Universidade de Aveiro Departamento de Biologia
2009

RAQUEL TEIXEIRA DE SOUSA

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OXIDATIVE STRESS AND CELL CYCLE DURING CORK OAK SOMATIC EMBRYOGENESIS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizado sob a orientação científica da Doutora Glória Catarina Cintra da Costa Pinto, Investigadora Auxiliar do CESAM e co-orientação da Profª Doutora Maria da Conceição Lopes Vieira dos Santos, Professora Associada com Agregação do Departamento de Biologia da Universidade de Aveiro/CESAM.

Dissertation submitted to the University of Aveiro to meet the requirements for obtaining the degree of Master in Toxicology and Ecotoxicology, held under the scientific guidance of Doctor Glória Catarina Cintra da Costa Pinto, CESAM Research Assistant and co-supervision of Professor Maria da Conceição Lopes Vieira dos Santos, Associate Professor with Aggregation of Department of Biology University of Aveiro/CESAM

Dedico este trabalho aos meus pais, avós e Rui.

I dedicate this work to my parents, grandparents and Rui.

"Deste modo ou daquele modo.
Conforme calha ou não calha.
Podendo às vezes dizer o que penso,
E outras vezes dizendo-o mal e com misturas,
Vou escrevendo os meus versos sem querer,
Como se escrever não fosse uma cousa feita de gestos,
Como se escrever fosse uma cousa que me acontecesse
Como dar-me o sol de fora.

Procuro dizer o que sinto
Sem pensar em que o sinto.
Procuro encostar as palavras à idéia
E não precisar dum corredor
Do pensamento para as palavras

Nem sempre consigo sentir o que sei que devo sentir
O meu pensamento só muito devagar atravessa o rio a nado
Porque lhe pesa o fato que os homens o fizeram usar.

Procuro despir-me do que aprendi,
Procuro esquecer-me do modo de lembrar que me ensinaram,
E raspar a tinta com que me pintaram os sentidos,
Desencaixotar as minhas emoções verdadeiras,
Desembrulhar-me e ser eu, não Alberto Caeiro,
Mas um animal humano que a Natureza produziu.

E assim escrevo, querendo sentir a Natureza, nem sequer como um homem,
Mas como quem sente a Natureza, e mais nada.
E assim escrevo, ora bem ora mal,
Ora acertando com o que quero dizer ora errando,
Caindo aqui, levantando-me acolá,
Mas indo sempre no meu caminho como um cego teimoso.

Ainda assim, sou alguém.
Sou o Descobridor da Natureza.
Sou o Argonauta das sensações verdadeiras.
Trago ao Universo um novo Universo
Porque trago ao Universo ele-próprio.

Isto sinto e isto escrevo
Perfeitamente sabedor e sem que não veja
Que são cinco horas do amanhecer
E que o sol, que ainda não mostrou a cabeça
Por cima do muro do horizonte,
Ainda assim já se lhe vêem as pontas dos dedos
Agarrando o cimo do muro
Do horizonte cheio de montes baixos."

***Alberto Caeiro**, in Deste modo ou daquele modo, Guardador de Rebanhos.*

O júri

Presidente

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Um muito obrigada a todos!

Raquel Teixeira de Sousa

Palavras-chave

Ciclo celular, indução de embriogénese somática (ES), enzimas antioxidantes, *Quercus suber* L., stress oxidativo

Resumo

A embriogénese somática (ES) é uma técnica de propagação in vitro muito utilizada para a regeneração de plantas. A ES apresenta algumas vantagens relativamente a outras técnicas de micropropagação e tem sido bastante reconhecida em programas de melhoramento. O sobreiro (*Quercus suber* L.) é uma espécie lenhosa com um grande valor económico em Portugal, tendo já sido alvo de estudos de indução de ES. Contudo, algumas fases da ES necessitam ainda de ser optimizadas, e por outro lado, desconhece-se o que medeia a transição de tecido não-embriogénico (TNE) para tecido embriogénico (TE). Pretende-se estudar a) o stress oxidativo e b) a dinâmica do ciclo celular durante a indução de ES de sobreiro, comparando TNE vs. TE.

Para indução de TE e TNE, embriões somáticos dicotiledonares maturos foram isolados e colocados em meio de cultura MS com 1,0 mg l^{-1} 2,4-ácido diclorofenoxiacético e 2,0 mg l^{-1} Zeatina. Para avaliar o nível de stress oxidativo na indução de ES, procedeu-se ao estudo de enzimas antioxidantes, como catalase (CAT), dismutase do superóxido (SOD) e peroxidase do guaiacol (G-POX) assim como de outros parâmetros de monitorização de stress oxidativo (peróxido de hidrogénio, prolina, malonildialdeído). Para avaliar o efeito dos tratamentos (meio de indução ou expressão) ao nível do ciclo celular recorreu-se à técnica de citometria de fluxo (FCM). Além das diferenças morfológicas, verificou-se que a actividade das enzimas antioxidantes diferem entre ambos os tecidos, sendo estatisticamente diferentes apenas para SOD. SOD e G-POX apresentaram tendência para valores mais elevados no TNE, enquanto CAT mostrou maior actividade no TE relativamente ao TNE. Apesar de não ter revelado diferenças significativas, TE mostrou maior tendência para níveis mais elevados de malonildialdeído e prolina. Da análise do ciclo celular verificou-se que a maioria dos núcleos se encontrou na fase G_0/G_1 independentemente do tecido e do tratamento. Não foram observadas diferenças significativas entre as fases S e G_2.

Este é o primeiro estudo que contempla a monitorização do stress oxidativo e do ciclo celular durante a indução de ES em sobreiro. Em conclusão, a partir dos dados apresentados neste estudo, existem algumas suspeitas quanto a possíveis diferenças entre TE e TNE durante a indução de ES em sobreiro relativamente ao stress oxidativo e ciclo celular, mas que necessitam de ser confirmadas, por exemplo aumentando o número de amostras para cada ensaio. Desta forma, é necessário proceder a mais estudos de modo a conhecer melhor o possíveis papeis do stress oxidativo durante a ES (causa/efeito), aprofundando o conhecimento deste processo para aplicação da produção de plantas em larga escala.

Keywords

Antioxidant enzymes, cell cycle, oxidative stress, *Quercus suber* L., somatic embryogenesis (SE) induction.

Abstract

Somatic embryogenesis (SE) is a technique for in vitro propagation which is used for regeneration of whole plant. The SE has some advantages over other techniques of micropropagation and has been recognized in breeding programs. Cork oak (*Quercus suber* L.) is a woody species with a great economic value in Portugal and has also been the subject of several studies in SE induction. However, some SE stages still require optimization and, on the other hand, factors that might mediate the transition from non-embryogenic tissue (NEC) to embryogenic tissue (EC) are still unknown. It was evaluated a) the oxidative stress status and b) cell cycle dynamics during cork oak SE induction, comparing NEC vs. EC.

For the induction of EC and NEC, mature dicotyledonary embryos were isolated and placed onto a MS culture medium supplemented with 1.0 mg l^{-1} 2,4-Dichlorophenoxyacetic acid and 2.0 mg l^{-1} Zeatin. To access the level of oxidative stress in SE induction, it was proceeded the study of antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) and guaiacol-peroxidase (G-POX) as well as other parameters for oxidative stress monitoring (hydrogen peroxide, proline, malonyldialdeyde). To evaluate the effects of treatments (induction or expression medium) on cell cycle, flow cytometry (FCM) was performed. Besides the morphological differences, it was found that the activity of antioxidant enzymes differ in both tissues, being statistically different only for SOD. SOD and G-POX showed a bias to higher values in NEC, while CAT revealed greater activity in EC than NEC. Although no significant differences were detected, EC showed a bias to higher levels of proline and malonyldialdeyde. Analysis of cell cycle highlighted a largest amount of nuclei in G_0/G_1 phase regardless of the tissue and treatment. No significant differences were observed between S and G_2 phases.

This is the first study that addresses the monitoring of oxidative stress and cell cycle during cork oak SE induction. In conclusion, from the data presented in this study, there are some clues that may indicate differences between NEC and EC during SE induction in cork oak in relation to oxidative stress and cell cycle. However, this study should be confirmed in e.g. by increasing samples amount in each assay. Moreover, more studies should be fulfilled to unveil the possible roles of oxidative stress during SE (cause/effect), deepening the knowledge of the process to improve plant large-scale production.

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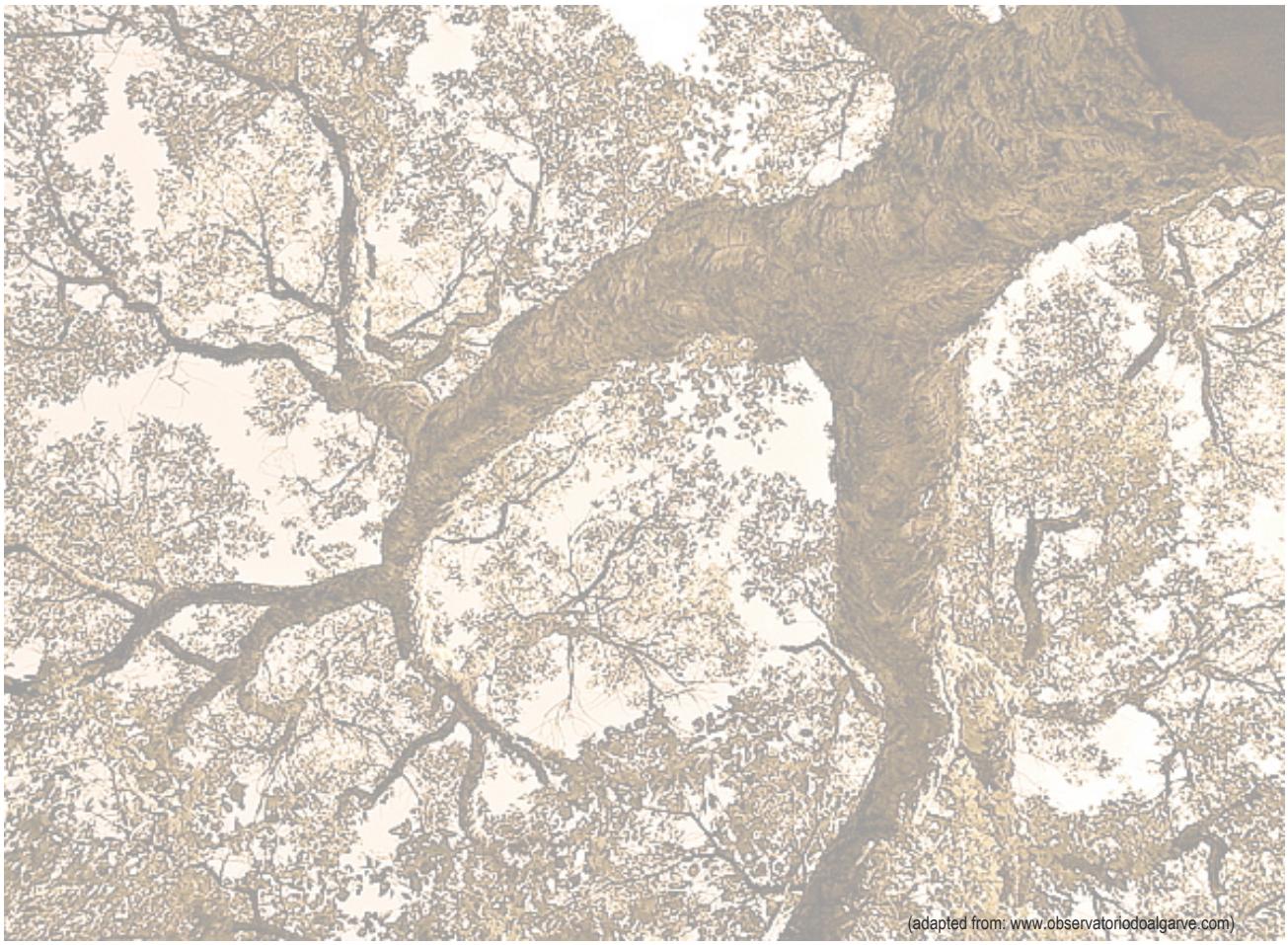
SIMBOLOGY TABLE

$^1O_2$	SINGLET OXYGEN
2,4-D	2,4 – DICHLOROPHENOXYACETIC ACID
AA	ASCORBIC ACID OR ASCORBATE
APX	ASCORBATE PEROXIDASE
CAT	CATALASE
CKs	CYTOKININS
CV	COEFFICIENT OF VARIATION
DNA	DEOXYRIBONUCLEIC ACID
E	EMBRYOGENIC (CELLS)
EC	EMBRYOGENIC CALLUS
FCM	FLOW CYTOMETRY
FSC	FORWARD SCATTER
G-POX	GUAIACOL-PEROXIDASE
G1	GAP 1 PHASE (CELL CYCLE)
G2	GAP 2 PHASE (CELL CYCLE)
GSH	GLUTATHIONE
H_2O_2	HYDROGEN PEROXIDE
M	MITOSE PHASE (CELL CYCLE)
MDA	MALONYLDIALDEYDE
MS	MURASHIGE AND SKOOG (CULTURE MEDIUM)
MSWH	MS PGRS-FREE MEDIUM OR EXPRESSION MEDIUM
NE	NON-EMBRYOGENIC (CELLS)
NEC	NON-EMBRYOGENIC CALLUS
O_2^-	SUPEROXIDE ANION
OH^-	HYDROXIL RADICAL
PGRs	PLANT GROWTH REGULATORS
POX	PEROXIDASE
PUFAs	POLYUNSATURATED FATTY ACIDS
ROS	REACTIVE OXYGEN SPECIES
S	SYNTHESIS PHASE (CELL CYCLE)
SE	SOMATIC SMBRYOGENESIS
SOD	SUPEROXIDE DISMUTASE
SSC	SIDE SCATTER
TBARS	THIOBARBITURIC ACID REACTIVE SUBSTANCES



(adapted from: www.observatoriodoalgarve.com)

PART I

A BROAD INTRODUCTION

STATE OF THE ART

I. SATE OF THE ART

"Life, a state of high organization or low entropy, can be maintained for a while by the consumption of a highly organized form of chemical energy (food) and, in the case of photosynthetic organisms, light energy, this energy is either converted to a less organized form of energy (heat) or utilized to perform work. However, the approach towards ultimate thermodynamic equilibrium is certain for every organism – death or decay." (Palmer, 1995)

The cork oak (*Quercus suber* L.)

Cork oak (*Quercus suber* L.) species belongs to the subgenus *Cerris*, genus *Quercus* and amember of the family Fagaceae (Toribio et al. 2005) and comprises more than 600 species, most of them trees, characterized by their fruits (acorns) (Pinto et al. 2002). The worldwide distribution of the cork oak is confined to the centre and western Mediterranean basin, including Spain, Portugal, France, Italy and countries of the North Africa (Pinto et al. 2002). Portugal is the leader of cork production used worldwide and the cork transformation industry is of great economical importance (Silva et al. 2005). Cork is the bark of the oak, which is a natural, renewable and sustainable raw material product of economic interest for a range of applications (Hernandéz et al. 2003b; Silva et al. 2005). *Quercus suber* (Fig.1.1) is a protected species in Portugal (FAO 1996). The cork oak demonstrates a relevant role in avoiding soil erosion, more particularly in poor and extremely acid soils of abrupt topography where it is one of the very few tree species that can survive. Therefore, several works have been performed in order to study this valuable material and to improve gene-conservation programs (Toumi and Lumaret 1998). Moreover, many efforts have been done for increasing the knowledge on in vitro techniques for breeding programs and large-scale tree production optimization.

Figure 1.1: Cork oak tree (*Quercus suber*) (adapted from photolost.blogspot.com).

In vitro tissue culture: Somatic embryogenesis

Propagation technologies for clonal production have been proposed as imperative tools in increasing breeding possibilities for oaks (Hernández et al. 2003b). Briefly, there are strategies to improve plant production and characteristics regarding agricultural and economical interests, which are based on sexual reproduction or asexual reproduction (cloning). The former introduces more genetic variability and thus might confer increased productivity and disease resistance. The latter, also named vegetative multiplication, is

obtained without production of seed. So, it allows the maintenance of good-quality characteristics of genotypes. These methods include grafting, suckering, cuttings and other procedures.

