887	1	1	1
OPS19	810	1	1	1
OPS19	700	1	1	1
OPS19	623	1	1	1
OPS19	555	1	1	1
OPS19	529	1	1	1
OPS19	480	0	1	1
OPS19	438	1	1	1
OPS19	379	1	1	1
OPS19	346	1	1	1
OPS19	278	1	1	1
OPS19	237	1	1	1

OPC19	720	1	1	1
OPC19	673	1	1	1
OPC19	571	1	1	1
OPC19	511	1	1	1
OPC19	490	1	1	1
OPC19	442	1	1	1
OPC19	386	1	0	1