Universidade de Aveiro Departamento de Biologia
2007

Glória Catarina Cintra da Costa Pinto

REGENERAÇÃO DE PLANTAS DE *Eucalyptus globulus* POR EMBRIOGÉNESE SOMÁTICA

Eucalyptus globulus PLANT REGENERATION VIA SOMATIC EMBRYOGENESIS

Universidade de Aveiro Departamento de Biologia
2007

Glória Catarina Cintra da Costa Pinto

REGENERAÇÃO DE PLANTAS DE *Eucalyptus globulus* POR EMBRIOGÉNESE SOMÁTICA

Eucalyptus globulus PLANT REGENERATION VIA SOMATIC EMBRYOGENESIS

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Conceição Santos, Professora Associada do Departamento de Biologia da Universidade de Aveiro e co- orientação científica da Doutora Lucinda Neves da empresa Silvicaima (Constância Sul).

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio

Dedico esta Tese a dois indivíduos “Plus”:

Ao meu pai **Adelino Pinto** e ao meu avô **Orlando Cintra**

E também à minha filha **Catarina**,

mais uma prova que 2003 foi um bom ano para a produção de embriões.

o júri/ the jury

Presidente/ President:

Prof. Dr. José Rodrigues Ferreira da Rocha
professor catedrático do Departamento de Electrónica, Telecomunicações e Informática da Universidade de Aveiro

Vogais/ Members:

Prof. Dr. Olinda da Conceição Pinto Carnide
professora catedrática do Departamento de Genética e Biotecnologia da Universidade de Trás-os-Montes e Alto Douro

Prof. Dr. Amadeu Mortágua Velho da Maia Soares
professor catedrático do Departamento de Biologia da Universidade de Aveiro

Prof. Dr. Maria da Conceição Lopes Vieira dos Santos
professora associada do Departamento de Biologia da Universidade de Aveiro (**orientadora**)

Prof. Dr. Maria Margarida Moutinho Girão Oliveira Barroso
professora auxiliar com agregação da Faculdade de Ciências da Universidade de Lisboa

Prof. Dr. Jorge Manuel Leal Pataca Canhoto
professor auxiliar com agregação da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

Dr. Mariano Toribio
Investigador do Instituto Madrileño de Investigacíon e Desarrollo Rural, Agrário y Alimentario (IMIDRA)- Madrid

Dr. Lucinda Oliveira das Neves
Responsável pelo Desenvolvimento de Material Vegetativo da empresa SILVICAIMA (**co- orientadora**)

agradecimentos/ acknowledgements

This PhD Thesis is the summary of a collaboration Project started in 1999 between the Laboratory of Biotecnology and Cytomics (Department of Biology, University of Aveiro) and the Pulp mill CELBI (actually Silvicaima).

First I would like to thank São, for the friendship, for all the nice work that we have done together. For the good but also for the bad moments spent together since 1998, for the hours spent discussing Science, but also for those that we spent arranging, rearranging and other –angings the furniture of the laboratory. Most importantly for all the trust on myself.

I would like to express my gratitude to my major professors, Conceição Santos (University of Aveiro) and Lucinda Neves (Silvicaima) for giving me the necessary guidance, support and encouragement to reach my goals and for their caring attitude during my PhD program. They were both great mentors and made genuine efforts towards my professional success. For the valuable advices, reviews and comments to each chapter/manuscript of this Thesis. They are the living proof, how two very different persons can successfully work together.

A special thanks to Clara Araújo from Silvicaima for the coordination and stimuli of this collaboration project.

CELBI are thanks for the financial support to this dissertation research, for the project concerning somatic embryogenesis in *Eucalyptus globulus* and for providing all plant material.

The foundation for Science and technology (FCT) is thanked for supporting this work providing the PhD fellowship no. SFRH/BD/9003/2002.

I would also like to acknowledge the Natural Resources Canada, Canadian Forestry Service - Canadain Wood Fibre Centre (Canada) for giving me the great opportunity to discuss my results concerning genetic control with Yill Sung Park and Jan M. Bonga. It was also the opportunity to meet two excellent scientific persons and to work in a program where somatic embryogenesis is already a practical application in forestry. Thanks are also due to and Ian MacEacheron for helpful suggestions in Lab and in the field.

My deepest gratitude goes to Armando Costa, for his technical support and help in the laboratory, but especially for his kindness and friendship and for being always present.

I wish to express my gratefulness to all those friends that have been by my side giving me encouragement through all the years, special the colleagues from the Laboratory of Biotecnology and Cytomics. I would also like to thank João Loureiro and Sónia Silva for their assistance, friendship and patience doing cytometry and microscopy. A special thanks to João Loureiro for being always available to help and for the teamwork.

I would like to express my sincere gratitude to all my family for their unconditional support and encouragement in particular to my parents Adelino e Maria Cecilia and my grand-father Orlando Cintra.

Finally, I would like to thank my husband João Matias for his understanding through the years and to my little daughter Catarina (Tata), who has given me all the strength to finish this dissertation.

palavras-chave

aclimatização, antioxidantes, controlo genético, embrião somático primário, embrião somático secundário, embriogénese somática, embriogénese somática repetitiva, estudos histológicos e ultraestruturais, *Eucalyptus globulus*, meio de cultura, Myrtaceae, ploidia, regeneração de plantas, reguladores de crescimento

resumo

A embriogénese somática (ES) foi investigada em *Eucalyptus globulus* com vista a desenvolver um protocolo padrão de micropropagação, desde a indução à aclimatização.
A propagação clonal de *E. globulus* recorrendo a ES tem o potencial de ir ao encontro das necessidades industriais de material de alta qualidade e uniforme, e de rapidamente capturar os benefícios dos programas de melhoramento, sendo encarada como um método efectivo de conseguir ganhos genéticos num curto espaço de tempo.
O Capítulo I faz uma revisão da importância de *E. globulus*, assim como de alguns aspectos mais importantes relativos à micropropagação via embriogénese somática, e dedica uma secção ao conhecimento actual sobre o uso da embriogénese somática no género *Eucalyptus*. Finalmente, os objectivos de investigação desta Tese são apresentados.
O Capítulo II centra-se no processo de ES primário, sobretudo nas condições que influenciam a indução do potencial embriogénico. Este Capítulo está dividido em 5 grandes estudos: No Capítulo II.1 o tipo de explante, a fonte de carbohidratos e a exposição a reguladores de crescimento na embriogénese somática é realçada. Neste Capítulo publica-se, pela primeira vez, a regeneração de plantas por embriogénese somática nesta espécie. No Capítulo II.2 é discutida a influência da composição salina dos meios e a adição de antioxidantes. No Capítulo II.3 é efectuada uma comparação preliminar entre a composição salina do meio de cultura e o conteúdo mineral dos explantes (embriões zigóticos). No Capítulo II.4 são apresentados estudos histocitológicos e de acumulação de reservas em *E. globulus* durante a indução, expressão e diferentes estádios de desenvolvimento do embrião somático primário. Esta informação sobre as alterações observadas contribui para uma melhor compreensão do processo de ES primária. Finalmente, e com base nos resultados obtidos no Capítulo II.1 e II.2, está agora disponível um protocolo padrão (standard) para induzir ES primária e regeneração de plantas a partir de embriões zigóticos que permitiu os estudos de controlo genético apresentados no Capítulo II. 5. Neste estudo a frequência de ES foi estudada em 13 famílias de polinização aberta durante três anos consecutivos e a indução de ES provou ser uma característica variável entre as 13 famílias e entre os anos de produção de semente testados. Baseado nestes resultados, os estudos de controlo genético foram conduzidos recorrendo a um cruzamento dialélico com cinco árvores parentais; os resultados obtidos sugeriram que a indução de ES está sobre controlo de efeitos genéticos aditivos.

resumo (Cont.)

No capítulo III, os estudos apresentados direccionaram-se para a ES secundária. O estabelecimento da ES secundária só foi possível depois de se obter a ES primária nesta espécie. Tal como é descrito no Capítulo III.1, este processo repetitivo de ES mostrou-se reprodutível levando à obtenção de grande quantidade de material embriogénico. Os resultados obtidos, relativamente ao nível de ploidia e conteúdo de DNA deste material embriogénico, comprovaram que o protocolo utilizado na proliferação de embriões somáticos secundários não afectou estes parâmetros. De forma a optimizar a manutenção/ multiplicação e conversão destes embriões em plantas, alguns factores como a composição do meio basal, reguladores de crescimento e intensidade luminosa foram investigados no Capítulo III.2. No capítulo III.3, é estudado o último e crucial passo do processo ES, a aclimatização. A aclimatização de plantas derivadas de ES é acompanhado por estudos histocitológicos e por análises de variação de ploidia.

Finalmente, no Capítulo IV são apresentadas as conclusões da Tese de Doutoramento, onde alguns aspectos inovadores deste processo de ES nesta espécie recalcitrante são realçados.

keywords

acclimatization, antioxidants, culture medium, *Eucalyptus globulus*, genetic control, histological and ultrastructural studies, Myrtaceae, plant growth regulators, plant regeneration, ploidy, primary somatic embryo, repetitive somatic embryogenesis, secondary somatic embryo, somatic embryogenesis

abstract

Somatic embryogenesis (SE) was investigated in *Eucalyptus globulus* in order to develop a standard plant micropropagation protocol from induction to plant acclimatization. Clonal propagation of *E. globulus* through somatic embryogenesis has the potential to meet the increasing industrial demands for high quality uniform materials and to rapidly capture the benefits of breeding programs, being regarded as an effective method for achieving higher genetic gains in a shorter time.