In the last decades, there has been a rapid progress in the development of in vitro culture techniques with the main objective of regenerating plants for clonal propagation purposes. These in vitro culture techniques allow the development of different regenerative pathways in a reproducible way from several plant tissues, with application to several genotypes resulting in true-to type material (Kim 2000). Briefly, in vitro plant culture consists in growing, under aseptic conditions, certain parts of the plant known as explants. The growth usually occurs in airtight containers containing nutritional media pre-sterilized. These cultures are kept under controlled light and temperature conditions (Benson 2000).

Vegetative propagation by in vitro methodologies (micropropagation techniques) presents several advantages in comparison to traditional methods. These in vitro methodologies could be the best way to preserve elite cork oak genotypes, more resistant to disease, among other ecological and economical interests (Komamine et al. 1995). There are two main pathways to in vitro regeneration of large number of woody plants with industrial interest: micropropagation by nodal/axillary cuttings and somatic embryogenesis (SE). This thesis will focus in SE.

SE is the process by which somatic cells differentiate into somatic embryos, which morphologically resemble zygotic embryos. They are bipolar and bear typical embryonic organs, the radicle, hypocotyl and cotyledons. Compared with other in vitro propagation methods, SE offers several advantages. A separate rooting step is not required, because somatic embryos have both a shoot and a root meristem. In addition, SE usually forms propagules fast and at high rates per explant. Furthermore, the SE process can be automated, meaning that it will eventually become cheaper than other clonal propagation techniques currently in use (Park et al. 1998). Finally, a well-established SE protocol will allow embryogenic clonal lines to be cryopreserved while corresponding trees, obtained from these lines, are tested in the field as is currently being done for conifers (Park 2002). Thus, high value clonal varieties can be developed by retrieving cryopreserved clones, that show best field performance and can then be propagated (Park et al. 1998). Both advanced breeding programs and commercial forestry can therefore benefit from the use of elite clones. In the area of forest biotechnology, this propagation method is regarded as a system for genetic manipulations and of choice for mass propagation of superior forest tree genotypes (Kim 2000; Wilhelm 2000). Actually, cork oak is one of the few forest dicotyledonous species in which complete plant regeneration from adult trees has been obtained (Pinto et al. 2002; Toribio et al. 2005). In *Q. suber*, the majority of the studies in SE refer to the induction of SE from zygotic or very young material. Initially, many regeneration studies have been accomplished from juvenile material, e.g., stem fragments (El Mâataoui et al. 1990), zygotic embryos (Bueno et al. 1992; Manzanera et al. 1993), leaves of seedlings (Fernández-Guijarro et al. 1995; Toribio et al. 2005), 3-years-old plants (Pinto et al. 2001). Since the last reviews from Chalupa (1995), Manzanera et al. (1996) and more recently Wilhelm et al. (2000), progress has been achieved in *Quercus* genus SE, especially using

mature tissue and developing reproducible SE protocols (Hernández et al. 1999, 2001, 2003a, 2003b; Toribio et al. 1999, 2005; Hornero et al. 2001; Pinto et al. 2002; Lopes et al 2006) and recently even using somatic embryos cryopreservation (Fernandes et al. 2008).

The selected explants, the genotype, the culture medium and the growth regulators have a major influence in the induction of embryogenic cultures and in plant conversion (Jimenez 2005). Although there are several protocols available to induce SE from mature leaves, they have several similarities. Besides the differences in medium composition and type of the plant growth regulators (PGRs) they are in accordance that the presence of both auxin and cytokinin were essential to induce SE. The most widely used auxin sources are NAA (Naphthalene acetic acid) or 2,4-D (Dichlorophenoxyacetic acid), while BAP (Benzylaminopurine) or Zeatin are the most frequently used cytokinins (Taiz and Zeiger 2002; Jimenez 2005). Independently of the explant tissue (leaves or zygotic embryos) cork oak somatic embryos show the same developmental pattern. Somatic embryos appear almost directly on the surface of leaves, without a defined pattern of organization, and soon start to show secondary or repetitive embryogenesis. The full expression of embryogenesis occurs when leaves are transferred to PGRs-free medium, in which more leaves would show their embryogenic ability, and somatic embryos their potential to multiply themselves by secondary embryogenesis (e.g. Pinto et al. 2002; Hérnandez et al. 2003a; Toribio et al. 2005).

Repetitive or secondary SE is a phenomenon whereby new somatic embryos are initiated from other somatic embryos. The process of SE is normally not synchronized (Wilhelm 2000; Toribio et al. 2005) (see Fig. 1.2). Therefore, a broad spectrum of different developmental stages can be found simultaneously. In an optimized procedure, this step can be automated by liquid culture (e.g. in bioreactors (Shohael et al. 2007)). In cork oak, secondary embryogenesis takes place continuously on media without PGRs, giving a recurrent process that lasts for years without apparent decline of multiplication ability, merely by monthly subculture to the fresh medium (Toribio et al. 2005). This phenomenon is frequently reported as typical in *Quercus* species (Fernadez-Guijarro et al. 1995; Cuenca et al. 1999; Wilhelm 2000; Pinto et al. 2002). This can be an efficient system to increase embryogenic callus and thus conversion frequencies (Pinto et al. 2002). Besides, somatic embryos may reach their full cotyledonary stage without any specific treatment of differentiation, allowing the isolation of somatic embryos (Toribio et al. 2005). In cork oak, secondary embryos are formed mostly next to the root pole and more rarely in the cotyledons (El Maâtaoui et al. 1990; Bueno et al. 1992, 2000; Fernandez-Guijarro et al. 1995; Puigderrajols et al. 1996; Pinto et al. 2002; Hernández et al. 2003a). In general, a typical cotyledonary somatic embryo is a bipolar structure flanked by two cotyledons that may have a transparent to opaque appearance according to the degree of maturation (Fernandez-Guijarro et al. 1995; Pinto et al. 2002; Hernández et al. 2003a). In the literature, it is often found references to several abnormal morphologies, such as the presence of one or more than two cotyledons in somatic embryos or even fused embryos (Pinto et al. 2002; Hernández et al. 2003b). Despite the occurrence of morphologically abnormal SE, Pinto et al (2002) reported that the plants derived from these SE showed no morphological variability when compared with

those obtained from conversion of normal dycotiledonary embryos. Even though SE is considered to be the best tissue culture-based method of regenerating forest plants, particularly cork oak, some limitations hamper operational applications (Merkle 1995; Hernández et al. 2003a). Indeed, one of the major limitations of embryogenic cultures induction is woody species low conversion rates that are not proper for industrial purposes (Pinto 2002).

Physiological and molecular events underlying SE as well as the dedifferentiation mechanisms remain hardly known (del Pozo et al. 2005; Fehér 2008). The acquisition of embryogenic competence is probably dependent on a range of special circumstances essentially determined by the given physiological state of the cells which is in turn dictated by its genotype and environmental conditions (del Pozo et al. 2005).

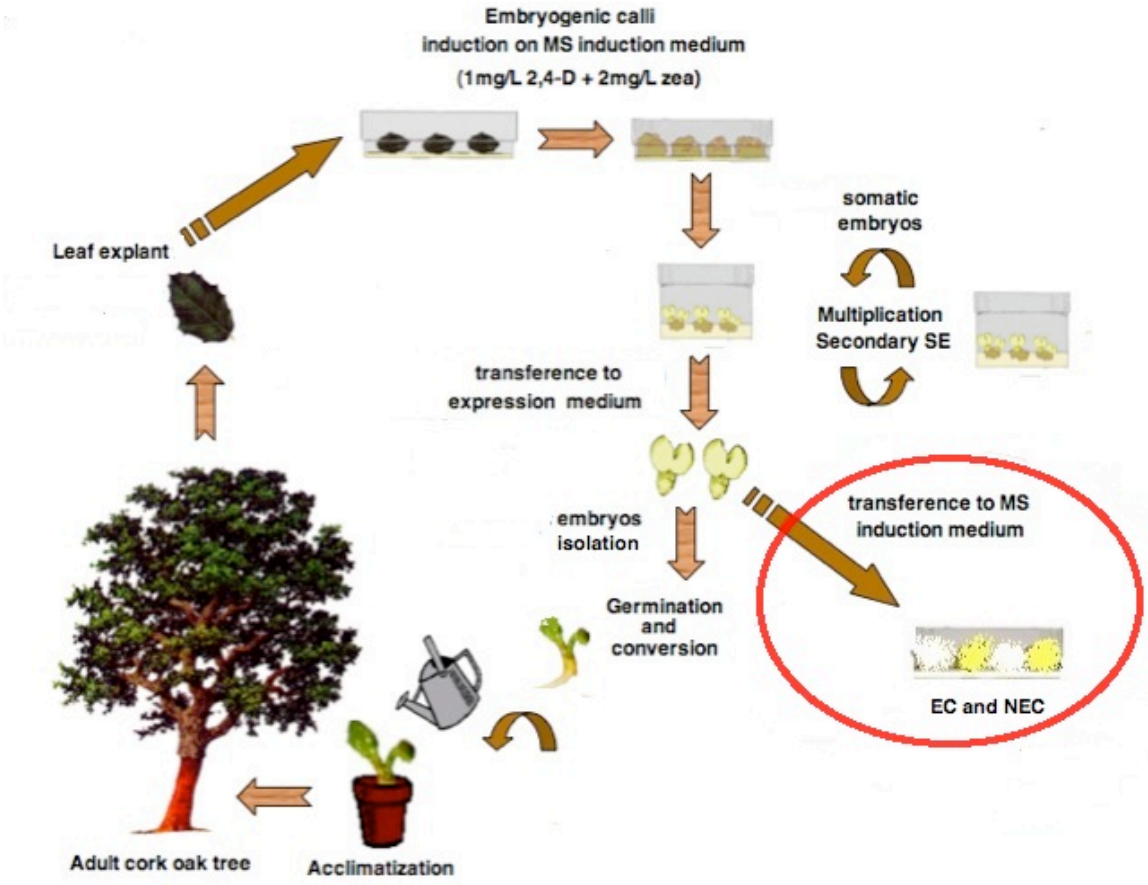


Figure 1.2: Schematic diagram of *Q. suber* somatic embryogenesis (SE) induction protocol. The area red lined is the strategy adressed for this study. PGRs (plant growth regulators); EC and NEC (embryogenic and non-embryogenic callus (adapted from Pinto 2002).

Multidisciplinary studies have been recently applied for a better understanding of underlying processes of SE, such as transcriptomic (Stasolla et al. 2004; Yin et al. 2007; Mantiri et al. 2008), metabolomic (Dowlatabadi et al. 2009) and proteomic (Winkelmann et al. 2006; Yin et al. 2007). In particular quantitative analyses of gene

expression and their putative roles in the process are of utmost importance to understand SE studies (Fehér et al. 2003; Zeng et al. 2007).

Embryogenic and non-embryogenic callus

During SE induction it is frequently observed a concomitant emergence of two types of callus on the induction medium. The non-embryogenic callus (NEC) is characterized as friable, whitish and easily distinguished from the yellowish and compact embryogenic callus (EC). The absence of competence for SE demonstrated by non-embryogenic (NE) tissue is not yet completely understood and data is missing regarding biochemical and molecular events during this process (Nieves et al. 2003). Furthermore, even NE cells appear to have no considerable function, some authors defend that there is some type of signaling between these cells and competent ones. Moreover, the conversion to embryogenic (E) cells might be enhanced by the presence of certain compounds produced by NE cells in culture (e.g. arabinogalactan proteins) (MacCabe et al. 1997).

Nieves et al. (2003) have investigated the levels of certain metabolites in both lines of EC and NEC of sugarcane (Saccharum sp. var CP-5243). They identified significant differences in some amino acid contents, including free L-proline, which has been related to the promotion of SE (Armstrong et al. 1985). Moghaddam and Mat Taha (2005) through image analysis by electronic microscopy also reported important discrepancies between E and NE sugar beet calluses (*Beta vulgaris*). These authors defended that SE is only possible in tissues with low levels of endoplasmatic reticulum and polysomes. As it was already reported for carrot (*Daucus carota* L.) (Pasternak et al. 2002), E cells are densely cytoplasmed (Kadota et al. 2005) and usually exhibit many starch grains, an intact cell wall and a higher nucleus to cytoplasm ratio. Unlike, NE callus reveals critical alteration in cytoskeleton, loosening of cell-to-cell connection and probably different cell wall composition (Mogaddam and Mat Taha 2005). These observations might lead to the findings of abnormal cellular behaviors manifested by NEC, which have lost the ability to cooperate with adjacent cells for plant regeneration.

Oxidative stress: a general introduction

As a consequence of aerobic life, reactive oxygen species (ROS) are formed by partial reduction of molecular oxygen. Under normal physiological conditions there is a balance between the formation of ROS and the protective antioxidant mechanisms of cells. However, many environmental stresses including drought, temperature, flooding and post-anoxia stress, a range of gaseous pollutants (ozone, nitrogen oxides, volatile organic compounds, etc), nutritional imbalances, heavy metals, pathogens attack and herbicides have been indicated to increase oxidative stress, leading to overproduction of ROS that overcomes the cellular antioxidant capacity (Arora et al. 2002; Vinocur and Altman 2005) (see Fig. 1.3).

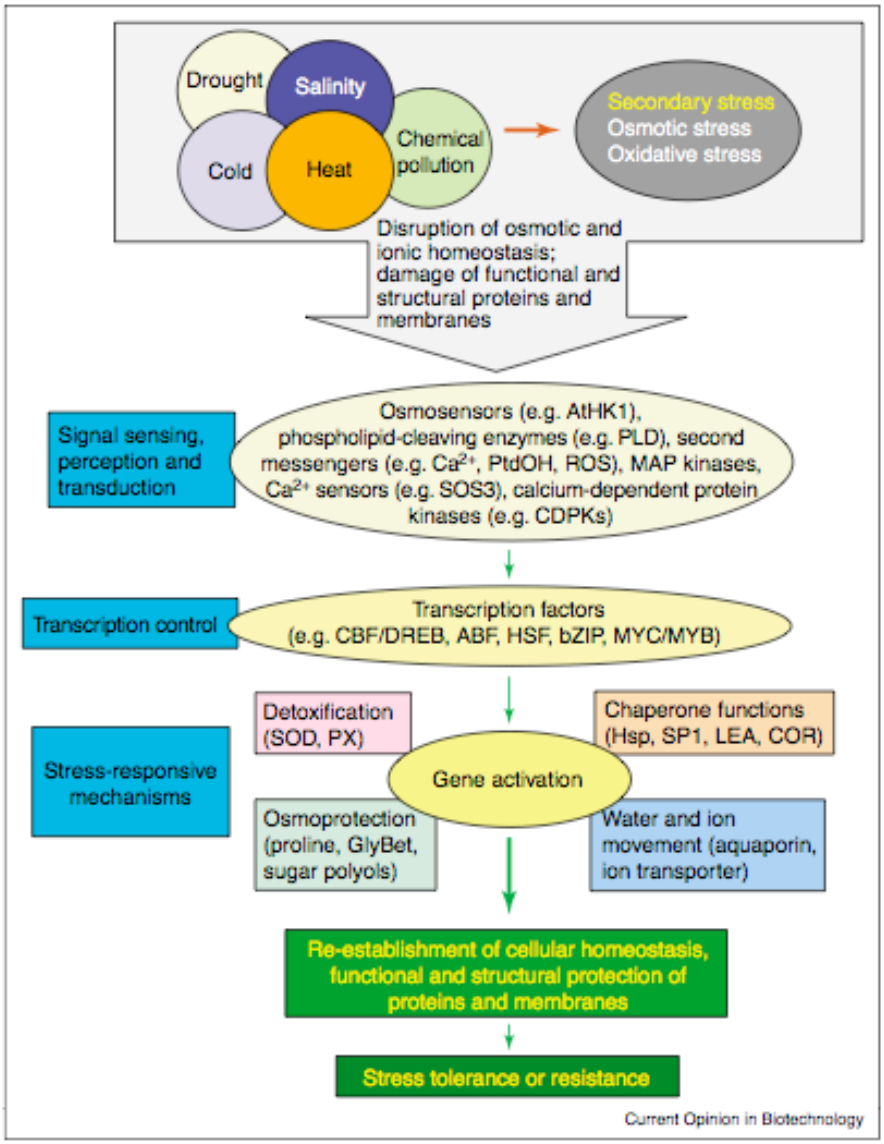


Figure 1.3: Schematic representation of plant response to abiotic stress. Response mechanisms and main intervenient The initial stress signals trigger the downstream signaling process and transcription controls, which activate stress-responsive mechanisms to re-establish homeostasis and to protect and repair damaged proteins and membranes (adapted from Vinocur and Altman 2005).