Chapter I revises the importance of *E. globulus*, as well as some of the most important aspects of SE process, and gives particular emphasis on the state of the art of SE in *Eucalyptus* genus. Finally, the research objectives of this Thesis are presented.

Chapter II is focused on primary SE process, mostly on the conditions influencing induction of SE potential. This chapter is divided in five main studies. In Chapter II.1, the roles of explants type, carbohydrate source and exposure to exogenous plant growth regulators on SE are particularly emphasized. This was the first report on plant regeneration by primary SE in this species. In Chapter II.2 the influence of several salt medium composition and the addition of antioxidants on SE potential are discussed. In Chapter II.3 a preliminary comparison of salt medium composition and the mineral content of the explants (zygotic embryos) are performed. In Chapter II.4 histocytological and reserve accumulation studies are presented in order to describe changes observed in *E. globulus* SE, during induction, expression and different developmental stages of primary somatic embryos, providing valuable information on the primary SE process. Finally, based on the results obtained in Chapter II.1 and II.2, a standard protocol to induce primary SE from zygotic embryos with plant regeneration is now available allowing studies concerning genetic control presented in Chapter II.5. In this study the frequency of SE induction was investigated among 13 open pollinated families during three consecutive years and SE induction proved to be a variable character among families and the years of seed production tested. Based on these results genetic control was studied using a diallel mating design with five parent trees and results suggest that SE induction is under control of genetic additive effects.

abstract (Cont.)

In Chapter III, the studies presented are focused on secondary SE. The establishment of secondary SE was possible after attaining primary SE in this species. As described in Chapter III.1, this repetitive SE process has shown to be reproducible leading to large amounts of embryogenic material and showed to be, for ploidy and DNA content, a true-to-type SE process. In order to optimize the multiplication and conversion of these somatic embryos into plantlets, some factors such as basal medium composition, growth regulators and light intensity are investigated in Chapter III.2. In Chapter III.3, plant acclimatization (the last step of any SE protocol) was investigated. The acclimatization of SE- derived plants was followed by histo-cytological studies before ploidy analysis.
Finally, in Chapter IV the general conclusions of the present PhD Thesis are presented, where the innovative aspects of the developed SE process in this recalcitrant species are highlighted

“O homem primeiro tropeça, depois anda, depois corre e um dia voará.”

Bartolomeu de Gusmão (O Padre Voador)

My mother at work (seen by my three years old daughter…**Catarina**)

Abbreviations:

½ MS: half strength MS medium

2,4-D: 2,4-dichlorophenoxyacetic acid

ABA: Abcisic acid

ANOVA: Analysis of varience

B5: Gamborg medium

$B5_{WH}$: Gamborg medium without growth regulators

BAP: 6-benzylaminopurine

CP: Control pollinated

CV: Coefficient of variation

DKW: Driver Kuniyuki Walnut medium

DKWWH: DKW medium without growth regulators

DTE: Dithioerythritol

DTT: Dithiothreitol

GCA: General combining ability

GA_3: Gibberellic acid

IAA: Indole-3-acetic acid

PI: Propidium iodide

FCM: Flow cytometry

IBA: Indole-3-butyric acid

KIN: Kinetin

Log: Logarithmic

MS: Murashige and Skoog medium

MSWH: MS medium without growth regulators

MVF: Multi- varietal forestry

NAA: α-naphtalene acetic acid

OP: Open-pollinated

PEG: polyethylrne glycol

pg: Picograms

PGR: Plant growth regulators

PIPES: Piperazine-N,N'-bis-2-ethanesulfonic acid

PVP: Polivinylpirrolidone

PVPP: Polivinylpolypirrolidone

SCA: Specific combining ability

SD: Standard desviation

SE: Somatic embryogenesis

SEM: Scanning electron microscopy

TEM: Transmission electron microscopy

v/v: Volume/volume

W/v: Weight/ volum

WPM: Woody plant medium

WPMWH: WPM medium without growth regulators

ZE: Zygotic embryo

INDEX

Jury

Acknowledgments

Abstract (in Portuguese)

Abstract (in English)

Quotations

Abbreviations

CHAPTER I

Somatic embryogenesis: The case study of *Eucalyptus globulus* Labill.

Introduction 3

Genome Size 4

Botany 4

Clonal forestry of *Eucalyptus* 6

Micropropagation 7

Somatic embryogenesis (SE) applications in clonal forestry 8

General characteristics of SE 9

Factors influencing SE induction 11

- Genotype and explant type 11
- Medium composition, carbohydrates, PGRs, and other culture conditions 13
- Other factors 15

Somatic embryogenesis induction in *Eucalyptus* genus 16

Repetitive SE 19

Maturation, germination and conversion of somatic embryos 21

Acclimatization and genetic fidelity of the emblings 22

Research objectives 25

References 26

CHAPTER II

Induction of somatic embryogenesis in *Eucalyptus globulus* L.

II 1: Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill.

Abstract 37
Introduction 38
Materials and methods 39
Results 40
Discussion and Conclusions 44
References 46

II 2: Factors influencing somatic embryogenesis induction in *Eucalyptus globulus* Labill.: Basal medium and antioxidants
Abstract 51
Introduction 52
Materials and methods 53
Results 56
Discussion 63
Conclusions 68
References 69

II 3: Importance of media mineral composition on the induction of somatic embryogenesis in *Eucalyptus globulus* Labill.
Abstract 77
Introduction 78
Materials and methods 79
Results 80
Discussion and Conclusions 83
References 84

II 4: Histocitological studies of primary somatic embryogenesis in *Eucalyptus globulus* Labill.
Abstract 89
Introduction 90
Materials and methods 91
Results 93
Discussion 103
Conclusions 106

References 108

II 5: Genetic control of somatic embryogenesis induction in *Eucalyptus globulus* Labill.

Abstract 113

Introduction 114

Materials and methods 116

Results 119

Discussion 123

Conclusions 126

References 129

CHAPTER III

Secondary somatic embryogenesis

III.1 Analysis of the genetic stability *of Eucalyptus globulus* Labill. somatic embryos by flow cytometry

Abstract 135

Introduction 136

Materials and methods 138

Results and Discussion 140

Conclusions 148

References 149

III 2: Factors affecting maintenance, proliferation and germination of secondary somatic embryos of *Eucalyptus globulus* Labill.

Abstract 155

Introduction 156

Materials and methods 158

Results 161

Discussion 166

Conclusions 169

References 170

III.3: Acclimatization of secondary somatic embryos derived plants of *Eucalyptus globulus* Labill: an ultrastructural approach

Abstract 175

Introduction 176

Materials and methods 177

Results 182

Discussion 189

Conclusions 193

References 194

Chapter IV
Concluding remarks

Concluding Remarks 201

Challenges for the future 203

Chapter I

General Introduction

Somatic embryogenesis:

The case study of *Eucalyptus globulus* Labill.

Part of this chapter is in preparation to be submitted, as a review on *Eucalyptus* somatic embryogenesis, to a SCI journal

Introduction

Eucalyptus species are among the most widely planted trees in the world. Eucalypts are native from Australia and the offshore islands to its north. They occur over a wide range of environmental conditions, from sea level to alpine tree line and high rainfall to semi-arid areas, and vary in form, from shrubs to giant trees (Williams and Woinarski 1997).

Eldridge *et al.* (1993) suggested a ranking of the ten most important *Eucalyptus* around the world including: *E. grandis*, *E. camaldulensis*, *E. tereticornis*, *E. globulus*, *E. urophylla*, *E. viminalis*, *E. saligna*, *E. deglupta*, *E. exserta*, and then either *E. citriodora*, *E. paniculata* or *E. robusta*. Of these, the first four are by far the most important. Among these species, *E. globulus* was the first to become known outside Australia as an ornamental and plantation tree.

Eucalyptus globulus is the main *Eucalyptus* species planted in Portugal and was introduced in our country in the XIXth Century. Spain and Portugal greatly increased their areas of *E. globulus* in the 1980s, although in Spain *E. camaldulensis* was very popular by that time (for a review see Eldridge *et al.* 1993). Presently, *Eucalyptus* ssp. are the third more representative species in Portugal and occupied an area of 672 140 ha (DGF/ IFN 2001) in 2001. Furthermore, the preliminary results of a recent report (preliminary report DGF/ IFN 2006) confirm an increase of these areas.

Eucalypts are renowned for their fast growth rate, straight form and growth ability in a wide variety of climates and soils, and for their quality for both solid wood products and pulp production. For the pulp industry, *Eucalyptus* pulps are preferred due to their lower production cost and their excellent bulk, softness, flexibility, opacity and porosity, which make them particularly suitable for printing and writing grades of paper. *E. globulus* has excellent fibber morphology for pulp production and has several advantages compared to other *Eucalyptus* species such as the need of less chemicals to obtain the bleached pulp, due to lower lignin content (Eldridge *et al.* 1993, Doughty 2000).

For all these reasons, continued expansion of the planting of fast-growing eucalypts seems inevitable because of their ability to help meet the worldwide demand for wood, pulp and fuel (MacRae and Van Staden 2000).