ROS are molecules, which have an unpaired electron, thus are highly reactive, that interact non-specifically with a variety of cellular components (Ashraf 2009). All aerobic organisms are totally dependent upon redox reactions and the transfer of single electrons and many life processes (e.g. oxidative respiration, photorespiration, photosynthesis, lipid metabolism and cell signalling) involve free radical intermediates, molecular oxygen and activated oxygen species such as the superoxide radical ($O^{2\cdot}$), hydrogen peroxide (H_2O_2), singlet oxygen (O_2^1) and eventually hydroxyl radical ($OH^{\cdot}$) (Cassells and Curry 2001; Scandalios 2005) (see Table 1.1). Uncontrolled ROS production may ultimately attack macromolecules such as polyunsaturated fatty acids (PUFAs) of the chloroplast membranes, leading to toxic breakdown products and

trigger lipid peroxidation (Benson 2000; Ledford et al. 2005). Actually, the main concern about ROS is the possible reactions with biological molecules (see Table 1.2). Peroxidation injury of the cell membrane leads to leakage of cellular contents, rapid desiccation, loss of cell function and, eventually to a breakdown in structural integrity which can lead to necrosis and death (Scandalios 1993; Benson 2000).

Table 1.1: Reactive Oxygen Species (ROS) of interest for oxidative stress (adapted from Scandalios 2005).

Name	Notation	Some comments and basic sources
Molecular oxygen (triplet ground state)	O_2; $^3\Sigma$	Common form of dioxygen gas
Singlet oxygen (1st excited singlet state)	$^1O_2$; $^1\Delta$	Photoinhibition; UV irradiation; PS II e^- transfer reactions (chloroplasts)
Superoxide anion	$O_2^{\bullet-}$	Formed in many photooxidation reactions (flavoprotein, redox cycling); Mehler reaction in chloroplasts; mitochondrial e^- transfer reactions; glyoxysomal photorespiration; peroxisomal activity; nitrogen fixation; reactions of O_3 and $OH^\bullet$ in apoplastic space; defense against pathogens; oxidation of xenobiotics
Hydrogen peroxide	H_2O_2	Formed from $O_2^{\bullet-}$ by dismutation; photorespiration; ß-oxidation; proton-induced decomposition of $O_2^{\bullet-}$; defense against pathogens
Hydroxyl radical	$OH^\bullet$	Decomposition of O_3 in apoplastic space; defense against pathogens; reactions of H_2O_2 with $O_2^{\bullet-}$ (Haber-Weiss); reactions of H_2O_2 with Fe^{2+} (Fenton); highly reactive with all macromolecules
Perhydroxyl radical	$O_2H^\bullet$	Protonated form of $O_2^{\bullet-}$; reactions of O_3 and $OH^\bullet$ in apoplastic space
Ozone	O_3	UV radiation or electrical discharge in stratosphere; reactions involving combustion products of fossil fuels and UV radiation in troposphere

Table 1.2: Some damages induced by Reactive Oxygen Species (ROS) on biomolecules (adapted from Scandalios 2005).

Oxidative damage to lipids
- Occurs via several mechanisms of ROS reacting with fatty acids in the membrane lipid bilayer, leading to membrane leakage and cell death.
- In foods, lipid peroxidation causes rancidity and development of undesirable odors and flavors.

Oxidative damage to proteins
- Site-specific amino acid modifications (specific amino acids differ in their susceptibility to ROS attack)
- Fragmentation of the peptide chain
- Aggregation of cross-linked reaction products
- Altered electrical charge
- Increased susceptibility to proteolysis
- Oxidation of Fe-S centers by $O_2^{\bullet-}$ destroys enzymatic function
- Oxidation of specific amino acids "marks" proteins for degradation by specific proteases
- Oxidation of specific amino acids (e.g., Try) leads to cross-linking

Oxidative damage to DNA
- DNA deletions, mutations, translocations
- Base degradation, single-strand breakage
- Cross-linking of DNA to proteins

ROS are produced basically within the chloroplast, mitochondria, plasma membrane, microbodies

(peroxisomes and glyoxisomes) and cell walls (see Table 1.3), during metabolic pathways as photosynthesis and photorespiration, which is the most obvious oxygenation pathway in the chloroplast (Arora et al. 2002). To prevent extensive oxidative damages, plants have developed specific mechanisms to avoid this stress outcome during normal metabolic processes, like the photosynthetic electron transport system (Arora et al. 2002).

Hydrogen peroxide (H_2O_2) has been considered as an interesting ROS. It has been accepted as a second messenger for signals generated by means of ROS as it easily diffuses through membranes and has a relatively long life (Arora et al. 2002). Moreover, many studies have suggested the existence of a close interaction between intracellular H_2O_2 and cytosolic calcium in response to biotic and abiotic stresses. In fact, environmental stress might trigger a rapid and transient increase in calcium influx, which boosts the generation of H_2O_2 (Grant et al. 2000; Yang and Poovaiah 2002). Yang and Poovaiah (2002) and other authors have proposed calcium/calmodulin (CaM) a controlling mechanism of H_2O_2 homeostasis in plants. They verified that increased cytosolic Ca^{2+} can down-regulate H_2O_2 levels by means of Ca^{2+}/CaM-mediated stimulation of catalase activity in tobacco leaves. This reactive oxygen species will be addressed along this study.

Table 1.3: Site location of ROS generation. Description of components and species involved active oxygen species formation and scavenging (adpated from Scandalios 1993).

Subcellular Location	Type of Active O_2 Species	Source of Active O_2 Species	Enzymic Scavenging Systems	Products	Nonenzymic Scavenging Systems
Chloroplast	Superoxide	PSII	SOD	H_2O_2	Fd
	H_2O_2	Enzymic	Ascorbate peroxidase	Dihydroascorbate	Carotenoids
				GSH	Xanthophylls
				$NADP^+$	
Mitochondria	Superoxide	Electron transport and	SOD	H_2O_2	
	H_2O_2	enzymic	Peroxidase	H_2O	
			CAT (CAT-3, maize)	H_2O, O_2	
Cytosol	Superoxide	Enzymic	SOD	H_2O_2	
	H_2O_2		CAT	H_2O, O_2	
			Peroxidase	H_2O	
Glyoxysome and peroxisome	H_2O_2	β-Oxidation (G)	CAT	H_2O, O_2	
		Photorespiration (P)			

Many parameters can be used to characterize and monitor the extent of oxidative stress: evaluation of plant membranes integrity (Heath and Packer 1968), measurement of redox potencial and of stress related metabolites (H_2O_2, ascorbic acid, glutathione), lipid peroxidation estimation through thiobarbituric acid reactive substances, evaluation of antioxidant enzymes, enzymes associated with cell cycle, enzymes of the SOS response like poly(ADP-ribose)-polymerase, screening for heat-shock proteins (HSP) and pathogenesis-related protein proteins (PR) (Cassells and Curry 2001).

Considering the potential ROS oxidation of biomolecules (e.g. DNA) (see Table 1.2), related direct/indirect damages may have severe consequences to cells. Several techniques might be applied to assess ROS-

induced DNA/chromosome injuries, such as, flow cytometry, microdensitometry (measure changes in chromosome number and DNA content), fluorescent in situ hybridization (FISH) (look for somatic recombination) or others that detect DNA sequence mutations such as microsatellites, restriction fragment length (RFLP), amplified fragment length polymorphism (AFLP) (Cassells and Curry 2001).

Plant protection mechanisms against oxidative stress: Antioxidant defense system

Environmental changes may condition cell signaling, with which the cell must deal and respond in a programmed manner, allowing the organism survival (Vandenabeele et al. 2000). Actually, higher plants possess a well-adapted apparatus that allow ROS-scavenging and protection to cell integrity against oxidative stress. ROS-scavenging is accomplished by a set of non-enzymatic antioxidants (glutathione, ascorbic acid, α-tocopherol, retinol) and enzymatic antioxidants (superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione-dependant enzymes, catalase (CAT), among others), in response to stress situations (Arora et al. 2002; Ashraf et al. 2009).

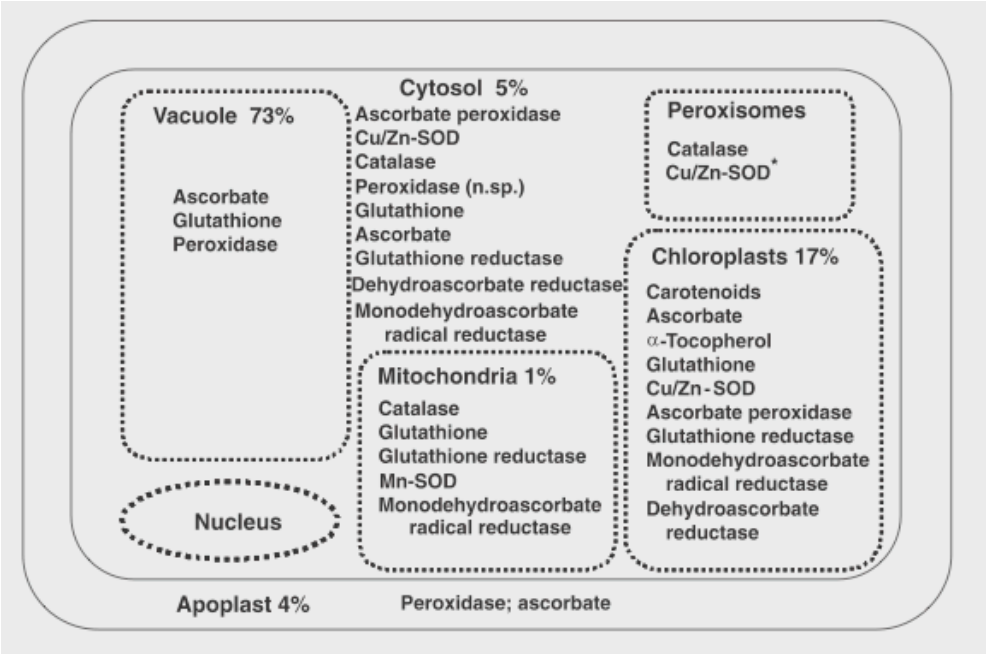


Figure 1.4: Main antioxidant components within a plant cell. These species are distributed through the cell, where are present in higher amounts in vacuoles and chloroplasts (adadpted from Scandalios 2005).

The antioxidant defense system in plats cells is manly constituted by superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione (GSH), ascorbate (vitamine C), tocopherol (vitamine E), carotenoids among others, which are also information-rich redox buffers and important redox signaling components that network cellular compartments (Fig.1.4). As higher plants are sessile, they develop into a series of pathways at different levels that combat with environmental stress, which produces more ROS (Shao

et al. 2008). In this study it will be focused just some stress-related compounds of interest, which are SOD, CAT, POX, L-proline and H_2O_2.

Antioxidant enzymes such as SOD, CAT, APX, POX (peroxidase), GR (glutathione reductase) and MDAHR (monodehydroascorbate reductase) are known to substantially reduce the levels of superoxide anion and H_2O_2 in plants (Ali and Alqurainy 2006; Ashraf 2009).

Superoxide dismutase (SOD; EC 1.15.1.1) was first isolated from bovine blood as a green copper protein (Mann and Keilin 1938) whose biological function was after discovered by McCord and Fridovich (1969). It is a metalo-enzyme and catalyzes the dismutation of superoxide (O_2^-) to molecular oxygen and H_2O_2 by removing superoxide and hence decreasing the risk of hydroxyl radical formation from superoxide via the metal-catalysed Fenton reaction. Since SOD is present in all aerobic organisms and in most subcellular compartments that generate activated oxygen, it has been assumed that SOD has a central role in the defense against oxidative stress (Shao et al. 2008). There are three main types of SOD already described in various plant species and prokaryotes, which are Mn-SOD (in mitochondria matrix, peroxisomes and prokaryotes) (Arora et al. 2002; Shao et al. 2008), Cu/Zn-SOD (in cytosol, chloroplasts, peroxisomes and apoplast) (Corpas et al. 2006; Iriti and Faoro 2008) and Fe-SOD (absent in animals and found mainly in chloroplasts) (Iriti and Faoro 2008).

Several reviews on SOD indicate that in both prokaryotes and eukaryotes, oxidative stress induces or enhances the activity of SOD (Scandalios 1993). These increases arise from several stress responses to herbicide treatments, high temperature and light exposure, ozone, drought, chilling, anoxia and pathogenic injury. In model plants, such as *Arabidopsis thaliana* and *Nicotiana tabacum*, it is possible to enhance the levels of key components of ROS metabolism and antioxidants by genetic manipulation. Studies on transformed plants expressing increased activities of single enzymes of the antioxidant defense system indicate that it is possible to deliberate a degree of tolerance to stress (Arora et al. 2002). In view of these data it is plausible that high levels of oxidative stress may result in high SOD protein turnover, resulting in the requirement for new SOD enzyme synthesis to maintain SOD levels sufficient for effective protection.

Catalase (CAT) (EC 1.11.1.6) is a heme-containing enzyme that catalyses the disproportion H_2O_2 into water and oxygen. The enzyme is found in all aerobic eukaryotes and is important in the removal of H_2O_2 generated in glyoxysomes, peroxisomes where H_2O_2-generating enzymes have been found (Vandenabeele et al. 2000). Peroxisomes contain high amounts of catalase, however, its properties seems to be inefficient to remove low concentrations of H_2O_2 (Arora et al. 2002).

Peroxidases (EC 1.11.1.7) are a single-polypeptide chain and heme-containing enzymes that are present in large amounts in higher plants and are usually expressed as several isoforms (Balen et al. 2003). Isoperoxidases are thought to join in a wide range of physiological processes like H_2O_2 detoxification (Yamasaki et al. 1997), cell elongation (Liszkay et al. 2004), cell wall synthesis (De Marco et al. 1999) and

differentiation and the plant response to stress (Bolwell et al. 2002). They catalyze the typical reaction 2 AH + H_2O_2 → 2 A + 2 H_2O, presenting an array of reducing substrates (AH) (Ghamsari et al. 2007). They are categorized into two main groups: guaiacol peroxidases (G-POX) named after their usual reducing substrate, guaiacol (o-methoxyphenol), which are located in cytosol, vacuole, apoplast (cell wall and extracellular medium), but are absent in organelles (Ghamsari et al. 2007). The physiological functions of G-POX are still hardly known, however, they are also assumed to be involved in a series of processes related to plant growth and development. It was found that this enzyme is expressed during all developmental stages of saffron plant (*Crocus sativus*) suggesting important roles during this morphogenic process (Ghamsari et al. 2007). Furthermore, many other functions such as cell wall lignifications, cell wall stiffening, auxin metabolism and root elongation have been reported for G-POX. The other group is ascorbate peroxidase (APX) as it oxidizes ascorbic acid. They are found in chloroplast, microbodies and cytosol and their main function is to scavenge H_2O_2 and defense against oxidative stress in plant cell (Shigeoka et al. 2002).

The antioxidant system is also constituted by non-enzymatic components that are fundamental for ROS scavenging and buffer cell pH between physiological levels. They are organic molecules such as ascorbate (AA) (vitamin C) that is the most abundant hydrophilic antioxidant in plants (Iriti and Faoro 2008). It is also present in chloroplasts cytosol, vacuole and apoplastic space of leaf cells in high concentrations. Glutathione (GSH) is a tripeptide (Glu-Cys-Glyi) and is the major low molecular weight thiol compound in most plants. It acts as disulphide reductant to protect thiol groups of enzymes, regenerate ascorbate and react with singlet oxygen and hydroxyl radicals (Shao et al. 2008). It is a great antioxidant because it can react chemically with singlet oxygen, superoxide and hydroxyl radicals and therefore function directly as a free radical scavenger. Moreover, GSH may stabilize membrane structure by removing acyl peroxides formed by lipid peroxidation reactions.

The accumulation of some organic compatible compounds in plants such as polyamines (diamine putrescine, triamine spermidine and tetramine spermin) and L-proline play significant roles in plant adaptation to a variety of environmental stresses. As reported earlier, the increase of free polyamines in several plant species under stresses seems to result in stress tolerance (Wei et al. 2009). L-proline is usually regarded as an osmoprotection agent (adjusting the intracellular osmotic potential) (Tatar and Gevrek 2008) and also as ROS-scavenging compound that prevents oxidative damage. There is some controversy regarding proline function in plants. Some authors suggest that its accumulation is an indicator of drought injury (Zlatev and Stoyanov 2005) and others that it confers stress tolerance to oxidative damage induced by water stress (Vendruscolo et al. 2007), even though it is still unveiled.