Genome Size

Eucalypts are diploid plants with a haploid chromosome number of 11 (Eldridge *et al.* 1993, Potts and Wiltshire 1997). Grattapaglia and Bradshaw (1994) estimated, by flow cytometry, the genome size of several eucalypt species and their hybrids. Using chicken erythrocytes as standard, they estimated a haploid genome size ranging from 370 to 700 million base pairs (Mbp). Species from the subgenus *Symphyomyrtus* had on average a haploid genome size of 650 Mbp with *E. globulus* and *E. dunnii* at the lower end of the scale (530 Mbp) and *E. saligna* at the higher end (710 Mbp) (Grattapaglia and Bradshaw 1994). In Chapter III.1 of this PhD Thesis, the DNA content of *E. globulus* was re-estimated using a plant internal standard (*Lycopersicum esculentum* Mill., recently renamed *Solanum lycopersicum* L.). Furthermore, the genome sequencing of this species would bring more information on this issue (Poke *et al.* 2005). In a recent review concerning genomics of *Eucalyptus*, Poke *et al*. (2005) highlighted that the genome size of *Eucalyptus* is larger than some of the plant species which had, recently, their genomes sequenced: *Arabidopsis thaliana* with 125 Mbp, *Oryza sativa* spp. (420 Mbp - 466 Mbp) and *Populus trichocarpa* with approximately 473 Mbp. In comparison to gymnosperms, such as *Pinus*, eucalypts have a considerably smaller estimated genome size, as reported by Bogunic *et al.* (2003) who estimated, by flow cytometry, that the genome size of five *Pinus* species ranged from 20,830 to 26,920 Mbp.

Botany

The genus *Eucalyptus* is a member of the Myrtaceae family, is composed of more than 700 species (Brooker, 2000) and is divided in eight subgenera, with the subgenus *Symphyomyrthus* containing the majority of species in the genus.

Eucalyptus globulus was one of the earliest of the eucalypts to be brought into cultivation. This species was formally described by Labillardière in 1799 from a specimen that he collected in south- eastern Tasmania in 1792 (Eldridge *et al.* 1993).

Plate 1: **a**) Unopened flower with the characteristic lid. **b**) Flower of *E. globulus*. **c)** Fruit of *E. globulus*. **d**) Branch of *E. globulus* showing flower buds at different stages of development and adult leaf type.

The genus name derives from the Greek (eu- well; Kalypto- I cover), from the characteristic lid or operculum which covers the unopened flowers (Plate 1.a). Flowers are bisexual and pollination is dependent, in most cases, on insect vectors (Plate 1.b). *Eucalyptus* is a predominantly out breeding, although most species have some degree of self-compatibility. Fruits are woody capsules at maturity (Plate 1.c, d) and contain several seeds, some of which are not viable. In *E. globulus*, it takes around 4 month from pollination until seed is mature (Eldridge *et al.* 1993).

The age at which *Eucalyptus* reaches maturity varies both among and within the species. The onset of reproduction is often associated with the transition from juvenile to adult foliage (Plate 1.d). In *E. globulus*, some trees reach maturity at one year, other can take three years. Juvenile leaves of young seedlings (<1 year old) are typically blue-grey in color, dorsiventral in structure, hypostomatous, and approximately horizontal in orientation. In contrast, adult leaves of mature trees (>5 years old) are dark green, isobilateral, amphistomatous and pendent (James *et al.* 1999).

Clonal forestry of *Eucalyptus*

Eucalyptus species are naturally propagated by seed with varying degrees of establishment and competitive success (Watt *et al.* 2003a). In fact, before the recent progress with mass vegetative propagation, all new *Eucalyptus* plantations were originated from seeds.

Although industrial needs demand an increase of *Eucalyptus* forest productivity, *E. globulus* is still in an early stage of domestication. Most of the genetic parameters reported to date are based on open-pollinated progenies (Lopez *et al.* 2002). However, due to inbreeding depression from selfing and/or related mating, genetic parameters derived from open pollinated eucalypt populations may be inaccurate. Most *E. globulus* breeding programs are now moving to control-pollinated assessment, which will allow more accurate estimations of genetic parameters and the separation of additive from non-additive genetic effects (Silva *et al.* 2004). This PhD Thesis results from collaboration with Celbi that was a worldwide leader in using control-pollination on a commercial scale in *Eucalyptus* to produce large quantities of seed for commercial plantation establishment (Leal and Cotterill 1997). Celbi plantations establishment since 1996 has been based on outstanding full-sib *E. globulus* families produced by mass-pollination using a refinement of the “one-stop” (Harbard *et al.* 1999) pollination systems (Cotterill *et al*. 2000).

Vegetative propagation is a widely used technique in tree breeding to manage breeding populations more efficiently (Eldrige *et al.* 1993) and is utilized in breeding strategies to produce improved plant stock (clonal forestry) more rapidly than conventional seed orchard procedures (Mullin and Park 1992). A major advantage of clonal forestry is the complete use of genetic potential of desired genotypes. Another advantage is the short-term ability to quickly capture a greater proportion of additive and non- additive genetic variation. Clonal propagation enables retention of most of the genetic potential of elite

selected plantations, including the non- additive components of genetic variance in new generation. Besides, in sexual propagation non-additive combinations are mostly lost due to genetic combinations (Mullin and Park 2002). Eldridge *et al.* (1993) emphasized that seed will continue to play a major role in plantation establishment and, because clonal propagation neither creates nor improves new genotypes in the next generations, clonal programs of *Eucalyptus* must be accompanied by intensive breeding ones. The preferred method for vegetative propagation is by rooted cuttings, a strategy already used with success in several clonal propagation programs (e.g. Celbi in Portugal, for a review see Watt *et al.* 2003a). Nevertheless, the vegetative propagation of *Eucalyptus* through cuttings is limited by the variability of rotting ability among the clones and the decrease of rooting potential with aging of parent plants (Eldridge *et al*. 1993, Watt *et al.* 2003).

Clonal propagation through in vitro methods can provide alternative vegetative multiplication methods to overcome difficulties found in conventional techniques.

Micropropagation

Micropropagation has the potential to provide very high multiplication rates of selected tree genotypes, with resulting short-term silviculture gains. Some of the earliest reports on in vitro culture of *Eucalyptus* spp. date back to the 1960s and over the last decade some progress has been made in developing complete plant regeneration protocols. In there review on *Eucalytus* micropropagation, Le Roux and Van Staden (1991) reported that between 1968 and 1991 only 30 out of 204 publications included protocols for plant regeneration. Since then, 29 out of 65 new publications reported plant regeneration (for a review see Watt *et al.* 2003a). According to these authors, *Eucalyptus* asseptic cultures have been established from a wide range of explant sources (e.g. seeds, seedlings, shoots, flowers and lignotubers). Explants from both juvenile and mature trees have been used for micropropagation.

Micropropagation through axillary proliferation and adventitious shoot proliferation on nodal explants has been successful (Cid *et al.* 1999, Glocke *et al.* 2006). The most common culture medium is MS medium (Murashige and Skoog 1962) with a low auxin/cytokinin ratio is most commonly used for shoot multiplication (Watt *et al.* 2003a, b). Nevertheless, other salt media compositions are reported, such as WPM (Lloyd and

McCown 1981) for the ornamental *Eucalyptus* cv. 'Urrbrae Gem' (Glocke *et al.* 2006) and JADS medium for *E. grandis* (Correia 1995).

To stimulate shoot elongation, gibberellic acid was added to some media (Cid *et al.* 1999, Glocke *et al.* 2006). Concerning in vitro rooting step, various media have been used for root initiation; often, a few days-pulse of indole-3-butyric acid (IBA) followed by subculture to IBA-free medium is used, and regenerated plants have already been transferred to field (Azmi *et al*. 1997, Glocke *et al.* 2006). In *Eucalyptus* in vitro cultures, hyperhydricity and shoot senescence still remain problems (Louro *et al*. 1999, Whitehouse *et al.* 2002) as also reported for other species.

Multiplication of selected *Eucalyptus* via axillary bud proliferation is currently employed for large-scale multiplication in numerous research and commercial laboratories as reported for other forest species (Watt *et al.* 2003a, b).

Plant regeneration from indirect organogenesis, somatic embryogenesis and protoplast was also successful for some species (for reviews see Le Roux and van Standen 1991, Watt *et al.* 1999, 2003a, Hajari *et al.* 2006). Optimization of these protocols is crucial not only for plant regeneration improvement but also for application of genetic engineering strategies to the genus *Eucalyptus* where in vitro techniques are presently being applied to achieve genetic transformation (Tournier *et al.* 2003, Poke *et al.* 2005). In fact, the main reason why this approach is not yet being used more widely at industrial level is the lack of well developed (reliable and low cost) in vitro plant regeneration protocols as also reported for other softwood trees (MacKay *et al.* 2006)

Somatic embryogenesis (SE) applications in clonal forestry

Although high rates of plant propagation of *Eucalyptus* from axillary shoots proliferation can be easily achieved, successful cases in other tree species indicate that much higher multiplication rates can be potentially obtained via SE (Park *et al.* 2006). In fact, a great progress has been achieved in the regeneration of woody plants by SE (for a review see Merkle and Nairn 2005). There are several advantages of SE in clonal forestry such as: SE is amenable to high-throughput production necessary to reduce production costs, embryogenic cultures may be maintained practically indefinitely through cryopreservation (this long term maintenance of propagation potential is essential to have time to field-testing and selection of the best clones), flexibility to rapidly deploy suitable clones given

changing breeding goals and/or environmental conditions, ability to manage genetic diversity and genetic gain in the plantation. Besides, SE also allow mass production of selected clones from relatively small quantities of control pollinated seed from controlled crosses where outstanding parents are difficult to flower and/or only a small quantity of seed is produced as well as a strategy to speed up the deployment of outstanding families identified in progeny trials (for a review see Högberg *et al.* 1998 Park *et al.* 1998, Park 2002). Park *et al.* (2006) highlighted that the most advantage of SE is the deployment of genetically tested trees varieties, preferably, integrated in tree breeding programs, that these authors define as multi-varietal forestry (Park *et al.* 2006). Despite all these advantages, clonal forestry is highly dependent of an efficient clonal propagation that can mass produce genetically tested material.

General characteristics of SE

Somatic embryogenesis has been defined as a non-sexual developmental process that produces a bipolar embryo (presenting shoot and root meristems) from somatic tissue (e.g. Merkle *et al.* 1995, Dodeman *et al.* 1997). This process was reported as the best example of totipotency in plants (Thorpe 2000). Developmental stages similar to zygotic embryogenesis occur and yield an embryo with no vascular connection to the parent tissue (e.g. Zimmerman 1993, Von Arnold *et al.* 2002).

Different patterns for the in vitro origin of somatic embryos have been distinguished. These include direct production of somatic embryos from the explant cells called pre-embryogenic determined cells, and indirect production of somatic embryos from induced embryogenically determined cells in unorganized callus (Williams and Maheswaran 1986, Thorpe 2000). Pre-embryogenic determined cells are already destined for embryogenic development prior to explanting, requiring only growth regulators or favorable conditions to allow release into cell division and expression of embryogenesis. By contrast, indirect embryogenesis requires redetermination of differentiated cells, callus proliferation and the development of the embryogenically determined state. Growth regulators are required not only for re-entry into mitosis but also for determination of the embryogenic state (Thorpe 2000). It has been proposed that both processes are extremes of one continuous developmental pathway and distinguishing between direct SE and indirect SE can be

difficult, and both processes have been observed to occur simultaneously in the same tissue culture conditions (Williams and Maheswaran 1986, Canhoto *et al*. 1999).

According to several authors (e.g. Williams and Maheswaran 1986, Yeung 1995, Canhoto *et al.* 1999, Gaj 2004) somatic embryos may be originated from a single cell or from a small group of cells that differentiate into an organized structure under still poorly characterized circumstances. By that reason, anatomical studies should be performed not just to confirm if the structures are in fact embryos, but also to distinguish the unicellular (often the preferable route for plant regeneration) or multicellular origin of somatic embryos, and to characterize cells involved in embryogenic induction (Canhoto *et al.* 1999).

With some exceptions in Myrtaceous species (e.g. Canhoto *et al.* 1996, 1999), the paucity of cytological, histological and ultrastructural information of different aspects associated with the induction and development of somatic embryos from explants is notorious in this family and in *Eucalyptus* genus in particular. For *Eucalyptus*, most of works described that somatic embryos showed morphological resemblances with zygotic embryos at various stages (e.g. Muralidharan *et al.* 1989, Watt *et al.* 1999), although some of them do not define clearly the different phases of development. According to Watt *et al*. (1991), *E. nitens* embryogenic cells present typical characteristics found in other embryogenic systems (dense cytoplasm, small volume, prominent nucleus and small vacuole). Furthermore, histological analyses showed somatic embryos at different development stages, though no other details were given on embryo histology nor on embryo origin (Watt *et al.* 1991). Similar observations were made in *E. grandis* (Lakshmi Sita 1986 according to Canhoto *et al.* 1999) and in *E. globulus* (Trindade 1996). Bandyopadhyay *et al.* (1999) examined the ultrastructure of *E. nitens* somatic embryos, comparing them with mature zygotic embryos. Also, Arruda *et al.* (2000) demonstrated, at morphological and histological levels, that calcium favored the morphogenic route for somatic embryogenesis in *E. urophylla*.

In the Chapter II.4 histocytological analyses were carried out during different stages of primary SE process, from explant to emblings (SE-derived plants) regeneration. These analyses of histocytological changes during the primary SE process open perspectives to a better understanding and control of this process in this species and in *Eucalyptus* in general.

Somatic embryogenesis is a complex process that has been traditionally divided in two main stages: a) induction, where tissues acquire (direct or indirectly) embryogenic competence, and b) expression, where competent cells develop into somatic embryo structures. This expression stage is usually divided in proliferation, histodifferentiation, maturation and germination/ convertion phases (e.g. Merkle *et al.* 1995). The characteristics phases of somatic embryos are usually reported to be similar to those present in zygotic embryos (i.e. globular, heart-shaped, torpedo-shaped and cotyledonar) (Jiménez 2005).

The success of any propagation system is visible in the quality of the final product, i.e. survival and growth of regenerated plants. From an academic point of view, the success of SE protocols may be materialized when emblings are achieved. However, for commercialization/ industry purposes, large scale production of emblings has to be achieved and the acclimatization phase must be integrated together with screenings of the embling quality (*e.g.* performance under ex vitro conditions, genetic fidelity). Emblings acclimatization and ploidy analysis will be integrated in this PhD Thesis (Chapter III.3).

Factors influencing SE induction

A prerequisite for the successful establishment of a SE system is the proper choice of plant material with the explants being a source of competent cells, and, on the other hand, determination of physical and chemical factors which switch on their embryogenic pathway of development (Gaj 2004). In fact, SE depends on different factors, such as: genotype, type, age, sanitary and physiological conditions of the explant-donor plant, and the external environment that include composition of media and physical culture conditions (e.g. light, temperature, pH, humidity, solid or liquid medium) (Merkle *et al.* 1995, Thorpe 2000, Phillips 2004). Interactions between these factors lead to the induction and expression of a specific mode of cell differentiation and development (Gaj 2004).

The influences of some of these factors on *E. globulus* SE were investigated and discussed later in different chapters of this PhD Thesis (Chapters II.1-3, II.5).

Genotype and explant type

The genotype effect is currently referred as a crucial factor in micropropagation and in particular in *Eucalyptus* SE induction, but no research was performed, up to the moment,

concerning the magnitude of genetic influence (e.g. Watt *et al.* 1999). The use of *E. globulus* SE in improvement programs of this species will depend, however, on the capacity of applying this process to a broad range of genotypes.

The genetic influence during the SE process is well known (Merkle e*t al.* 1995) and understanding genetic control is an important element in improving the SE process (Park *et al.* 1998). Depending on the type and magnitude of genetic variation, improved SE initiation may be introduced in recalcitrant genotypes (Park *et al.* 1998, Park 2002). Such genotypic differences in embryogenic capacity may reflect current differences in the ability to activate key elements in the embryogenic pathway (Merkle *et al*. 1995).

Chapter II.5 represents a big effort to clarify the genetic influence in *E. globulus* SE. Embryogenic capacities among the 13 *E. globulus* open pollinated families and variability in yearly production were studied. To fully understand this study it was crucial the information obtained in the previous chapters, which allowed the development of a standard SE protocol. The degree of genetic control during the SE was examined using controlled crossed families with a potential application to improve SE process and integration into breeding programs.

Most woody species have marked phase changes that result in a decline of their potential for micropropagation and in particular SE or (Bonga and Von Aderkas 2000). Although adult material is desirable, in most of the experiments on SE induction in *Eucalyptus*, juvenile material was used. In fact this is a general procedure in Myrtaceous species (for a review see Canhoto *et al.* 1999) and in woody species in general. The necessity of using juvenile material still represents the major limitation for woody species propagation by SE because the quality of the adult trees to be formed is still unknown.

Somatic embryogenesis can be induced in cultures derived from various explant types: seedlings and their fragments, petioles, leaves, roots, anther filament, shoot meristems, seeds, cotyledons, and immature and mature zygotic embryos (Dunstan *et al.* 1995, Gaj 2004). In *E. citriodora* (Muralidharan and Mascarenhas 1995) and *E. tereticornis* (Prakash and Gurumurthi 2005) decoated seeds were used as explant, which consisted in the embryo with intact cotyledon. Watt *et al.* (1991) used leaves from in vitro propagated shoots in *E. grandis* and in *E. dunnii* 3-day-old seedlings were used (Termignoni *et al.* 1996). According to Watt *et al.* (2003a), Termignoni and co-workers

(1998) obtained a successful protocol of SE with explants of mature trees although no details are given.

Concerning *E. globulus*, Trindade (1996) tested explants with different phases of development and found that partially germinated seeds were more suitable for SE than cotyledons or leaves from micropropagated plants. Also, in *E. globulus*, Nugent *et al*. (2001) used cotyledons from mature seeds to induce SE. In none of these studies however, complete somatic embryogenesis structures (having well-defined shoot and root poles) were achieved. Finally, Bandyopathyay *et al*. (1999) observed, for the same species, organized structures resembling somatic embryos, but their evolution was not achieved.

In chapter II.1 of this PhD Thesis several types of explants (mature zygotic embryos, isolated cotyledons, hypocotyls, leaves and stems) were used to induce SE in *E. globulus* and a successful protocol for plant regeneration was published for the first time (Pinto *et al.* 2002). Besides, other attempts were done to induce SE from mature plant material using floral buds (from several families and in different stages of development). For this, disinfected buds were sectioned to provide stamens for SE induction but, up the moment, only non-embryogenic callus was observed (data not shown).

When using seeds as starting material, these are often surface disinfected (surface-sterilized) and several procedures are reported depending on the species and type of material to be disinfected. The most common disinfectants used in *Eucalyptus* are alcohol, NaOCl and $HgCl_2$ (Le Roux and Van Satden 1991) usually with few drops of detergent (i.e. Teepol or Tween 20). Muralidharan and Mascarenhas (1995) washed seeds with water and a few drops of Teepol and after three washes with distilled water, and seeds were then treated with 0.05% $HgCl_2$ (w/v) for 5 min. and then rinsed with sterile water. Also a 2 minutes treatment with $HgCl_2$ (0.1%) (w/v) was used in *E. tereticornis* after immersion in 70% (v/v) of ethanol for 2 minutes (Prakash and Gurumurthi 2005). In *E. grandis*, 1% of sodium hipochlorite for 30 min or $HgCl_2$ (0.1%) for 5 min are routinely used for disinfection of explants from mature trees (Watt *et al.* 1999).