Conditions enhancing oxidative stress in in vitro culture

A number of well documented problems in physiological, epigenetic and genetic quality are associated with the culture of plant cell, tissue and organs in vitro, namely, absence or loss of organogenic and

embryogenic cell competence (recalcitrance), hyperhydricity and somaclonal variation (genetic and epigenetic variance) (Cassells and Curry 2001). Pasternak et al. (2002) states that early phases of SE depict the induction of many stress-related genes, which leads to the hypothesis that SE is an extreme stress response of cultured plant cells. After oxidative stress exposure, plant cells might acquire a less differentiated status (Pasternak et al. 2002). Many in vitro SE systems rely on the use of exogenous 2,4-D as an inducer, which evokes oxidative stress response (Fig. 1.5).

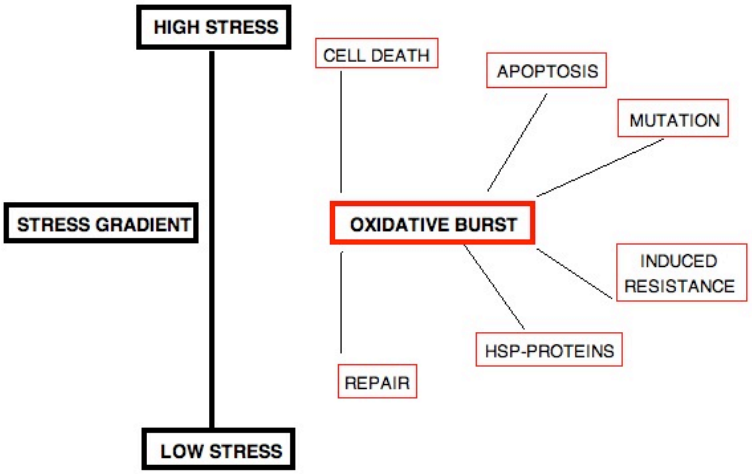


Figure 1.5: Oxidative stress-induced responses and main consequences. There are a significant difference between high and low oxidative stress exposure. HSP-proteins (Heat Shock proteins) (adapted from Cassells and Curry 2001).

Many previous data indicated that oxidative stress caused by radical oxygen species (ROS) promotes SE in many plant species (Luo et al. 2001; Pasternak et al. 2002; Caliskan et al. 2004; Ganesan and Jayabalan 2004). Some variations were identified as a result of plant tissue culture, which are somehow assumed to outcome aside the oxidative burst during explant preparation, and in culture, due to media and other environmental factors. Indeed, both in initiating cultures and in sub culturing, explant preparation (ex: hypochlorite) involves wounding of the tissues, which is known to cause oxidative stress (Cassells and Curry 2001). Other agents or factors that elicit oxidative stress in plant tissue culture are high salt stress, water stress, mineral deficiency, excess metal ions and overexposure to auxin or other plant growth regulators.

As it was already referred, auxin is believed to be one of the most important plant growth regulators in relation to cell division and differentiation, in particular in SE induction, acting as a prerequisite for mitotic activity and meristem formation (Casimiro et al. 2001; Marchant et al. 2002; Pasternak et al. 2002). In fact, moderated oxidative stress mimics auxin stimuli in SE (Pasternak et al. 2002, 2005), which might be addressed as an inherent part of 2,4-D induced SE. Low stress exposure might then result in higher constitutive resistance to subsequent stress, making controlled stress in vitro a reliable option to help overcome stress through cross tolerance.

Certainly, a particular level of oxidative stress is required to promote the formation of E cells and to trigger specific morphogenic pathways involved (Kairong et al. 1999; Fehér et al. 2003; Imin et al. 2005; Pasternak et al. 2005; Blázquez et al. 2009). In this context, ROS could entail the complex signal transduction pathway required to trigger the reprogamation of the gene expression pattern, cellular metabolism and totipotency

crucial for the embryogenic competence of somatic cells in in vitro tissue cultures (Blázquez et al. 2009). Other studies supported that oxidative stress is a component of micropropagation processes, including SE. It can be enhanced by supplementing oxidative stress-inducing agents like copper, menadione (vitamine K_3), paraquat (methyl viologen dichloride), or alloxan to the culture medium at sublethal concentrations (Pasternak et al. 2002) and affects cell cycle progression (Reichheld et al. 1999). Moreover, recently Pinto et al. (2008) reported for the recalcitrant woody species *Eucalyptus globulus* that adding antioxidants to the medium during induction and expression stages, could reduce explant/medium browning but also reducing SE induction and/or expression. These data also support previous data suggesting that regenerative pathways in plant tissue cultures as well as SE comprise a range of developmental processes in which H_2O_2 has been shown to be involved (Kairong et al. 1999; Tian et al. 2003). All these findings strongly support that certain levels of oxidative stress may function as SE promoters. Therefore, changes in antioxidant enzyme activities and oxidative-related metabolites have been considered as markers for the different stages of SE in plants (Bagnoli et al. 1998; Blázquez et al. 2009) and for oxidative stress screening.

Besides these findings, the major bottleneck of this speculation is still the understanding of the main causal mechanisms of oxidative stress-induced response in in vitro culture, and at what level this oxidative stress is required for promoting the process without causing severe damages to cell.

The plant cell cycle, growth regulators and oxidative stress: a general overview

The cell cycle (Fig. 1.6) represents cell division time and thus the range of morphological and biochemical events, which are responsible for cell proliferation (Dewitte and Murray 2003). In plants, cell proliferation is orchestrated by the same fundamental and conserved mechanisms that operate at the cell cycle of all eukaryotes. However, in comparison to animals, plants have a dissimilar development and specific cell-cycle molecules and regulators, as they may undergo indeterminate growth and organogenesis (Dewitte and Murray 2003).

Dewitte and Murray (2003) have supported the differences in cell-cycle controls, mainly due to developmental, environmental and growth hormone influences. The cell cycle encompasses four sequential and spatially organized processes. Mitosis (M phase) is the nuclear division phase, which comprises other four stages (prophase, metaphase, anaphase and telophase) and culminates with cytokinesis (cell division into two daughter cells). Thereafter cells arrive into the gap 1 (G_1), first growth phase, where they are metabolically active and increasing their size. It follows the synthesis phase (S) in which DNA replication takes place. In some species, like *Arabidopsis* they may go into an alternative cycle named endoreduplication. This involves repeated S phases with no subsequent mitosis, resulting in an increase at ploidy level (Dewitte and Murray 2003). The gap 2 (G_2), second growth phase, is when proteins and other relevant metabolites are synthesized to allow mitosis to occur. Sometimes, cells exit G_1 phase to a quiescent one (G_0). Here, cells remain metabolically active but no longer proliferate unless under appropriate external signals (Taiz and

Zeiger 2002; Schmidt et al. 2006). The G_1, S and G_2 phases constitute the so-called Interphase. Plants seem to be more complex in this type of metabolism. This is probably associated with their sessile lifestyle and to become well adapted to changing environmental conditions (Dewitte and Murray 2003).

The different stages of cell cycle must be narrowly coordinated with one another so that cell division occurs properly. This is ensured by a set of checkpoints during cell cycle, which prevent entry into the next stage until the preceding phase has been completely accomplished (Schmidt et al. 2006). There are three main checkpoints that arrest cell cycle. One major checkpoint arrests cells at G_2 when senses damaged or unreplicated DNA. Then, it activates a signaling pathway that may lead to cell cycle arrest, activation of DNA repair and, sometimes programmed cell death (PCD) (Schmidt et al. 2006). Holding cell cycle at G_1 phase also allows DNA reparation before entry into S phase. Likewise, S-phase checkpoint supplies a continual monitoring of the DNA integrity. This provides a quality control, preventing, e.g., the incorporation of incorrect bases within DNA strains (Cooper and Hausman 2004).

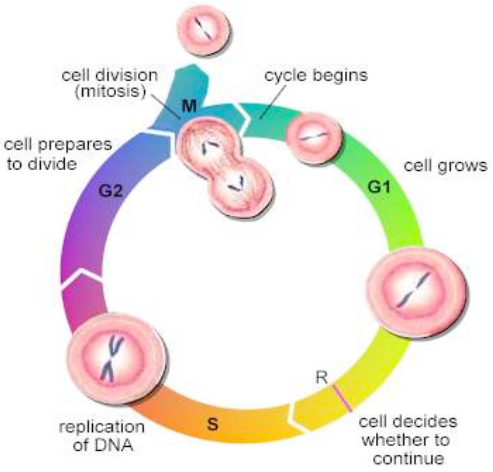


Figure 1.6: Generic diagram of the eukaryotic cell cycle. Here is shown the basic three phases of Interphase in which cell growth (G_1 and G_2) and DNA replication (S) occur and the begining of mitosis (chromossome distribution and cell division) (adapted from http://teachline.ls.huji.ac.il)

Among the many cellular processes that oxidative stress may modulate, cell cycle is one of them, as it was demonstrated that oxidative stress affect cell progression and cell division (Fehér et al. 2008). This was supported by Reichheld et al. (1999 vide Fehér 2008), who demonstrated that the generation of ROS also altered cell redox potencial, influencing cell cycle progression.

The influence of an oxidative agent on SE vs. its putative cytoxic effect is highly dependent on the agent concentration, duration pulse and site of action (Fehér et al. 2008). Moreover, it was found that the influence on cell cycle dynamics was associated with an inhibition of the activity of cyclin-dependent kinases, cell cycle gene expression, and a concomitant activation of stress genes (Reichheld et al. 1999). This is to say that defense responses against oxidative stress also depend on the phase of the cell cycle in plants, e.g. G_1 cells seem to be most sensitive to oxidative stress, inducing defense responses or programmed cell death to protect themselves in plants as well as in animals and fungi (Kadota et al. 2005).

Cell cycle is also intimately dependent on the presence of plant growth regulators (PGRs), which are used to promote cell multiplication and morphogenesis, and ultimately plant regeneration. The effects of PGRs on

cell cycle have been broadly studied. Although their consequences are not exactly understood, they are known to influence cell proliferation and morphogenic development. During in vitro culture there are two main types of PGRs more likely to influence cell proliferation, which are both cytokinins and auxins (Tamura et al. 1999; Dewitte and Murray 2003; del Pozo et al. 2005). They are well documented in controlling the transcriptional expression of an array of cell cycle genes (del Pozo et al. 2005) and crucial for undifferentiated cell proliferation (Tamura et al. 1999; Dewitte and Murray 2003; del Pozo et al. 2005).

Here, it was addressed only two growth factors of interest, cytokinins (Zeatin) and auxin-like 2,4-D (2,4 Dichlorophenoxyacetic acid). Exogenous auxin was suggested to influence the rates of cell division and cell elongation, and possibly, triggering specific differentiation (Tamura et al. 1999) and to influence cell cycle progression primarily by inciting the synthesis of determining cell cycle regulators. On the other hand, it seems that the application of cytokinins increase the amount of CDK protein (cyclin-dependant kinases), but only CK-mediated dephosphorylation induces CDK activation (Taiz and Zeiger 2002). It was suggested that cytokinins affect the G_1/S and G_2/M transitions, as well as on progression through S phase. It was described that the inhibition of isoprenoid biosynthesis at the G_2/M transition associated with release of the inhibition of mitosis by exogenous zeatin showed that this transition requires Zeatin-like cytokinins (Dewitte and Murray 2003). Cytokinin is as well associated with CDK activation at the G_2/M transition either by direct activation of a phosphatase or by downregulation of the WEE1 kinase (Dewitte and Murray 2003), which is also a major regulator of mitotic entry.

It may be kept in mind that the same PGR may participate on cell cycle at different stages and then several mechanisms correlate with each other at a given cell cycle transition (Taiz and Zeiger 2002; del Pozo et al. 2005).

Flow cytometry technique: general principles

"... the cytometric one cell is one biochemical cuvette concept, overcomes these limitations by combining the advantage of microscopic single cell observation with the advantage of multiparametric quantitative biochemical analysis of intact and fully functional cells…" (Valet, G., Max-Planck-Institut für Biochemie)

Flow cytometry (FCM) is a powerful and rapid technology for the random analysis of multiple parameters (chemical and physical) of individual cells within heterogeneous populations simultaneously at real time. These measurements are accomplished by passing particles through the interrogation point, which is surrounded by an array of photomultiplier tubes (PMTs). These analyses are performed by passing thousands of particles (in a cell suspension in movement) per second through a laser beam, and capturing the light that emerges from each particle as it passes through. Then, the data gathered is available to be statistically analyzed by the flow cytometry software to explore a range of information about cellular properties (e.g. size, complexity, integrity, phenotype) (adapted from http://probes.invitrogen.com/). The three fundamental

integrated systems of flow cytometer (Givan 2001; adapted from http://probes.invitrogen.com/) are (Fig. 1.7): fluidic, optic and electronics and computer systems. Initially, the sample in solution is initially injected into the flow cytometer and then it is presented to the interrogation point through a stream of particles, a process managed by the fluidics system (Rahman 2006). The interrogation point is where the laser and the sample intersect and the optics system collects the resulting scatter and fluorescence. This system is the central channel through which the sample is injected. For accurate data collection, cytomes must pass through the laser beam one at a time, thus the sample is injected into a flowing stream of sheath fluid or saline solution which is at a higher velocity. Sheath fluid that is pressurized at a reservoir provides the supporting vehicle for directing cells through the laser beam. This is usually buffer of a composition that is appropriate to the types of particles being analyzed (Givan 2001). Then the sample injection into that reservoir generates a drag effect and altering the velocity of the central fluid. This effect promotes a single file of particles (single flow) – hydrodynamic focusing.

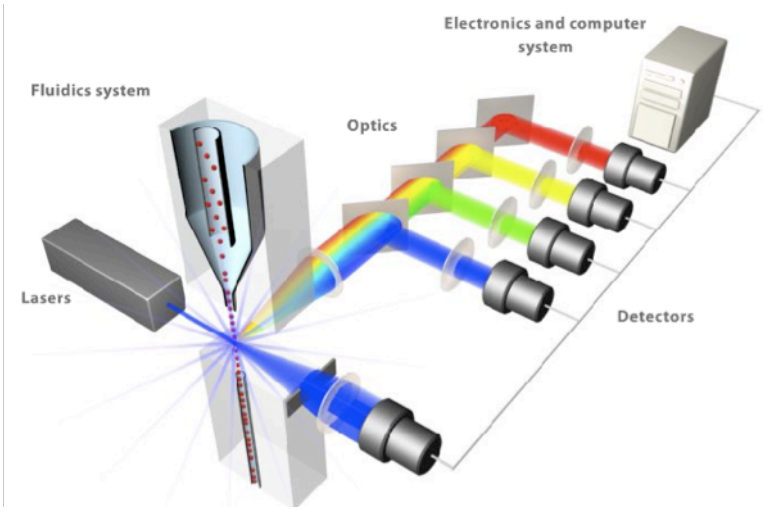


Figure 1.7: Common components of flow cytometers. Fuidics system, lasers, optics, detectors and electronics and computer systems (adapted from http://probes.invitrogen.com/).

The samples must then pass through one or more laser beam that has a circular, radially symmetrical cross-sectional profile. As the laser strikes the cell, the light is reflected, diffracted and/or refracted at all angles. It also can be converted to a different color if it has been absorbed by a fluorescent chemical (Givan 2001). The light gathered in lenses is then focused onto photodetectors, which convert light signal into an electrical impulse which intensity is proportional to intensity of light reached on the detector. The forward scatter (FSC) signal is thought as light of the same wavelength (color) as the incident laser beam that has been bent to a small angle from the direction of that original beam and is named a size or volume signal (Givan 2001; Rahman 2006). The intensity of this signal can also be considered to distinguish between cellular debris and living cells through the cell refractive index analysis (Givan 2001; Rahman 2006). The scattered light received by the photodetectors is translated into a voltage pulse, as the magnitude of the voltage pulse generated is often considered proportional to the particle size. On the other hand light measured at larger angles, as the side, is described side scatter (SSC). The side scatter light is focused through a lens system and is collected by a separate detector located 90º from the laser's path (adapted from http://probes.invitrogen.com/). The intensity of this signal provides

information about particle complexity (granular content) (Rahman 2006) and is related to the cell's surface texture and internal structure as well as to its size and shape (see Fig. 1.8).