Medium composition, carbohydrates, PGRs and other culture conditions

Nutrient medium composition obviously was an important factor in cell and tissue culture (for a review see Ramage and Williams 2002). In seeds, the endosperm or megagametophyte ensure proper nutritional, osmotic and hormonal environments that are

known to control embryo growth. The excision of zygotic embryos and their culture on an artificial medium, as well as the analysis of the composition of the developing seed can provide information to optimize the nutrient requirements for SE induction/ expression (Pullman *et al*. 2003). These kinds of reports dealing with woody species are, however, quite rare and the complete factorial design is complex (Bonga and Von Aderkas 1992). In plant tissue culture, the basis of all nutrient medium is a mixture of mineral salts (macro- and micronutrients), vitamins, amino acids supplemented with a source of carbon (e.g. Bonga and Anderkas 1992, Ramage and Williams 2002). The most extensively used medium for woody angiosperm somatic embryogenesis has been the nitrogen-rich medium of Murashige and Skoog (MS) (Murashige and Skoog 1962, in Dunstan *et al.* 1995) and the most reported in micropropagation of *Eucalyptus* genus (Le Roux and Van Staden 1991, Watt *et al.* 2003a).

Carbohydrates are included in all tissue culture media and probably play multiple roles during somatic embryogenesis. Sugars in plants generally serve mainly as: (1) sources of carbon and energy; (2) osmotica; (3) stress protectants; and (4) signal molecules (Lipavska and Dova 2004). The type and concentration of sugar used in media influences SE. Sucrose has been most frequently employed to induce SE in different plant species, but different species may have varying carbohydrate requirements for the development of somatic embryos. Carbohydrate supply during embryo maturation appears to be important for both embryo quality and quantity (Merkle *et al*. 1995). Its effect on induction, maintenance and maturation of somatic embryos has been investigated (Lipavska *et al.* 2000). The recommended concentrations vary in different cultures, usually ranging from 2 to 5% for *Eucalyptus* genus SE induction (Table 1). In other embryogenic systems other carbohydrates were tested as well (e.g., glucose, fructose, maltose, lactose, cellobiose, mannitol, sorbitol, myo-inositol) (e.g. Canho *et al.* 1999, Lipavska and Dova 2004).

Addition of plant growth regulators (PGRs) into the culture medium is the preferable strategy, and more documented one, to induce morphogenetic responses in vitro in any plant tissue culture system (Gaj 2004, Jiménez 2005). In this Thesis, the term plant hormone will be used to define the endogenous and naturally occurring substances in the tissues, while the expression PGR will refer to general compounds of synthetic origin.

The level of endogenous hormones is considered a crucial factor influencing embryogenic potential (Fehér *et al.* 2003, Gaj 2004). Also exogenously applied PGRs play

a critical role and can determine the embryogenic potential. Often auxins, such as 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) or naphthalene acetic acid (NAA), are used to reactivate the cell cycle and initiate embryo formation. In the present Thesis only the influence of exogenously PGR application will be studied.

Initiation of embryogenic cultures of some woody species requires continuous exposure to auxin (or in some cases, to other PGRs) to continue to produce secondary embryos, while in others, a short pulse of auxin (e.g. few days) is sufficient to induce secondary embryogenesis that will continue for years on a basal medium (Merkle 1995). Usually after removing the auxin, the somatic embryos switch to a program of development, maturation and germination, and repetitive embryogenesis ceases.

Cytokinins, such as kinetin (KIN) or benzylaminopurine (BAP), have been useful in initiating somatic embryo formation of some woody species (Dunstan *et al*. 1995) and they are usually supplied combined with auxin in the induction medium. However, PGRs as abscisic acid (ABA), ethylene, gibberellic acid (GA_3) and others also have regulatory roles that must not be ignored in some culture systems (Jiménez 2005)

Other factors

It has become widely recognized that somatic cells can acquire embryogenic potential as a result of different external chemical and physical stimuli often associated with stress conditions (Gaj 2004). In vitro culture conditions expose explants/cells to significant stresses, as they are removed from their original tissue environmental and placed on synthetic media in artificial conditions. In fact, wounding itself is a significant signal in the induction and dedifferentiation (Fehér *et al.* 2003). Stress is commonly recognized as an essential component of embryogenesis induced in microspore culture (i.e. androgenesis) and a positive influence of stress was also observed in cultures of somatic tissue where development of somatic embryos was induced (Dudits *et al.* 1995). Embryogenic competence of in vitro cultured somatic cells can be stimulated by various factors, such as osmotic pressure, pH, low or high temperature, starvation, mechanical wounding of explants or high auxin level. Light is one of the most important environmental signals, and the numerous effects of light on plant growth and development are widely known. The intensity, the spectrum/wavelength, and duration of the light supplied for in vitro cultures can influence morphogenic response and its efficiency and can be critical for SE. However,

molecular mechanisms involved in the stimulatory/regulatory effect of stress treatment on cell differentiation and morphogenesis remain unclear (for a review see Fehér *et al.* 2003, Gaj 2004).

Somatic embryogeneis induction in *Eucalyptus* genus

This section intends to summarize relevant findings related with somatic embryogenesis in *Eucalyptus*. There are several reports on *Eucalyptus* somatic embryogenesis induction in literature (for a summary see Table 1). Somatic embryogenesis and plant regeneration was reported for the first time from callus of seedlings of "*E.* x Liechow" (Ouyang *et al.* 1980, 1981, according to Le Roux and Van Staden 1991) from which they differentiated somatic embryos and regenerated plantlets. Boulay (1987, according to Le Roux and Van Staden 1991) achieved somatic embryogenesis with hypocotyl and internode calli derived from seedlings of *E. gunnii* on two different media and a variety of PGR concentrations. Chang-Le and Kirby (1990, according to Le Roux and Van Staden 1991) induced embryo-like structures in cultures of hypocotyls, cotyledons, and young seedling leaves of *E. botryoides*, *E. dunnii*, *E. grandis* and *E. rudis*, as well as from young leaves of cultured shoots of superior adult *E. grandis* clones. A sequential culture technique was used with Murashige and Skoog salts and RV (reference not given) vitamins and amino acids as medium. Slow growing green protuberances developed from cut surfaces of explants after 2 weeks in culture on a medium containing 1.1 mg 1^{-1} 2,4-D. These developed into adventitious shoots and embryo-like structures when transferred to medium with 1.1 m g 1^{-1} BAP.

Table 1: Summary os somatic embryogenesis induction in *Eucalyptus* genus

References (By year)	Species	Explant type	BM	Culture conditions	Sucrorse	PGR	Response	Emblings
Ouyang *et al.* 1980*	*"E.* x *leichow"*	In vitro seedlings, callus	nd	Light	nd	nd	Embryoids and plants	**Yes**
Lakshmi 1986*	*E. grandis*	Internodal segments (5-year-old- plants)	MS	16h L /25ºC	2%	NAA and BAP	Proembryos	No
Boulay 1987*	*E. gunnii*	Hypocotyl and internodes	MS	nd	nd	NAA and BAP	Embryogenic callus	No
Muralidharan and Mascaranhas 1987	*E. citriodora*	ZE	B5	Dark/27ºC	5%	NAA	Embryogenic callus, somatic embryos and emblings	**Yes**
Franclet and Boulay 1989*	*E. gunnii*	Leaf, hypocotyl, seedlings internodes	MS	nd	nd	NAA and BAP or NAA and KIN	Somatic embryogenesis	No
Muralidharan *et al.* 1989	*E. citriodora*	Mature ZE	B5	Dark/27ºC	5%	NAA	Embryogenic callus, somatic embryos and emblings	**Yes**
Qin chang-Li* 1990	*E. botyroides* *E. dunnii* *E. grandis* *E. rudis*	Cotyledons, hypocotyl, seedlings	MS (mod) and RV vitamins	nd	nd	2,4-D	Embryo-like structures	No
Watt *et al.* 1991	*E. grandis*	Young leaves in vitro shoots	MS	Dark/ 25ºC	3%	2,4-D	Somatic embryos and emblings	**Yes**
Trindade 1996	*E. globulus*	ZE	WPM	16 h L /26ºC	3%	BA and 10% coconut milk	Somatic embryos (globular and heart- shaped)	No
Termignoni *et al.* 1996	*E. dunni*	Seedlings (3 day- old)	B5	Dark/27ºC	2%	NAA and CH	Somatic embryos	No

Ruaud *et al.* 1997**	*E. nitens*	Mature zygotic embryos	nd	nd	nd	nd	Embryo-like structures	nd
Bandyopadhyay *et al.* 1999	*E. nitens* *E. globulus*	Seedlings (2-3 weeks)	MS	16h L /20ºC	3%	NAA and BAP	Embryo-like structures (ocasionally)	No
Nugent *et al.* 2001	*E. globulus*	Cotyledons/ hypocotyl	MS	16 h L/25ºC	3%	IBA	Somatic embryos	No
Pinto *et al.* 2002	*E. globulus*	Mature ZE	MS	Dark/24ºC	3%	NAA	Somatic embryos and emblings	**Yes**
Oller *et al.* 2004	*E. globulus*	Leaf of epicormic shoots	nd	nd	nd	IBA	Embryogenic callus	No
Prakash and Gurumurthi 2005	*E. tereticornis*	Mature ZE	MS	16h L /25ºC	3%	NAA for callus induction and BAP for SE formation	Somatic embryos and emblings	**Yes**

*According to Le Roux and Van Staden (1991)

** According to Watt *et al.* 2003

(BM: basal medium; L: light; nd: not defined; ZE: zygotic embryo; nd: not defined)

Somatic embryogenesis was reported on callus derived from shoots of 4-year-old trees of *E. grandis* on MS medium with 0.1 mg 1^{-1} NAA and 5 mg 1^{-1} KIN (Lakshmi *et al.* 1986, according to Le Roux and Van Standen 1991). Somatic embryos were also obtained by culturing friable callus in liquid medium containing 1 mg 1^{-1} each of BAP, KIN, NAA and 2.4-D and cultures were grown in a 16-h photoperiod at 25 ºC. Somatic embryogenesis was achieved in *E. citriodora* zygotic embryos grown on B5 medium with 3 $mg1^{-1}$ NAA and sucrose at 5% (Muralidharan and Mascarenhas 1987, Muralidharan *et al.* 1989). Recently, Prakash and Gurumurthi (2005) reported SE and plant regeneration in *E. tereticornis* from embryogenic calli from mature zygotic embryos. When calli were transferred to the respective callus induction medium (MS or B5 with 2,4-D or NAA) also containing BAP, somatic embryos developed after 1- 2 weeks. Somatic embryos germinated and converted in MS PGR-free medium and rooted plants were successfully acclimatized (Prakash and Gurumurthi 2005).

Somatic embryogenesis induction in *Eucalyptys globulus* was first described by Trindade (1996). Later, Bandyopadhyay *et al*. (1999) and Nugent *et al.* (2001) also reported SE induction and embryo formation although with low and no reproductive success. Recently Oller *et al.* (2004) reached the embryogenic callus phase using leaf callus in a basal medium with IBA.

In the Chapter II.1 of this Thesis the first report of emblings regeneration in *E. globulus* is described. In this work the effect of the explant, the type of PGRs and time of exposure was also investigated. In the Chapter II.2 and Chapter II.3 the importance of medium composition in *E. globulus* was highlighted, in order to assay optimization of primary SE in this species.

Repetitive SE

In contrast to primary SE induced from explant cells, repetitive, secondary or recurrent SE may also occur in the culture of somatic embryos, directly or through callus (for a review see Raemakers *et al.* 1995, Merlke *et al.* 1995) and this phenomenon is of potential importance for both mass clonal propagation and gene transfer technology (Thorpe 2000). A much higher efficiency of secondary SE over primary SE has been indicated for many plant species (Raemakers *et al.* 1995, Akula *et al*. 2000, Vasic *et al*. 2001, Nair and Gupta 2006). Some cultures are able to retain their competence for secondary embryogenesis for

many years and thus provide useful material for various studies, as described for *Vitis rupestris* (e.g. Martinelli *et al*. 2001).

Proliferation of embryogenic cells takes a number of forms and is influenced by a variety of factors, as those described above for induction phase and formation. In general embryogenic callus is maintained on a medium similar to that used for induction, being the use of liquid medium preferred for large-scale propagation (Von Arnold *et al*. 2002). From all known reports concerning SE in *Eucalyptus* just three of them report repetitive SE (Table 2).

The third part of this Thesis (Chapter III.1, III.2) highlights this phenomenon (Table 2).

Table 2: Repetitive somatic embryogenesis in *Eucalyptus* genus

Specie	reference	Explant	BM	Culture conditions	PGR and other supplements	Response	Somaclonal Variation	Period maintained	Emblings
*E. gunnii**	Boulay 1987	EC	MS	nd	Various combinations	Secondary EC	nd	nd	nd
E. citriodora	Muralidharan *et al*. 1989 Muralidharan and Mascarenhas 1995	Somatic embryos	B5	Dark /27ºC	5mgl^{-1}NAA CH/ glutamine	secondary somatic embryos	nd	36 month (in 1989) 9 years	**Yes**
E. globulus	Pinto *et al*. 2004 b Pinto *et al*. 2006	Somatic embryos	MS	Dark/ 24ºC	3mgl^{-1} NAA	secondary somatic embryos	No**	8 months	**Yes**

* According to Le Roux and Van Staden. (1991)

** Flow cytometry and microssatellites

(BM: basal medium, EC: embryogenic callus; CH: Casein hydrolisade; nd: not described)

Boulay (1987, according to Le Roux and Van Staden 1991) reported secondary somatic embryogenesis in *E. gunnii* by subculturing embryogenic calli. A high efficient protocol for SE was establish by Muralidharan *et al.* (1989) and Muralidharan and Mascarenhas (1995) allowing large-scale propagation of *E. citriodora*. According to these authors, the embryogenic potential was maintained for a period over 3 years, in the dark, on B5 medium containing 5 mg 1^{-1} NAA, casein hydrolysate (500 mg 1^{-1}), glutamine (500 mg l^{-1}) and 30 gl^{-1} of sucrose. Later, inositol was reported to be crucial for long term (9 years) embryogenic competence maintenance of the same cultures, when growing in liquid medium. Eventually, embryo development occurred on fresh B5 medium without PGRs and in the light (Muralidharan and Mascarenhas 1995).

An intensive study of the factors that can affect the maintenance of repetitive SE was performed in this PhD Thesis and, based on the results, a standard maintenance medium was formulated and used for routine maintenance (Chapter III.2). The occurrence of repetitive SE in *E. globulus* was reported for the first time in 2004 by Pinto *et al.* (2004a, b) on MS medium with 3 mg l^{-1} NAA, 30 g l^{-1} of sucrose and cultures maintained at 24ºC in dark. In the chapter III.1 of this PhD Thesis eight month old somatic embryos were used to investigate potential ploidy changes by flow cytometry, one of many screening tools to evaluate variability occurrence and study the true-to type propagation of *Eucalyptus globulus* via repetitive SE (Pinto *et al.* 2004b). A preliminary work with microsatellites was also performed and corroborates the results from flow cytometry (Loureiro *et al.* 2004)

Maturation, germination and conversion somatic embryos

Only mature embryos that have accumulated enough storage materials and acquired desiccation tolerance at the end of maturation seem to develop into normal plants (e.g. Merkle 1995). The addition to the culture medium of certain PGRs like ABA, as well as the increase of osmotic pressure (e.g. polyethylene glycol), desiccation and low temperature exposure may allow latter phases of SE, stimulating maturation and inhibiting precocious germination (Merkle 1995, Watt *et al.* 1999, Jiménez 2005).

Even when high quantities of somatic embryos are obtained, a bottleneck for large-scale propagation is the conversion of these somatic embryos in plants. Somatic embryos usually develop into small emblings, comparable to seedlings, on culture medium lacking PGRs. However, there are cases where auxin and cytokinin stimulate germination/convertion. Furthermore, a marked alteration in basal medium is often required. For some species, inclusion of extra compounds like glutamine and casein hydrolysate is also required (for a review see Von Arnold *et al*. 2002). All the factors that influence this step may also contribute to a better embling performance in what concerns development and later acclimatization to ex vitro conditions.

In *Eucalyptus,* mature somatic embryos usually do not develop in the presence of auxin. Besides, plant regeneration has usually been achieved in auxin free medium or occasionally in media containing cytokinins and/ or GA_3 (Table 3). In *E. citriodora*, mature embryos germinated easily when transferred to an auxin-free medium. After

isolation in a liquid medium, individual mature somatic embryos were transferred to germination medium (B5 medium with 20g l^{-1} sucrose) and 52% of the embryos germinated and developed healthy shoot and root systems. In this species the addition of ABA had a negative effect on embryogenic masses growth and embryos turned moribund with increasing concentration of ABA (Muralidharan and Mascarenhas 1995). In *E. dunnii* and in *E. grandis* embryo maturation and subsequent germination was also achieved (Watt *et al.* 1999). In *E. grandis* the effect on ABA, PEG (alone or in combination) and 3 hours of dissection did not show any success and the rate of emblings regeneration was low or absent, depending on the explant source (Watt *et al.* 1999). It is consensual that the regeneration of viable emblings is still a problem in many species (Merlke 1995), with *Eucalyptus* being no exception.

In the Chapter III.2 several factors (basal medium, PGR and light conditions) were studied in order to optimize the maintenance and germination of secondary somatic embryos.

Acclimatization and genetic fidelity of the emblings

From the point of view of fundamental research, the success of any SE system is usually achieved by the conversion of somatic embryos in emblings. However, and as referred above, the industrial value of these strategies requires that, besides embling large scale production, plants are successfully acclimatized to field conditions. As the in vitro propagated plants should be true-to-type, so that the advantages (e.g. high yield, uniform quality, shorter rotation period) of explant genotypes are maintained, somaclonal variation is in general undesirable being, therefore essential to verify the clonal fidelity and field performance of somatic embryo derived plants (e.g. Tremblay *et al.* 1999, for a review see Kaeppler *et al.* 2000).

In general, culture procedures, environment conditions, genotype, ploidy level and in vitro culture age are known to be often associated with the occurrence of somaclonal variation (e.g. Rani and Raina 2000). Morphological markers, chromosome analysis, breeding behavior, isoenzymes or DNA markers may be used to detect somaclonal variation. Early assessment of genetic fidelity at various cultural stages will help to identify the specific cultural condition(s) that induce variation (Rani and Raina 2000). Flow cytometry (FCM) has increasingly been chosen for analysis of major ploidy changes in

genetic variation assays. It thereby may replace other methods such as chromosome counting being that FCM provides unsurpassed rapidity, ease, convenience and accuracy. Until this moment, very few reports used this technique to assay somaclonal variation in woody plants (e.g. Santos *et al*. 2007).