Regarding signaling processing, flow cytometers use separate fluorescence channels (FL-) to detect different light emitted. The detectors accommodate a specific sort of optical filters, which block certain wavelengths while transmitting others. The three major filter types are “Long pass” that let through long wavelengths, “Short pass”, that set a limit to lower wavelengths and “band pass” filters which transmit light within a narrow range of wavelengths – a band width (Rhaman 2006).

FCM is broadly applied both to fundamental research and industry and presents a set of distinctive characteristics that make it an extremely useful scientific tool (Loureiro and Santos 2005): general easy sample preparation methodologies, non-destructive, capable of running a multiparametric analysis at the same time (real time). Indeed, FCM is now a changing and useful technique for modern cell biology research present almost at all laboratories.

The applications of FCM have proliferated (and continue to proliferate) rapidly both in the direction of theoretical and practical/applied science, with botany, molecular biology, embryology, biochemistry, marine ecology, genetics, microbiology, and immunology, for example, all represented, and in the direction of clinical diagnosis and medical practice, with hematology, bacteriology, pathology, oncology, obstetrics, and surgery involved (Givan 2001). They are used in many applications such as cell counting, immunophenotyping, green fluorescent protein (GFP) expression and ploidy analysis (adapted from http://probes.invitrogen.com/). The flow cytometer can perform structural and biochemical analyses like the volume and morphological complexity of cells, organelles (e.g. chloroplasts) and biomolecules such as DNA (cell cycle, amount) or RNA, enzyme activity, cell viability (e.g. FDA - fluorescein diacetate), and functional like intracellular pH, membrane potential (e.g. in mitochondria), redox reaction derived-species (e.g. ROS).

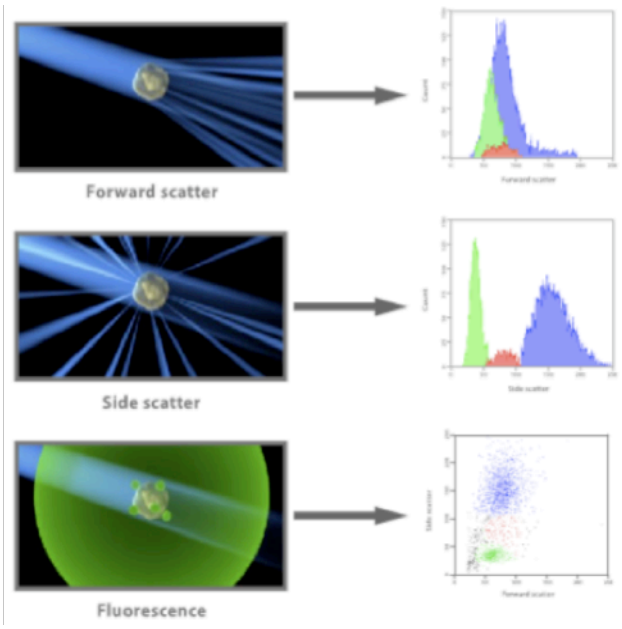


Figure 1.8: Schematic representation of scatter and fluorescence values. They are gathered by the flow cytometer as each particle intersects the laser beam. This allows the analysis of multiparameters of the particle and to distinguish subpopulations within the sample by size, complexity and fluorescence (adapted from http://probes.invitrogen.com/).

Fluorescent probes are effective in directly target a cytome of interest and enable its parameters to be measured more easily by the flow cytometer (Rhaman 2006). There are many fluorescent molecules

applicable to flow cytometry analysis among which some bind specifically nucleic acids in a range of ways such as intercalating (propidium iodide and ethidium bromide), AT-binding (DAPI, Hoechst 33258) and GC-binding (chromomycin). These molecules support flow cytometry for the study of basic mechanisms of the cell cycle and also the study of effects of different putative modulators and inhibitors (hormones, growth factors, toxins, etc.) and environmental conditions (including stress) (Loureiro and Santos 2005).

WORK PURPOSE

This study falls within the field of plant biotechnology and cytomics, presently undertaken by the Laboratory of Plant Biotechnology and Cytometry (University of Aveiro). It was intended to answer the biological questions "*Is oxidative stress involved in different morphological stages during SE induction in woody species?*", "*Do EC and NEC present different profiles of cell cycle dynamics?*". For this, two different developmental stages of SE (non-embryogenic calli and embryogenic) in *Q. suber* were compared regarding antioxidant enzyme activities and other oxidative stress-related parameters (H_2O_2, L-proline and lipid peroxidation). Moreover, and considering a putative direct/indirect role of oxidative stress in cell cycle, claimed in literature, the cell cycle dynamics was also evaluated in both tissues. For this, nuclei were evaluated by FCM and the profile among G_0/G_1: S : G_2 phases compared between all experimental conditions.

Giving an insight to these biological problems this work will provide valuable information of the possible roles (cause/effect) of oxidative stress during SE and may also contribute to better correlate it with cell cycle dynamics (intimately linked to morphogenic processes). This will then give further knowledge to basic questions of plant development field as "*What might happen in NEC to prevent cell differentiation?*"; "*Might the knowledge of oxidative stress status contribute to SE induction protocols improvement?*".

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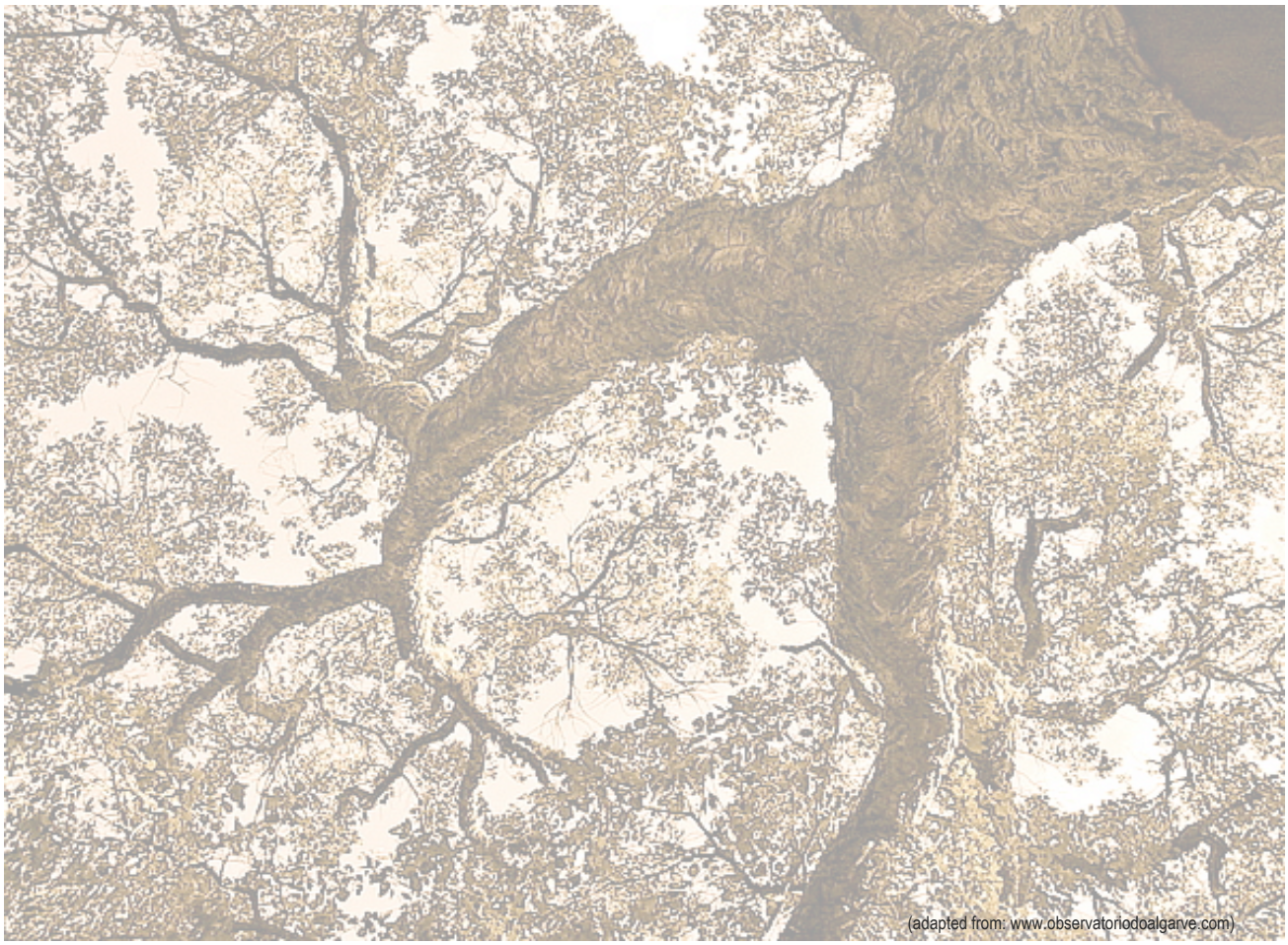
(adapted from: www.observatoriodoalgarve.com)

PART II

A CASE STUDY

EVALUATION OF ANTIOXIDANT ENZYMES AND CELL CYCLE PROFILE DURING SOMATIC EMBRYOGENESIS INDUCTION OF *Quercus suber* L.

II. A CASE STUDY

EVALUATION OF ANTIOXIDANT ENZYMES AND CELL CYCLE PROFILE DURING SOMATIC EMBRYOGENESIS INDUCTION OF *QUERCUS SUBER* L.

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Abstract

Cork oak (*Quercus suber* L.) is an important forest species, particularly in Southern Europe. Due to its economic and environmental values, the improvement of in vitro protocols has become an extremely important tool. For the induction of embryogenic (EC) and non-embryogenic callus (NEC), single mature somatic embryos were transferred onto MS induction medium. Both types of calli can be differentiated by their morphology, but little is known about the biochemical events that take place when somatic cells become competent to produce somatic embryos. Antioxidant enzymes (catalase, superoxide dismutase, guaiacol-peroxidase), metabolites (hydrogen peroxide), L-proline and lipid peroxidation were evaluated for monitoring and screening oxidative stress status. The comparative biochemical/physiological analysis between EC and NEC seems to suggest different behaviourss between them. Data revealed significantly higher levels of L-proline and total protein contents in EC, but not statistical differencences regarding MDA and H_2O_2 contents. Despite a trend to high levels of malonyldialdeyde (MDA), even though not significantly different. In contrast, NEC demonstrated an apparent increase of H_2O_2 levels rather than EC (no statistical significance). Regarding antioxidant enzymes, only SOD showed significant differences for NEC increased activity. EC suggested a trend to increase CAT and decreased G-POX activities. With respect to cell cycle analysis by flow cytometry (FCM), no significant differences were reported neither between tissues nor treatments. As expected, it was detected a dominance of nuclei amount in G_0/G_1 phase in both tissues, independently of the treatment. So far, this is the first report where oxidative stress status and cell cycle were monitored during cork oak SE induction. Changes in antioxidant enzyme activities might give support the speculation that oxidative stress is a driving force for cork oak SE induction. Furthermore, three main factors (concentration, duration of the pulse and site location) suggest that ROS species (e.g. H_2O_2) may affect cellular redox status, ultimatly preventing or not cell cyle progression.

Keywords: Cell cycle, cork oak, embryogenic callus (EC), non-embryogenic callus (NEC), oxidative stress, somatic embryogenesis (SE) induction.

Introduction

Cork oak (*Quercus suber* L.) is one of the most remarkable evergreen species of the Mediterranean ecosystem (Pinto et al. 2002; Hernández et al. 2003b). It represents a great economical value for portuguese cork transformation industry (Pinto et al. 2001; Silva et al. 2005). Nevertheless, the survival of *Q. suber* has been threatened in Portugal and it was given a protected status in this country in the last decades (FAO 1996). In vitro plant propagation technologies have been proposed as imperative tools in increasing breeding possibilities for oaks (Hernandéz et al. 2003).

Somatic embryogenesis (SE) explores plant totipotency and is the development switch of somatic cell to embryo (Zimmerman 1993). SE can serve as a model system to study the molecular, cytological, physiological/biochemical and developmental events of plant regeneration with accessible experimental manipulation (Zimmerman 1993). Over the past decades, research on cork oak SE mainly focused on physiological description and improvements in culturing technologies (Bueno et al. 1992; Hernández et al. 2001, 2003a; Pinto 2002; Toribio et al. 2005). Although cork oak SE induction and further regeneration processes have been previously reported (El Mâataoui et al. 1990; Manzanera et al. 1990; Bueno et al. 1992; Fernández-Guijarro et al. 1995; Hernandez et al. 1999, 2001, 2003a, 2003b; Toribio et al. 1999, 2005; Hornero et al. 2001a; Pinto et al. 2001, 2002) very little is known about the biochemical and oxidative stress status during early stages of this process. On the other hand, there are still technical limitations among different protocol steps that must be answered and optimized to guarantee SE practical application for large-scale production. The induction from adult plants is still recalcitrant and some authors also reportded the loss of embryogenic competence in several in vitro culture systems for many plant species (Benson 2000). Biochemical markers may be useful for early identification of embryogenic cultures before any morphogenic changes. It would allow the improvement of embryogenic culture conditions, monitoring the course of SE and discriminating the cultures for follow up the multiplication process (Hussein et al. 2006).

In recent years, there has been a growing interest in the functional significance of Reactive Oxygen Species (ROS) and the concomitant antioxidant response in growth, development and differentiation of plant cells (Foyer and Noctor 2005). It has been demonstrated that hydrogen peroxide (H_2O_2) embraced within the ascorbate-glutathione (ASC-GSH) cycle, is implicated in the maintenance of cell wall plasticity and the stimulation of organized cell division (De Gara et al. 1997). Both processes are required during the initial stages of SE. A link between ROS and plant morphogenic processes has been suggested (Obert et al. 2005) as its level and type are determining factors for the response they might be able to induce. ROS may trigger defense genes and adaptative responses at low concentrations while activating a genetically controlled cell death program at higher concentrations (Van Breusegem 2006). Pasternak et al. (2002) states that early phases of SE depict the induction of many stress-related genes, which leads to the hypothesis that SE is an extreme stress response of cultured plant cells. In culture, plants are exposed to oxidative stress resulting from: severe wounding, sub-culturing, unbalanced mineral composition of the medium, plant growth regulators

(PGRs) effects. Corroborating these statements, many previous data have indicated ROS to promote SE in several plant species (Luo et al. 2001; Pasternak et al. 2002; Caliskan et al. 2004; Ganesan and Jayabalan 2004) which has been associated with the activation of the cell cycle and cell dedifferentiation. A particular level of oxidative stress seems to be required to promote embryogenic (E) cells formation and to trigger its specific morphogenic pathways (Kairong et al. 1999; Fehér et al. 2003; Pasternak et al. 2005; Imin et al. 2005; Blázquez et al. 2009). Other studies supported the hypothesis that oxidative stress may be an implicit component of micropropagation processes (Cassells and Curry 2001), particularly concerning SE. The cellular stress response is an ubiquitous defense mechanism triggered when cells are confronted with stress. To prevent ROS harmful effects, plants activate antioxidant systems (enzymatic and non-enzymatic), including superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), peroxidase (POX) and ascorbate peroxidase (APX) and stimulate antioxidant molecules production such as ascorbic acid and glutathione for ROS-scavenging (Cassells and Curry 2001). For this, changes in antioxidant enzyme activities have been considered as markers for different stages of SE (Bagnoli et al. 1998; Blázquez et al. 2009) and for oxidative stress screening in several plants species. Many other parameters can also be applied to characterize and monitor the extent and/or effects of oxidative stress: measurement of redox potencial, stress-related metabolites (H_2O_2, ascorbic acid, glutathione), lipid peroxidation estimation through thiobarbituric acid reactive substances (TBARS), cell cycle, among others.

The study of cell cycle profile during morphogenic processes (e.g. SE) is also relevant as it controls organ/individual growth and differentiation processes (e.g. during regenerative pathways). Flow cytometry (FCM) has become a reliable and routine approach for these type of analyses as it is able to analyse individual cells within an heterogeneous population at high speed and short time (Doležel 1997) (e.g. nuclear events may reflect G0/G1: S: G2 phase ratios/dynamics during SE). Several studies on plant genome have been performed by FCM such as ploidity stability (e.g. Winkelmann et al. 1998; Endemann et al. 2001; Pinto et al. 2004; Loureiro et al. 2005; Lopes et al. 2006; Santos et al. 2007; Fernandes et al. 2009), nuclear DNA content (e.g. Galbraith et al. 2002), cell cycle dynamics (e.g Winkelmann et al. 1998). Recently, it has also been described an association between oxidative stress and auxin during cell cycle progression during in vitro culture (Fehér et al. 2008).