Besides, somatic embryo preservation, though important, was only reported for *E. citriodora* by using somatic embryo encapsulation (Muralidharan and Mascarenhas 1995). Together with criopreservation, this is therefore an open field in this genus and strongly depends on the development of reliable somatic embryogenesis protocols.

In vitro culture conditions may result in the formation of plantlets of abnormal morphology, anatomy and physiology. After ex vitro transfer, these plantlets may easily be impaired by sudden changes in environmental conditions and so a period of acclimatization is needed (for a review see Pospisilova 1999, Hazarica 2006). In this chapter, the recalcitrance of *Eucalyptus* for emblings regeneration was well demonstrated, and so, it is not surprising that only few works report plant hardening/acclimatization in this genus. Embling's acclimatization was reported for *E. grandis* (Watt *et al.* 1991), E. *citriodora* (Muralidharan *et al.* 1989, Muralidharan and Mascarenhas 1995) and *E. tereticornis* (Prakash and Gurumurthi 2005). In all these species, the basic acclimatization procedure implied a gradual reduction of the environmental relative humidity and the transfer to soil substrates (e.g. peat, perlite or sand). However, in those works, emblings' performance was only measured as survival rates. Up to moment, no studies are known concerning important aspects (such as histocytology, physiology, or genetics) that may occur during emblings acclimatization in *Eucalyptus* genus.

Chapter III.3 presents an ultrastructural approach of different steps of acclimatization. This work was performed after an evaluation of the ploidy level of emblings.

Table 3: Attemps to regenerate emblings in *Eucalyptus* genus and complementary studies

Species	References	Explant	BM	PGR or other supplements	Light conditions	Somaclonal Variation	Response	Acclimatization	Other studies
E. citriodora	Muralidharan *et al.* 1989	EC	B5	PGR free	Continous L	nd	Emblings	**Yes**	-
E. grandis	Watt *et al.* 1991	EC	MS/2	BAP, GA_3 and NAA	1 week dark followed by a 16 h L		Emblings	**Yes**	
E. citriodora	Muralidharan and Mascarenhas 1995	EC	B5	PGR free	Continous L	nd	Emblings	**Yes**	Somatic embryo encapsulation
E. dunnii	Termignoni *et al.* 1996	EC	B5	10% cocconut milk	16h L		Embryos with green cotyledons		-
E. globulus	Trindade 1996	Globular structures	B5	NAA and BAP	16h L	nd	Shoot proliferation	No	-
E. dunnii	Termignoni *et al.* 1998*	nd	nd	nd	nd	nd	Embryo maturation and germination	nd	nd
E. globulus	Pinto *et al.* 2002	Primary somatic embryos	MS	PGR free	Dark	nd	Emblings	no	-
E. globulus	Pinto *et al.* 2004a	Secondary somatic embryo	MS	PGR free	Dark	nd	Emblings	no	-
E. tereticornis	Prakash and Gurumurthi 2005	Primary somatic embryos	MS	PGR free	16h L	nd	Emblings	**Yes**	-

* According to Watt *et al.* (1999)

(nd: not defined; L: light; EC: embryogenic callus; BM: basal medium)

Research Objectives

As already explained, this work has been integrated in a collaboration work with Celbi and their strategic *Eucalyptus* breeding program. When this collaboration started (1999) the reports about *Eucalyptus globulus* SE potential were rare and, in fact, efficient protocols for plant regeneration of large genotypes by SE were missing.

During this PhD work emphasis has been given to identify the current trends and strategies for the establishment of a somatic embryogenic system, particularly in this economically important species.

In this context, the mail goal of the present work was to establish efficient protocols for in vitro plant regeneration by SE. This work is divided in the two main processes from which plant regeneration by SE could be achieved: primary and secondary SE. The specific objectives of this work were:

- To evaluate the species potencial concerning primary somatic embryogenesis, by testing different factors: explants type, PGRs combination and period of exposure (induction period) as well as carbohydrate presence, medium composition and antioxidants in the primary somatic embryogenesis and design a standard protocol for SE induction.
- According to the results of the first objective, to design a standard protocol for primary SE and follow histocytological changes occurring during this process (from explant to embling).
- To evaluate the genetic control of SE induction and year of seed production using the standard protocol.
- To develop a standard protocol of secondary SE (up to embling stage) and evaluate putative ploidy changes during the process.
- To acclimatize emblings to ex vitro conditions and follow some aspects concerning histocytology.

References

Akula A., Becker D. and Bateson M (2000) High-yielding repetitive somatic embryogenesis and plant recovery in a selected tea clone, 'TRI-2025', by temporary immersion. Plant Cell Rep 19:1140–1145

Arruda SCC, Souza GM, Almeida M, Gonçalves AN (2000) Anatomical and biochemical characterization of calcium effect on *Eucalyptus urophylla* callus morphogenesis in vitro. Plant Cell Tiss Organ Cult 63: 143- 154

Azmi A, Noin M, Landré P, Proteau M, Boudet AM, Chriqui D (1997) High frequency plant regeneration from *Eucalyptus globulus* Labill. hypocotyls: Ontogenesis and ploidy level of the regenerants. Plant Cell Tiss Organ Cult 51(1): 9-16

Bandyopadhyay S, Cane K, Rasmussen G, Hamill JD (1999) Efficient plant regeneration from seedlings explants of two commercially importante temperate eucalyptus species- *Eucalyptus nitens* and *E. globulus*. Plant Science 140: 189- 198

Bogunic FEM, Brown SC, Siljak-Yakovlev S (2003) Genome size and base composition of five *Pinus* species from the Balkan region. Plant Cell Rep 22(1):59–63

Bonga JM, Von Aderkas P (1992) In vitro culture of trees. Kluwer Academic Publishers, Dordrecht, 236p

Brooker MIH (2000) A new classification of the genus *Eucalyptus* L'Her. (Myrtaceae). Aust Syst Bot 13:79–148

Canhoto JM, Cruz G (1996) Histodifferentiation of somatic embryos in cotyledons of pineapple guava (*Feijoa sellowiana* Berg.) Protoplasma 191: 34-45

Canhoto JM, Lopes ML, Cruz GS (1999) Somatic embryogenesis in myrtaceous plants. In Somatic embryogenesis in woody plants Vol. 4. (Eds: SM Jain, PK Gupta, RJ Newton) Kluwer Academic Publishers, pp 293-338

Cid LPB, Machado ACMG, Carvalheira SBRC, Brasileiro ACM (1999) Plant regeneration from seedlings explants of *Eucalyptus grandis* x *E. urophylla*. Plant Cell Tiss Organ Cult 56 (1): 17- 23

Correia D, Gonçalves NA, Couto HYZ, Ribeiro MC (1995) Efeito do meio de cultura líquido e sólido no crescimento e desenvolvimento de gemas de *Eucalyptus grandis* x *Eucalyptus urophylla* na multiplicação in vitro. IPEF, Piracicaba, n. 48/49, p. 107-116

Cotterill PP, Araújo C, Lencart P (2000) Project 3D: Celbi's advanced-generation Eucalyptus globulus improvement. Celbi Investigation Report No. 75, Volume 8, pp. 67-178

Direcção Geral das Florestas (2001) Inventário Florestal Nacional. 3ª revisão 1995- 1998. Portugal continental. Direcção Geral Florestas. Lisboa

Dodeman VL, Ducreux G, kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. J Exp Bot 48 (313): 1493- 1509

Doughty RW (2000) The *Eucalyptus:* a natural and commercial history of the gum tree. John Hopkins University Press, Baltimore, MD

Dudits D, Györgyey J, Bögre L, Bakó L (1995) Molecular biology of somatic embryogenesis. In: Thorpe TA (ed) In vitro embyogensis in plants, Kluwer Academic Publishers, Dordrecht, pp 267-308

Dunstan DI, Tautorus TE, Thorpe TA (1995) Somatic embryogenesis in woody plants. In: Thorpe TA (ed) In vitro embyogensis in plants, Kluwer Academic, pp 471-538

Eldridge K, Davidson J, Harwood C, Van Wyk G (1993) Eucalypt domestication and breeding. Clarendon Press, Oxford

Ezhova TA (2003) Genetic control of totypotency of plant cells in na in vitro culture. Russian J Develop Biology 34 (4): 197- 204

Fehér A, Pasternak TP, Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74:201–228

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regulat. 43:27-47

Glocke P, Delaporte K, Collins G, Sedgley M (2006) Micropropagation of juvenile tissue of *Eucalyptus erythronema* x *Eucalyptus stricklandi* cv “Urrbrae Gem”. In Vitro Cell Delv Biol Plant 42 (2):139-143

Grattapaglia, D. & H.D. Bradshaw, 1994. Nuclear DNA content of commercially important Eucalyptus species and hybrids. Can. J. For. Res. 24: 1074–1078.