The aim of this work is to characterize the oxidative status and cell cycle dynamics during the first phases of cork oak SE induction. Non-embryogenic callus (NEC) and embryogenic callus (EC) were induced and a comparison was made with respect to: antioxidant enzyme systems (SOD, CAT, G-POX), lipid peroxidation, L-proline and H_2O_2. Cell cycle analyses by FCM were also monitored for EC and NEC on induction and expression medium. A better understanding of SE would allow the development of new in vitro culture strategies for plant propagation.

Material and Methods

Plant material and somatic embryogenesis induction

Initial cuttings were obtained from a 60-years-old cork oak tree (Companhia das Lezírias, S.A. at South of Portugal) and the leaf explants were treated and induced according to the protocol established by Pinto et al. (2002). Briefly for SE induction, desinfected leaves were placed on Murashige and Skoog (1962) medium (MS) with 30 g l^{-1} sucrose, 3 g l^{-1} gelrite, pH 5.8, 1.0 mg l^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D) and 2.0 mg l^{-1} zeatin (Zea), in the dark at 24±1°C. When EC aroused, they were isolated and transferred to fresh MS medium without PGRs (in this report so-called MSWH) for somatic embryos development up to cotyledonary stage (expression stage). Then, they were transferred to a photoperiod of 16 h and exposed to a light intensity of 98±2 µmol m^{-2} s^{-1}. Then, the embryogenic lines were multiplied by repetitive SE on the expression-proliferation medium (MSWH), which was routinely renewed every 30 days and maintained under the same growth chamber conditions.

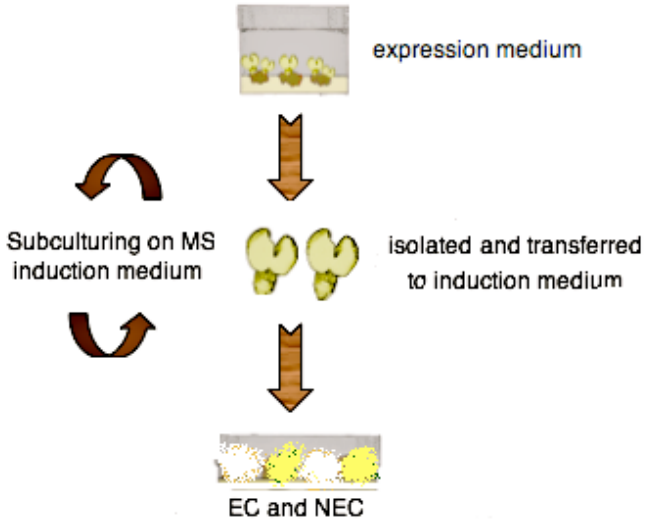

Figure 2.1: Diagram of plant source preparation (adapted from Pinto 2002).

For the induction of EC and NEC single mature dicotyledonary somatic embryos were transferred onto MS induction medium for 12 weeks (routinely renewed), until the production of yellowish embryogenic masses able to be converted into somatic embryos and translucent masses of NEC. EC was identified on a morphological basis under a stereomicroscope and manually separated from NEC. Both tissues were used for protein extraction and antioxidant enzymes assays, lipid peroxidation and H_2O_2 assay. Figure 2.1 shows the schematic procedure of plant source preparation. For cell cycle analysis by FCM, both tissues were used in two different conditions: NEC and EC on induction medium and NEC and EC on MSWH (expression medium).

Determination of Lipid Peroxidation (MDA)

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation, which is one of the products resulted from the reaction with thiobarbituric acid. According to the protocols established by Salem et

al. (2005), both EC and NEC of cork oak (250 mg of fresh weight - FW) were homogenized with 2.5 ml of TCA (0.1% w/v) and then centrifuged at 10000 g for 20 min at 4 ºC. Thereafter 500 μl of supernatant was mixed with 2 ml of 0.5% (w/v) thiobarbithuric acid (TBA) in 20% (w/v) trichlotoacetic acid (TCA). The reaction mixture was heated at 95 ºC for 30 min in a boiling water bath. After cooling in an ice bath, a second centrifugation was accomplished at 10000 rpm for 10 min at 4ºC. The supernatant was collected to spectrophomometric (Thermo Scientific, Genesys 10 UV Scanning) analysis both at 532 nm (specific absorbance) and 600 nm (inespecific absorbance). The MDA concentration was determined by extinction coefficient of 0.155 $M^{-1}cm^{-1}$

L-Proline content assay

L-proline content was determined as described by Hamid et al. (2003) with some variations. So, 100 mg of FW were homogeneized in 1.5 ml of 3% sulphosalicylic acid with a mortar and a pestle. Then the preparation was centrifuged at 10000 g for 10 min at room temperature (21 ºC). The supernatant (100 μl) was collected to react with 2 ml of glacial acetic acid and 2 ml of freshly prepared ninhydrin acid (1.25 g ninhydrin warmed in 30 ml glacial acetic and 20 ml (6 M) phosphoric acid until dissolved) for 1 h at 100 ºC. The reaction stops in an ice bath and the extraction proceeds by the adition of 1 ml of toluene. The chomophore phase with toluene is removed and warmed to room temperature and then its optical density was measured at 520 nm. The proline content was estimated using a standard curve of 1.0-100 μg ml^{-1}.

Hydrogen peroxide content assay

H_2O_2 was extracted from EC and NEC as descrebed by Zhou et al. (2006), with some variations. Samples with 500 mg FW was ground to powder in a mortar with pestle together with 5 ml of 5% TCA and 0.15 g activated charcoal and centrifuged at 10000 g for 20 min at 4ºC. The supernatant was collected and adjusted to pH 8.4 with 25% ammonia and divided into two aliquots of 1 ml, one of which was setted up as blank (addition of 8 μg CAT at room TºC for 10 min for H_2O_2 consumption). To both, 1 ml of colorimetric reagent was added (10 mg 4-aminoantipyrine, 10 mg phenol, 5 mg peroxidase (150 U mg^{-1}) in 50 ml of 100 mM acetic acid buffer pH 5.6). The reaction mixture (final vol. 2 ml) was incubated for 10 min at 30ºC and the absorbance was estimated spectrophotometrically after 30 sec at 505 nm . The H_2O_2 content was determined using a standard curve of 0.1-50 μM H_2O_2.

Protein content and antioxidant enzymes assay

Total protein assay

Total soluble protein contents were estimated according to the Coomassie Protein Assay (Bradford 1976), using the Bio-Rad assay kit (microassay protocol) with bovine serum albumin (BSA) as a calibration standard at concentrations of 0-0.7 mg ml^{-1}. Blank contained 1 ml Blue Coomassie dye and 20 μl NaCl.

Extraction and estimation of superoxide dismutase, catalase and peroxidase

Enzyme extract for superoxide SOD (EC 1.15.1.1), CAT (E.C. 1.11.1.6) and POX (EC 1.11.1.9) were performed according to Sairam et al. (2000) and Almeselmani et al. (2006). Then, the tissue was firstly prepared by grinding the callus (0.5 g) in 10 ml of 100 mM potassium phosphate buffer, pH 7.5 containing 0.5 mM EDTA. For SOD quantification it was measured the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) and a final volume of 3 ml for the reactional solution was applied. According to Almeselmani et al. (2006) it was prepared a reaction mixture containing 13 mM metionine, 63 µM nitro-blue tetrazolium chloride (NBT) (Cervilla et al. 2007) freshly prepared (in water heated till 500 ºC, centrifuged and stored in cold and dark), 0.1 mM EDTA, 50 mM phosphate buffer (pH 7.8), 50 mM Na_2CO_3 and 100 µl of extract. The reaction started by the addition of 2 µM riboflavin (freshly prepared in boiling water for 2 min and stored in cold and dark) and placing the the samples under 15 W fluorescent light for 15 min at 30 cm. Thereafter the reaction was stoped by turning off the light and placing them in the dark. Control tubes were setted up without enzyme and blank ones without irradiation. The increase in absorbance due to formazan formation was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT in light of control sample.

The measurement of CAT was settled for the final volume of 2 ml of reaction mixture, according to Almeselmani et al. (2006). This contained 0.1 M phosphate buffer pH 7.0, 6 mM H_2O_2 and 50 µl of enzyme extract, which starts the reaction. After 5 min the reaction stops by the addition of 4 ml titanium reagent (1 g titanium dioxide, 10 g potassium sulphate and 150 ml concentrated H_2SO_2). Therefore the aliquots are centrifuged at 5000 x g during 10 min at room temperature. The optical density of the resultant supernatant was measured at 415 nm and catalase was assayed by the quantification of the residual H_2O_2. The blank and the control consisted on mixture without enzyme and H_2O_2 (non-specific degradation), respectively**.** CAT content was estimated using a standard curve of 1.0-100 µg ml^{-1}.

Also according to Almeselmani et al. (2006), POX was determined using guaiacol as the electron donor substracte by measuring the reaction mixture at 470 nm during 3 min. This one contained combined 10 mM of phosphate buffer (pH 6.1), 96 mM guaiacol, 50 µl of enzyme and 12 mM of H_2O_2 which starts the reaction. Blank include complete reaction mixture without H_2O_2 and control without enzyme. The G-POX content was estimated using a standard curve of 0.005-1 µg $ml^{-1.}$

Cell cycle analysis by FCM

Flow cytometric analyses of nuclear DNA werer executed to monitor cell cycle progression. Present assays were performed in accordance to the protocol established by Galbraith et al. (1983) and Loureiro et al. (2005). Approximately 25 mg of tissue was directly collected from the cultured medium and “chopped” with a razor blade in a Petri plaque containing 500 µl of Woody Plant Buffer (WPB) (0.2 M Tris.HCl, 4 mM $MgCl_2.6H_2O$, 2 mM EDTA $Na_2.2H_2O$, 86 mM NaCl, 10 mM sodium metabisulfite, 1 % PVP-10, 1 % (v/v) Triton

X-100, pH 7.5) (Loureiro et al. 2007). The suspension was filtered in a nylon mesh to discard most of residues. Thereafter, nuclei were stained by 25 µl of propidum iodide (PI, Fluka, Buchs, Switzerland), which binds specific and stoichiometrically (1:4) to double stranded nuclei acids (as DNA). The mixture was supplemented with 25 µl of RNAse (Sigma, St. Louis, Mo.) to accurate the analysis by removing other interferent nucleic acids. A schematic representation of this methodology is shown is in Fig. 2.2. The estimation of relative fluorescence intensity of PI-stainded nuclei was conducted by the Beckman Coulter EPICS® XL™ Flow Cytometer (Beckman Coulter, Inc.; Hialeah, Florida, USA) with an argon laser cooled with air, operating at 480nm. The percentage of cells in the G0/G1, S and G2 phase were calculated with the "gating region" function of XL-II Software. The results were obtained in the form of three graphics: linear-fluorescence light intensity (FL), forward angle (FS) versus side angle (SS)-light scatter and FL pulse integral versus FL pulse height. This last cytogram was used to eliminate partial nuclei and other debris, nuclei with associated cytoplasm and doublets. Flow cytometry measurements were carried out for both tissues (EC and NEC) and treatments (EC_{ind} and NEC_{ind} – on MS induction medium; EC_{MSWH} and NEC_{MSWH} - on PGRs-free medium/expression medium).

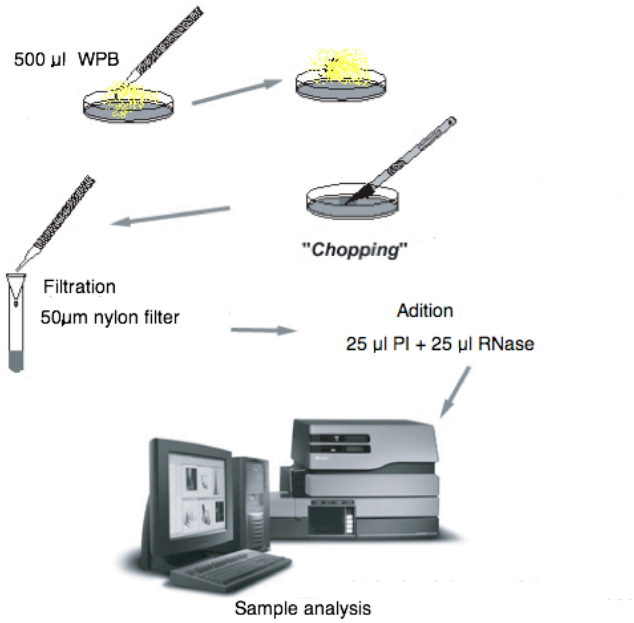

Figure 2.2: Method of sample preparation for cell cycle analyses in the Beckman Coulter EPICS® XL™ Flow Cytometer (Beckman Coulter, Inc.; Hialeah, Florida, USA) (argon laser cooled with air, operating at 480 nm). WPB (Woody Plant Buffer). PI (propidium iodide) was used as a specific fluorochrome to DNA staining (adapted from Pinto 2007).

Statistical Analysis

For antioxidant enzymes and metabolites analysis a t-test was conducted to compare differences between its amount in both callus tissues (EC and NEC) (*N*=3). Data are presented as mean±SEM (standard error) and statistical analysis was performed using a one-way ANOVA or two-way ANOVA (GraphPad Prism 4.0 for MAC OS X, 2005, GraphPad Software, Inc.) to analyse possible differences among cell cycle phases between EC and NEC cork oak within treatments with or without PGRs (*N*=2). A multiple comparison Tukey-Kramer test was applied to determine which groups were different at $P \leq 0.05$ with a confidence level of 95%.

Results and Discussion

Morphological analysis

A method for induction and cyclic proliferation of embryogenic cultures of cork oak was previously setted up in laboratory (Pinto et al. 2002). When cultured in MS induction medium, containing 2,4-D and Zeatin, the single mature somatic embryos generated two different healthy and active proliferating callus (Fig. 2.3). These calluses were screened on a morphological basis in order to identify the embryogenic-type. EC was yellowish and compact, NEC appeared friable (large and vacuolated cells), and white. Both types of callus were easily separated by hand under a stereomicroscope. It was verified the embryogenic potential of these two different materials by culturing for 8 weeks on proliferation medium without PGRs. Under these conditions, only EC was able to produce large amounts of somatic embryos within an assynchronous process. These observations are consistent with other literatures for these species (e.g. Pinto et al. 2002; Toribio et al. 2005; Loureiro et al. 2005; Lopes et al. 2006).

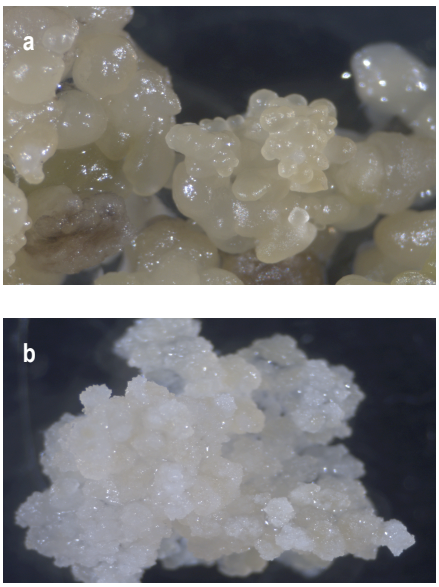

Figure 2.3: EC (a) and NEC (b) of *Q. suber* cultured in vitro on MS induction medium.

Lipid peroxidation

The MDA content (a biochemical indicator of lipid peroxidation) measured for both callus is presented in Fig. 2.4. Data suggested higher levels of MDA in EC (2.14±0.44 mmol mg^{-1} FW) in comparison to NEC (1.80±0.43 mmol mg^{-1} FW), even though means are not significantly different at P≤0.05 (Table 2.1). An increase in peroxidation reactions at early stages of SE of flax (*Linum usitatissimum* L.) from immature zygotic embryos was pointed out as a possible consequence of in vitro culture during culture initiation and routine subculture (Pret'ová et al. 2005). This parameter showed a positive correlation with the progression of SE in saffron (*Crocus sativus*) (Blázquez et al. 2009). In this species, the MDA content was significantly lower in the initial stages of somatic embryogenesis with a clear increase of almost 50% at the end of the process in saffron (Blázquez et al. 2009). Increased levels of

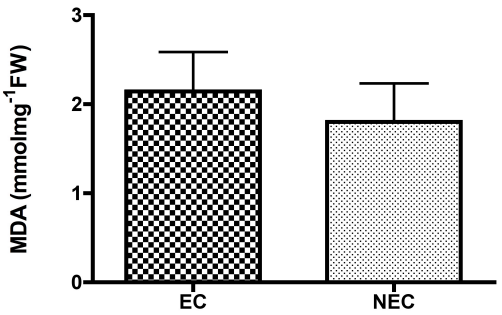

Figure 2.4: Evaluation of lipid peroxidation by MDA content estimation on EC and NEC of *Q. suber* (cultured on MS induction medium). The data represent the mean±SEM (*N*=3); FW (fresh weight).

lipid peroxidation were also reported by Adams et al. (1999) in cell cultures of carrot (*Daucus carota* L.) during the differentiation of somatic embryos in where increased levels of lipid peroxidation products were detected in EC compared to NEC ones. Also Hao et al. (2006) confirmed lower levels of MDA in NEC than in EC of naked oat (*Avena nuda*) in studying the role of salicylic acid in enhancing SE.

Hence, the observed trend of cork oak EC to present higher levels of MDA than NEC, corroborates literature, despite further studies are needed with larger number of samples, to support this assumption. These data, also suggest that along with higher embryogenic potential, oxidative stress may play a dual role, leading to both cytotoxic and cytoactive effects coexisting in a delicate equilibrium. These factors might promote differentiation of embryogenic cells toward somatic embryo formation as suggested by Weber (2000) and Fehér et al. (2008).

Table 2.1: Data results of MDA, L-Proline and H_2O_2 content of both EC and NEC of *Quercus suber*.

	MDA[a] (mmol mg $^{-1}$ FW)	L-Proline[a] (μg mg^{-1} FW)	H_2O_2[a] (μM mg^{-1} FW)
EC	2.14±0.44	19.12±3.0	0.20 ± 0.05
NEC	1.80±0.43	1.46±0.27	0.24 ± 0.06
P≤0.05[b]	NS	**	NS

[a] Data are given as Mean±SEM (*N*=3). [b] *P≤0.05, significantly different according to t-test for comparsion of means (GraphPad Prism 4.0 for MAC OS X, 2005). MDA (malonyldialdeyde); H_2O_2 (hydrogen peroxide).

L-proline content

Proline quantification is one of the most popular stress biomarker and is used to evaluate the impact of different types and levels of stresses such as water stress (Vendruscolo et al. 2007; Tatar and Gevrek 2008), salt stress (Poustini et al. 2007) and UV (Tian and Lei 2007). Rao et al. (1995) suggested that proline accumulates in stressed plants and its production is associated with enhancing stress tolerance, favouring in vitro regeneration. Proline accumulation was also reported in other SE research as in Nieves et al. (2003). They stated that EC of sugar cane (*Saccharum* sp.) had a greater free proline content than NEC. Theses results supported the present data obtained for cork oak,

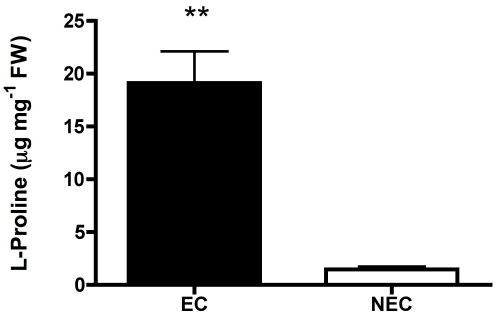

Figure 2.5: Evaluation of L-Proline content on EC and NEC of *Q. suber* (on MS induction medium). The data represent the mean±SEM (*N*=3); FW (fresh weight).**P≤0.05.

which also had significantly higher levels of proline content in EC (see Table 2.1 and Fig. 2.5). Greater levels of proline in EC have been indicated to promote SE and also confere stress tolerance in several literatures (Nieves et al. 2003). These authors also pointed out that greater concentration of free proline in EC may be important factors in the metabolic processes that led to SE in sugarcane.

Hydrogen Peroxide (H_2O_2) content

No statistical differences were found for H_2O_2 contents between NEC and EC (Fig 2.6). However, a bias of higher levels of H_2O_2 in NEC (0.24±0.06 μM mg^{-1} FW) was found, suggesting a slight decrease of this ROS species during SE in cork oak.

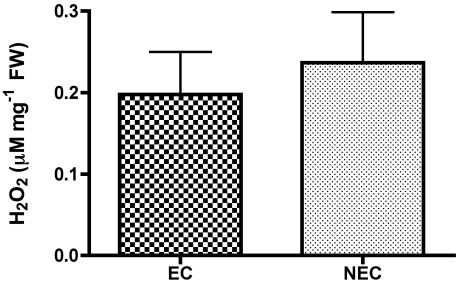

Figure 2.6: Evaluation of H_2O_2 content estimation on EC and NEC of *Q. suber* (on MS induction medium). The data represent the mean±SEM (*N*=3); FW (fresh weight).

Regenerative pathways, e.g. SE, in plant tissue cultures comprise a range of developmental processes in which H_2O_2 has been shown to be involved (Bagnoli et al. 1998; Kairong et al. 1999; Tian et al. 2003). A modification in endogenous H_2O_2 content during morpohogenic responses was described for ice plant (*Mesembryanthemum crystallinum)* (Libik et al. 2005). This increase was regarded as one of the early morphogenic responses (to abiotic stresses), and therefore related with the regeneration ability, by regulating the expression of some genes involved in those morphogenic processes (Libik et al. 2005). More recently, Hao et al. (2006) recorded high levels of H_2O_2 in EC of naked oat rather than in NEC, and even suggested this ROS species to be a signaling molecule involved in plant in vitro regeneration. As a possible explanation, they pointed out that those high levels might be favorable for SE by means of H_2O_2-inducing programmed cell death, which is relevant for the establishment of polarity at early stages of plant embryogenesis (Bozhkov et al. 2005; Libik et al 2005). This literature apparently contradicts the absence of variation of H_2O_2 in cork oak EC vs. NEC, raising several questions: a) Does H_2O_2 contents variation during EC process depend on the plant species?; b) Is H_2O_2 the only ROS species involved in SE process?; c) Does H_2O_2, and/or other ROS, change during the different stages of SE? To answer these questions, further investigations should be conduced in cork oak (e.g. increasing the number of samples; surveying other ROS and other stages of SE differentiation) and in other woody species (e.g. eucalyptus, elm).

Protein content and antioxidant enzymes estimation

Total soluble protein content was estimated according to Bradford (1976) and it was 2-fold higher in EC (4.27±0.31 μg mg^{-1} FW) than NEC (1.87±0.20 μg mg^{-1} FW) significantly different at P≤0.05 (Table 2.2). Other

authors also reported higher levels of total protein in embryogenic callus, for example in sugarcane (*Saccharum* sp.) (Nieves et al. 2003) and during SE of *Eurycoma longifolia* (Hussein et al. 2006). These improved total protein contents in EC may lead to the assumption that this might be associated to higher metabolic activity, which in turn can be responsible for molecular events encouraging to SE (Hussein et al. 2006).

Table 2.2: Data results of antioxidant enzyme activities (SOD, CAT and G-POX) and protein contents on both EC and NEC of *Q. suber*.

	Total protein[a] (μg mg^{-1} FW)	SOD[a] (U μg^{-1} prot)	CAT[a] (μg μg^{-1} prot)	G-POX[a] (μg μg^{-1} prot)
EC	4.27±0.31	0.30±0.10	40.20±2.23	0.34±0.04
NEC	1.87±0.20	0.69±0.06	20.36±8.10	0.74±0.32
P≤0.05[b]	**	*	NS	NS

[a] Data are given as Mean±SEM (*N*=3). [b] *P≤0.05, ** P≤0.01, significantly different according to t-test for comparsion of means (GraphPad Prism 4.0 for MAC OS X, 2005). SOD (superoxide dismutase); CAT (catalae); G-POX (guaiacol peroxidase).

In respect to antioxidant enzymes, in the current experiments SOD had a significantly higher activity in NEC at P≤0.05 (see Table 2.2 and Fig 2.7). SOD has a central role in the defense against oxidative stress (Shao et al. 2008) and catalyzes the dismutation of superoxide anion to O_2 and H_2O_2. Contrarily to SOD activity, CAT and G-POX did not present significant differences between EC and NEC (see Table 2.2 and Fig. 2.8). SOD and CAT are the most efficient antioxidant enzymes as they serve, in tandem, as front-line antioxidant defenses (Scandalios 1993; Shao et al. 2008), and CAT and peroxidases later scavenge the reaction products (H_2O_2) of SOD activity . Despite not statistically different, the apparent bias to lower H_2O_2 levels found in cork oak EC may be associated to the high trends of CAT activity in the same tissue. Libik et al. (2005) verified the opposite situation in SE in callus culture of ice plant (*Mesembryanthemum crystallinum*), speculating that those higher levels of H_2O_2 in morphogenic callus as a result of oxidative imbalance might increase the expression of some genes responsible for the induction of morphogenic processes. In the case of cork oak, a comparative study between seedlings and microcuttings cultured in vitro (epicormic shoots and stem sprouts) demonstrated higher levels of CAT and SOD activities (Racchi et al. 2001). Supporting present findings, Shohael et al. (2007), who studied antioxidant responses in development of *Eleutherococcus senticosus* somatic embryos, verified the lowest levels of CAT activity in NEC comparing to further differentiated stages. Blázquez et al. (2009) recorded increased levels of SOD activity at a globular stage of saffron embryo development, pointing out that changes in SOD isoforms (not quantified here) could characterize different developmental stages during SE of different plant species.

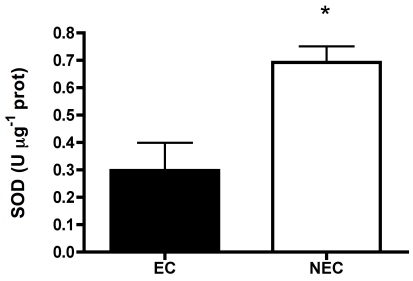

Figure 2.7: Evaluation of SOD activity on EC and NEC of *Q. suber* (on MS induction medium). The data represent the mean±SEM (*N*=3); *P≤0.05.

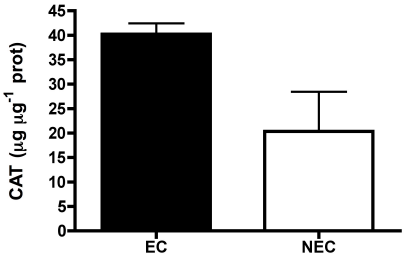

Figure 2.8: Evaluation of CAT activity on EC and NEC of *Q. suber* (on MS induction medium). The data represent the mean±SEM (*N*=3); *P≤0.05.

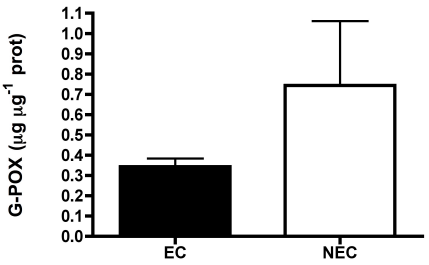

Figure 2.9: Evaluation of G-POX activity on EC and NEC of *Q. suber* (con MS induction medium). The data represent the mean±SEM (*N*=3); *P≤0.05.

Peroxidases are one of the most studied enzymes in plants and the fact that they have been found in all major divisions of plants denotes the functional importance of these proteins. Nieves et al. (2003) reported a peroxidase activity 35-fold higher in NEC than in EC in sugarcane SE in contrast with Hussein et al. (2006), who verified the opposite in *Eurycoma longifolia*. In embryo development of *Eleutherococcus senticosus,* in a bioreactor, it was also reported a marked boost in G-POX activity in EC in comparison to the lowest levels in NEC (Shohael et al. 2007). For cork oak POX activity no significant differences were detected between EC and NEC, but again a trend can be observed, with higher activity in NEC tissues (see Table 2.2 and Fig. 2.9). Despite this trend needs further confirmation and studies, it seems to be in agreement with previous results for alfalfa (Hrubcová et al. 1994) and sugarcane (Nieves et al. 2003). These authors suggested that POX lower activity in embryogenic tissues might be related to the restriction of cell wall development at early stages of embryo formation. It is suggested that peroxidase might play important regenerative functions and maintaining the size and shape of protoderm cells during somatic embryogenesis (Cordewener et al. 1991). Furthermore, POX has been indicated as a possible biomarker of somatic embryos determination (Hussein et al. 2006).

Differentiation and development of E cells in SE of *Lycium barbarum* L. are mostly regulated by three antioxidant enzymes, SOD, POX and CAT (Kairong et al. 1999). These findings strongly insinuate that antioxidant enzymes are deeply involved in the metabolism during SE development, promoted by the imposition of stress treatments (Aderkas and Bonga 2000). In this context, ROS could entail the complex signal transduction pathway required to trigger the reprogrammation of the gene expression pattern and cellular metabolism crucial for the embryogenic competence of somatic cells in in vitro tissue cultures (Blázquez et al. 2009). The data presented here for cork oak, suggests an involvement of proteins during the process, including the participation of some antioxidant enzymes during SE. The role of such enzymes (e.g. APX, GR) needs further studies for better clarification of their role(s) in these tissues during the whole SE process.

Cell cycle analysis by FCM

To examine differences between cell cycle profiles, nuclei events occurring at each G_0/G_1, S and G_2 phase were evaluated by the GraphPad Prism 4.0 Software for both callus lines of *Q. suber* (EC and NEC) and both treatments during induction or expression phase. The results are summarized in Table 2.3. and were obtained in the form of FL (Fig.2.10). EC had coefficient of variance (CV) values ranging from 0.26% to 4.23% and NEC from 0.51% to 6.23%. On this type of study, the importance of showing CV values was first pointed out by Marie and Brown (1993). These values reflect the quality of the applied methodology and its range depends on biological material source. A CV value below 5% is considered good (Galbraith et al. 2002), but for traditionally recalcitrant species like *Quercus* sp. these low CV values might be hardly achieved (Winkelmann et al. 1998; Zoldos et al. 1998; Endemann et al. 2001)

Recently, standard protocols for this and other recalcitrant species were developed (e.g. Pinto et al. 2004; Loureiro et al. 2005), giving reliable histograms with low CV values. However, these authors highlighted the possible interference of cytosolic compounds with PI-DNA. Higher CV values manifested in woody plants analyses might be related to excessive browning of samples, nuclei degradation and pH instability (Zoldos et al. 1998) or to the applied dye, i.e. if it binds to specific base pairs or intercalate in nucleic acid strains, as the former often shows lower CVs than the latter (Galbraith et al. 2002). Even so, the latter, such as propidium iodide (PI) seems to give an accurately nuclei DNA content evaluation as reported previously (Loureiro et al. 2005). Recently, Loureiro et al. (2007) developed a new buffer, WPB (Woody Plant Buffer) that minimized cytosolic compounds (e.g. phenols) interference in e.g. woody species. Using this new buffer, the present data showed fairly good CV values (Table 2.3) demonstrating a good applied methodology.

With respect to cell cycle events, no significant differences at $P \leq 0.05$ were detected between treatments neither between tissues (EC and NEC). The percentage of events occurring at G0/G1 phase for NEC_{ind}, EC_{ind}, NEC_{MSWH} and EC_{MSWH} were, respectively, 76.09±5.01%, 81.74±2.70%, 71.50±7.12% and 80.70±3.29%. These data clearly showed significant differences for G0/G1 peak at $P \leq 0.001$ in comparison to S and G2

phases, independently of tissue or treatment. No significantly differences were detected between S and G2 phases at P≤0.05 in each assay.

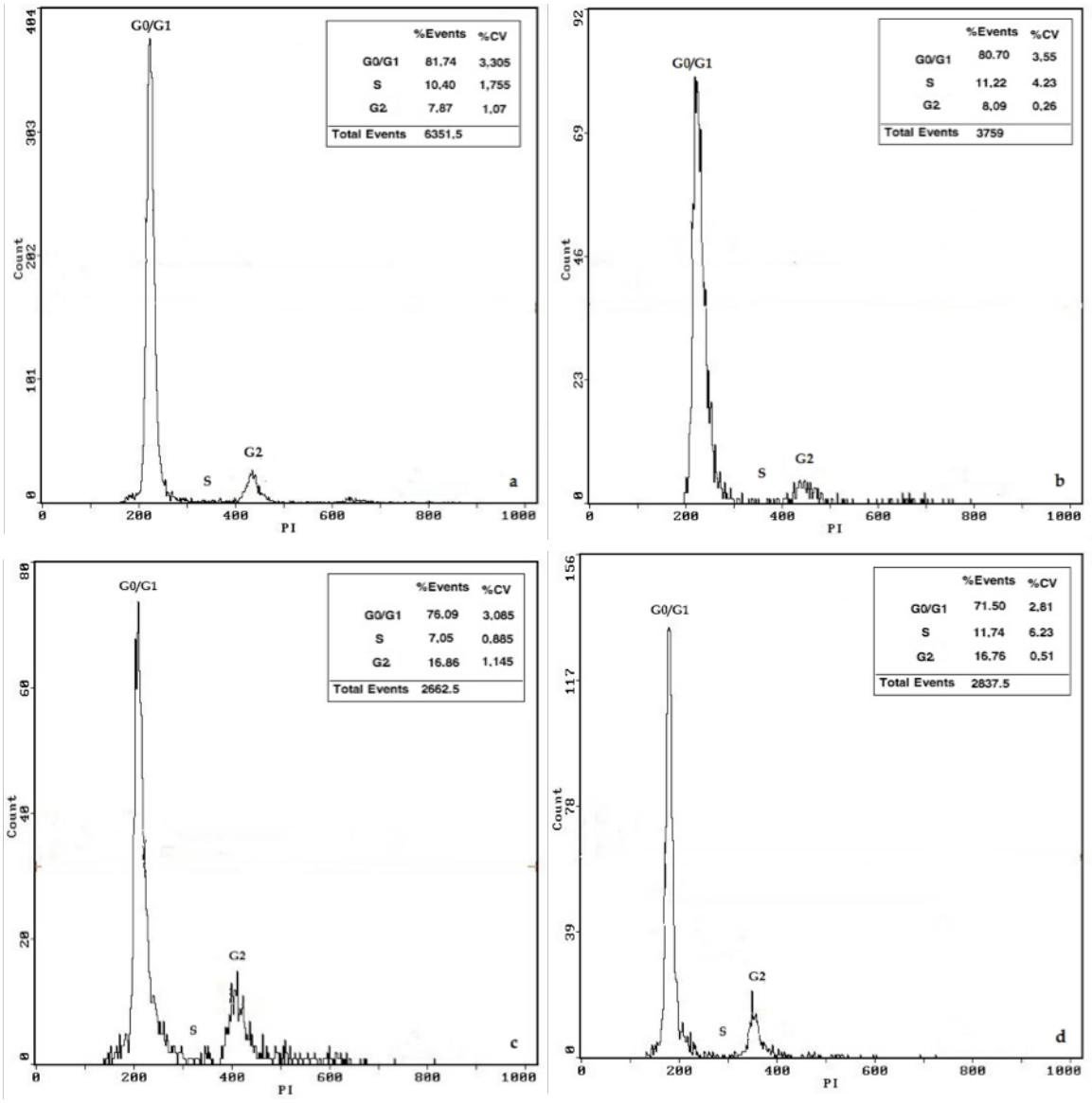

Figure 2.10: Flow cytometric cell cycle analysis of subcultured *Quercus suber* diploid callus tissue. Data are shown as linear-fluorescence light intensity (FL) histograms by Beckman Coulter EPICS® XL™ Flow Cytometer (Beckman Coulter, Inc.; Hialeah, Florida, USA) and as means of percentage of events (*N=2*). (a): EC_{ind} (EC on induction medium); (b): EC_{MSWH} (EC on expressiom medium); (c) NEC_{ind} (NEC on induction medium); (d) NEC_{MSWH} (on expression medium)

Table 2.3: Cell cycle phases (G_0/G_1, S and G_2) analysis of EC and NEC of *Q. suber*.

Plant Material Source[a]	Cell Cycle Phase	% Events Mean±SEM[b]	Total Events	CV (%)	Tukey-Kramer Test[c]
EC_{ind}	**G_0/G_1**	81.74±2.70	6351.5	3.31	***
	S	10.40±3.61		1.76	NS
	G_2	7.87±0.92		1.07	NS
EC_{MSWH}	**G_0/G_1**	80.70±3.29	3759	3.55	***
	S	11.22±2.19		4.23	NS
	G_2	8.09±1.11		0.26	NS
NEC_{ind}	**G_0/G_1**	76.09±5.01	2662.5	3.09	***
	S	7.06±3.02		0.90	NS
	G_2	16.86±1.99		1.15	NS
NEC_{MSWH}	**G_0/G_1**	71.50±7.12	2837.5	2.81	***
	S	11.74±4.97		6.23	NS
	G_2	16.76±2.16		0.51	NS

[a]NEC_{ind} and EC_{ind} on MS induction medium; NEC_{MSWH} and EC_{MSWH} on expression medium.
[b]The values are given as mean and standard error (SEM) of the number of events occurring during each phase for each sample (*N*=2). The mean coefficient of variation (CV) of *Q. suber* nuclei in histograms is also given.
[c]***Significantly different according to the multiple comparison Tukey-Kramer test at P≤0.001; NS (no significance at P≤0.05).

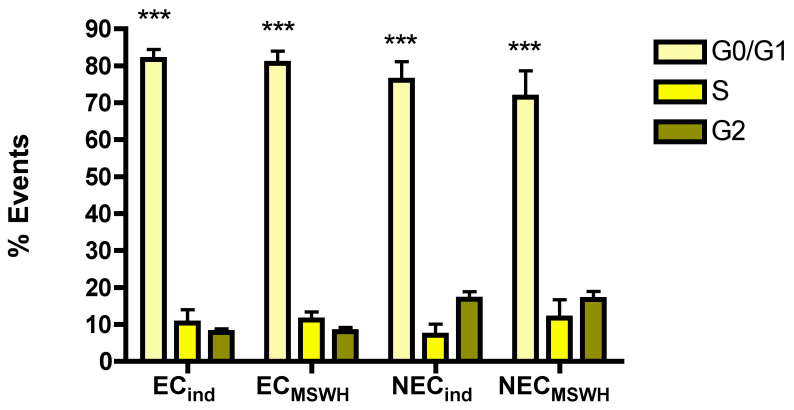

Figure 2.11: Cell cycle analyses in both EC and NEC of *Q. suber*. Here are given the number of events occurring at each cell cycle phase (G_0/G_1, S, G_2) for each sample (***P≤0.001 means significantly different, Tukey-Kramer Multiple Comparison test in GraphPad Prism 4.0 Software). NEC_{ind} and EC_{ind} on MS induction medium; NEC_{MSWH} and EC_{MSWH} on expression medium.

Koroleva et al. (2004) reported a yield in cyclin D expression accelerates cell cycle progression at two key stages of the cell cycle, indicating its expression to affect the length of both G_0/G_1 and S/G_2 transition phases. By this way, probably any external factor affecting the expression of D-cyclin would prevent cells in G_0/G_1 stage to proceed along cell cycle. From the analysis of Figure 2.11, in EC it is possible to observe an apparently decrease in G_0/G_1 phase when cultured on expression medium. This is to notice that defense responses against oxidative stress also depend on the phase of the cell cycle in plants, i.e. G_1 cells seem to be most sensitive to oxidative

stress to induce either defense responses or programmed cell death (Kadota et al. 2005). Winkelmann et al. (1998) verified a parallel development in the three cycle phases of both EC and NEC lines of *Cyclamen persicum* Mill. on induction medium. The withdrawal of PGRs from the medium was reported to reduce cell division activity, probably accompanied by changes in cell physiology related to the differentiation process. These authors also realized that after 5 years cultivation on callus induction medium (high levels of 2,4-D), the early stages of SE *Cyclamen* callus line preserved its embryogenic capacity probably by its manifested diploid stability over the years (Winkelmann et al. 1998). In a recent study, Fehér et al. (2008) proposed that moderate oxidative stress/ROS, in concert with auxin, may play a concentration-dependent synergistic effect on differentiated plant cells and cell cycle entry (G_0-to-G_1) in leaf alfalfa protoplasts. Furthermore, cumulative studies support the hypothesis that ROS may function as intracellular messengers during cell proliferation, differentiation and cell death, as reviewed by Sauer et al. (2001 vide Fehér et al. 2008). Although, ROS are "double-edged sword", their role is mainly dependent on concentration, pulse duration and site of action (Fehér et al. 2008).

In present work, NEC exhibited an apparently trend to increased nuclei events in G2 phase than EC in both treatments, despite not significantly different at $P \leq 0.05$ (Table 2.3). This may highlight the proliferation pattern as a feature of NEC (e.g. meristematic nodules) comparatively to EC. As suggested by Moghaddam and Mat Taha (2005) in sugar beet callus, higher cell activity of the NE cells should be interpreted as an index for cell proliferation and not embryogenesis. Winkelmann et al. (1998) also give support that higher percentage of events on G_2 phase of NE cell line of *Cyclamen persicum* on PGRs-free medium might evidence a predisposition to cellular proliferation. Regarding morphological and biochemical patterns, Moghaddam and Mat Taha (2005) also concluded that some improper hormonal balances might eventually increase cell volume, induce incomplete cell division, loosening of cell-to-cell connection and latter conducting to the generation of NE cells that lost the ability to cooperate with other cells for regeneration.

In conclusion, despite EC and NEC did not demonstrate statistical differences for all parameters, it coud be reported an apparent different behaviours regarding oxidative stress status and cell cycle events during cork oak SE induction. However, these speculations must be confirmed by further researches. In order to understand the possible roles of ROS in in vitro cultures, it could be identified what might mediate the cause/effect transitions between beneficial vs. deleterious effects of this type of molecules. So that, assembling the knowledge in SE process might give reliable information for plant large-scale production optimization.

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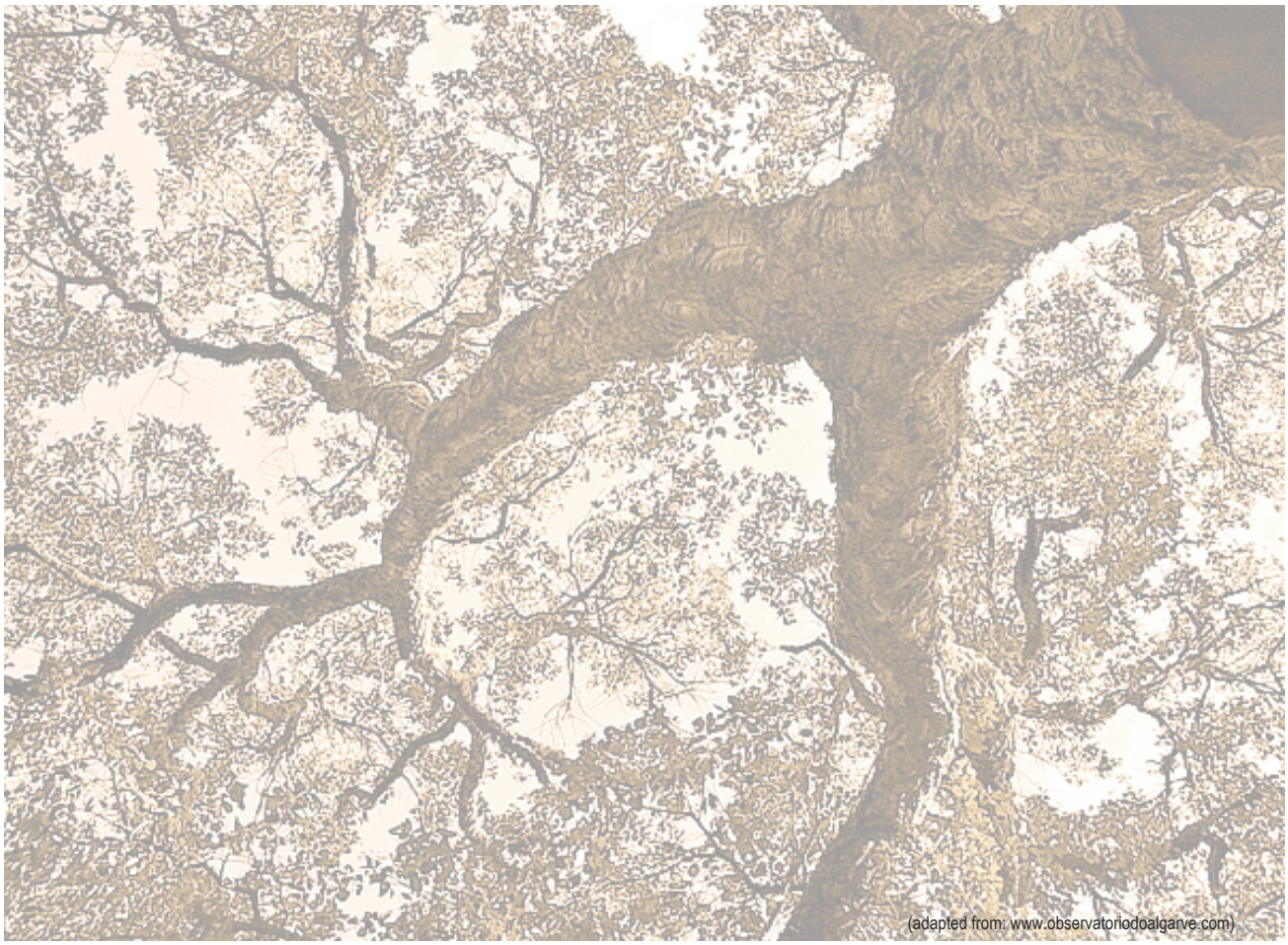

(adapted from: www.observatoriodoalgarve.com)

PART III

CONCLUSION

III. CONCLUSION

Somatic embryogenesis is a suitable tissue culture method of asexual propagation used in horticulture, agriculture and in forestry breeding programs as a means of rapidly multiplying elite genotypes. In vitro culture embraces a series of manipulations which could induce major changes in metabolism, physiology and development (Benson 2000), that may lead to raising ROS generation (Cassels and Curry 2000; Benson 2000).

This was the first report on oxidative stress and cell cycle status during cork oak SE induction and contributed to a better understanding of SE induction in *Q. suber*. Deep knowledge of SE could explain and help to improve and overcome existing limitations (e.g. genotypic dependency of the embryogenic response, the initiation of cell embryogenic suspension cultures and poor plant regeneration), especially in respect to its induction at early stages. Albeit some analyzed parameters did not reveal significant differences, the present work demonstrated an apparent difference between cork oak EC and NEC behaviours regarding oxidative stress and cell cycle status during SE induction tissues. Besides, it was also reported that SE requires a certain level of oxidative stress to promote the formation of embryogenic cells and to trigger its specific morphogenic pathway (e.g. moderated oxidative stress mimics auxin stimuli in SE (Pasternak et al. 2002, 2005); synergistic effect of oxidative stress and auxin in cell cycle activation (Fehér et al. 2008)). More studies should be performed to clarify this hypothesis and other antioxidant enzymes should be evaluated. It is hipotethysed that ROS may act as a component of the complex signal transduction pathways required to induce the reprogamation of the gene expression pattern and cellular metabolism essential for the embryogenic competence of somatic cells in cork oak callus cultured in vitro. Besides, the acquisition of embryogenic competence of differentiated cells probably consists on the “release from suppression rather than the induction of SE” (Fehér 2008). This is to say that SE may occur if the genes responsible for the embryogenic development program are released from chromatin-mediated gene silencing vegetative cells. From these considerations it is reasonable to assume that multiple cellular pathways might be controlled by a set of gene regulatory network during SE (Zeng et al. 2007) and methylation gene research should be implemented to clarify what happened in the particular case of cork oak.

For further researches some strategies must be addressed to complement the present study and expand the knowledge in developing improved in vitro culture strategies for woody species SE induction as follows: increase sample amount per assay; supplement MS induction medium with oxidative agents such as H_2O_2 (at sublethal and different concentrations) as it might confer a more protective status against stress or even activate certain morphogenic pathways, promoting SE; investigate if H_2O_2 or other ROS endogenous levels change during different SE stages or if H_2O_2 contents variation during SE process depend on the plant species; study a set of PGRs concentrations; study a larger range of antioxidant molecules both enzymatic (e.g. GR, APX, MDHAR/DHAR) and non-enzymatic (e.g. ascorbic acid, glutathione); analyze oxidative stress

and cell cycle status over time; apply other different parameters/techniques to estimate oxidative stress (e.g. intracellular redox potential, estimation of changes in DNA base methylation). Moreover, it should be highlighted that the data presented in this Master Thesis were part of a project/study where all SE process will be monitored from induction to plant acclimatization. This would allow a deepening and more comprehensive discussion about the whole process.

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