Hajari E, Watt MP, Mycock DJ, McAlister B (2006) Plant regeneration from induced callus of improved *Eucalyptus* clones. S Afri J Bot 72: 195- 201

Harbard JL, Griffin AR, Espejo J. (1999) Mass Control pollination of *Eucalyptus globulus*: A pratical reality. Can. J. For. Res. 29 (10): 1457-1463

Hazarika BN (2006) Morpho- physsiologycal disorders in in vitro culture of plants. Sci Hortic 108:105- 120

Högberg K-A, Ekberg I, Norell L, Von Arnold S (1998) Integration of somatic embryogenesis in a tree breeding programme: a case study with *Picea abies*. Can J For Res 28: 1536-1545

James SA, Smith WK, Vogelmann TC (1999) Ontogenetic differences in mesophyll structure and chlorophyll distribution in *Eucalyptus globulus* ssp. *globulus* (Myrtaceae) Amer J Bot 86 (2): 198- 207

Jiménez V (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Reg 47:91-110

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology 43: 179-188

Le Roux JJ, Van Staden J (1991) Micropropagation and tissue culture of *Eucalyptus* – a review. Tree Physiol 9: 435–477

Leal AMC, Cotterill PP (1997) Mass control-pollination of *Eucalyptus globulus*. Proc., IUFRO Symposium on Silviculture and Genetic improvement of *Eucalyptus*. Salvador, Brazil-August

Lipavska H, Svobodova H, Albrechtova J, Kumstýrová L, Vágner M, Vondráková Z (2000) Somatic embryogenesis in Norway spruce: carbohydrate status during embryo maturation. In Vitro Cell Dev Biol Plant 36:260–267

Lipavska H, Dova HK (2004) Somatic embryogenesis in conifers: the role of carbohydrate. In vitro Cell Dev Biol Plant 40:23-30

Lopez GA, Potts BM, Dutkowski GW, Apiolaza LA, Gelid P (2002) Genetic variation and inter-trait correlations in *Eucalyptus globulus* base population trials in Argentina. For Genet 9:223–237

Loureiro J, Lopes T, Pinto G, Santos R, Siva S, Santos C (2004a) Analysis of genetic stability of *Eucalyptus globulus* Labill. somatic embryos by Flow cytometry and microsatellites. In " *Eucalyptus* in a changing world" (Eds. NMG Borralho, JS Pereira, C Marques, J Coutinho, M Madeira, M Tomé) pg 529- 531. Proc. IUFRO Conf. Aveiro, 11- 15 Oct.

Louro RP, Dos Santos AV, Machado RD (1999) Ultrastructure of *Eucalyptus grandis* X Eucalyptus *urophylla*. I. Shoots cultivated in vitro in multiplication and elongation-rooting medium. Int J Plant Sci 160 (2): 217- 227

MacKay JJ, Becwar MR, Park YS, Corderro JP, Pullman GS (2006) Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genet Genom 2:1–9

MacRae S, Van Staden J (2000) Trangenic Eucalyptus. In Biotechnology in agriculture and forestry 44: transgenic trees. In Bajaj YPS (ed.), Springer, pp 88- 114

Martinelli L, Candioli E, Costa D, Poletti V (2001) Morphogenic competence of *Vitis rupestris* S. secondary somatic embryos with a long culture history. Plant Cell Rep 20:279–284

Merkle A, Nairn JC (2005) Hardwood tree biotechnology. In Vitro Cell Dev Biol Plant 41:602-619

Merkle SA (1995) Strategies for dealing with limitations of somatic embryogenesis in hardwood trees. Plant Tiss Cult Biotechnol 1:112-121

Merkle SA, Parrott WA, Flinn BS (1995) Morphogenic aspects of somatic embryogenesis. In: Thorpe TA (ed) In vitro embryogenesis in plants, pp 155-203

Mullin TJ, Park YS (1992) Estimating genetic gains from breeding strategies for clonal forestry. Can J For R 22:14-23

Muralidharan EM, Gupta PK, Mascarenhas AF (1989) Plantlet production trough high frequency somatic embryogenesis im long term cultures of *Eucalyptus citriodora*. Plant Cell Reports 8:41- 43

Muralidharan EM, Mascarenhas AF (1987) In vitro plantlet formation by organogenesis in *E. camaldulensis* and by somatic embryogenesis in *Eucalyptus citriodora*. Plant Cell Rep 6:256–259

Muralidharan EM, Mascarenhas AF (1995) Somatic embryogenesis in *Eucalyptus*. In: Somatic embryogenesis in woody plants, vol 2_ Angiosperms, Jain SM, Gupta PK, Newton RJ (eds) Kluwer Academic Publishers, Dordrecht, pp 23–40

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

Nair RR and Gupta SD (2006) High- frequency plant regeneration trough cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum* L.). Plant Cell Rep 24:600–707

Nugent G, Chhandler SF, Whitemn P, Stevenson T (2001) Somatic embryogenesis in *Eucalyptus globulus*. Plant Cell, Tiss Organ Clut 67: 85-88

Oller J, Toribio M, Celestino C, Toval G (2004) The culture of elite adult trees in a genetic improvement programme through *Eucalyptus globulus* labill. clonal micropropagation. In " Eucalyptus in a changing world" (Eds. NMG Borralho, JS Pereira, C Marques, J Coutinho, M Madeira and M Tomé) pg 539- 540. Proc. IUFRO Conf. Aveiro, 11- 15 Oct.

Park YS (2002) Implementation of conifer somatic embyogenesis in clonal florestry: technical requirements and deployment considerations. Ann For Sci 59: 651-656.

Park YS, Barrett JD, Bonga JM (1998) Appications of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell Dev Biol Plant 34:231-239

Park YS, Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K, Bonga JM (2006) Initiation of somatic embryogenesis in *Pinus bankesiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. Plant Cell Tiss Organ Cult 86: 87- 101

Phillips G (2004) In vitro morphogenesis in plants- recent advances. In Vitro Cell Dev Biol Plant 40:342-345

Pinto G, Loureiro J, Lopes T, Santos C (2004b) Analysis of the genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry. Theor Appl Genet 109: 580– 587

Pinto G, Santos C, Neves L, Araújo C (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21(3):208-213

Pinto G, Silva S, Coutinho J, Araujo C, Neves L, Santos C. (2006) Importance of media mineral composition on the induction of somatic embryogenesis in *Eucalyptus globulus* Labill. 2º Simpósio Iberoamericano de *Eucalyptus globulus*. 17- 20 of October. Pontevedra, Spain

Pinto G, Silva S, Santos C, Neves L, Araújo C (2004a) Somatic embryogenesis of *Eucalyptus globulus* Labill. and assessement of genetic stability. In " *Eucalyptus* in a

changing world" (Eds. NMG Borralho, JS Pereira, C Marques, J Coutinho, M Madeira and M Tomé) pg 543- 544. Proc. IUFRO Conf. Aveiro, 11- 15 Oct.

Poke FS, Vaillancourt RE, Potts BM, Reis JB (2005) Genomic research in *Eucalyptus*. Genética 125:79-101

Pospísísilová J, Tichá I, Kadlecek P, Haisel D, Plazákova S (1999) Acclimatization of micropropagated plants to ex vitro conditions. Biologia Plantarum 42 (4):481-497

Potts BM, Wiltshire RJE (1997) Eucalypt genetics and genecology. In: JE Williams and JCZ Woinarski (eds), Eucalypt ecology: individuals to ecosystems. University Press, Cambridge, UK pp. 56–91

Prakash MG, Gurumurthi K (2005) Somatic embryogenesis and plant regeneration in *Eucalyptus tereticornis* Sm. Cur Sci 88 (8): 1311- 1316

Pullman GS, Montello P, Cairney J, Xu N, Feng X (2003) Lobolly pine (*Pinus taeda* L.) somatic embryogenesis: maturation improvements by metal analysis of zygotic and somatic embryos. Plant Sci 164:955-969

Raemakers CJJM, Jacobsen E, Visser RGF (1995) Secondary somatic embryogenesis and aplications in plant breeding. Euphytica 81:93–107

Ramage CM, Williams RR (2002) Mineral nutrition and plant morphogenesis. In Vitro Cell Dev Biol Plant 38:116- 124

Rani V, Raina SN (2000) Genetic fidelity of organized meristem-derived micropropagated plants: a critical reappraisal. In Vitro Cell Dev Biol Plant 36:319-330

Ruaud J-N, Churchill K, Pepper S (1997) Somatic embryogenesis initiation in eucalyptus nitens. Acta Horticulture 447:185-186

Santos C, Loureiro J, Lopes T, Pinto G (2007) Genetic fidelity analyses of in vitro propagated cork oak (*Quercus suber* L). In Protocols for micropropagation of woody trees and fruits, Vol 7 (eds. S. Mohan Jain and H. Häggman) Springer, Dordrecht pp 63- 87

Sedjo RA (2001) Biotechnology in forestry: considering the cost and benefits. Resour Future 145: 10-12

Silva JC, Borralho NMG, Potts BM (2004) Additive and non-additive genetic parameters from clonally replicated and seedling progenies of *Eucalyptus globulus.* Theor Appl Genet 108:1113–1119

Teasdale RD (1996) Priorities for application of biotechnology to plantation improvement. In. MR Ahuja *et al* (eds.) Somatic cell genetics and molecular genetic of trees, Kluwer Academic Publishers, Netherlands, pp 69-78

Termignoni RR, Wang PJ, HU CY (1996) Somatic embryo induction in *Eucalyptus dunnii*. Plant Cell Tiss Organ Cult 45:129- 132

Thorpe TA (2000) Somatic embryogenesis: morphogenesis, physiology, biochesmestry and molecular biology. Korean J Plant Tiss Cult 27:245:258

Tournier V, Grat S, Marque C, El Kayal W, Penchel R, de Andrade G, Boudet A-M, Teuliéres C (2003) An efficiente procedure to stably introduce genes into an economically important pulp tree (*Eucalyptus grandis* x *Eucalyptus uroplylla*). Transgenic Research 12 (4):403-411

Tremblay L, Levasseur C, Tremblay FM (1999) Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability. American J Bot 86: 1373-1381

Trindade H (1996) *Eucalyptus globulus* Labill: systems for in vitro regeneration. PhD Thesis. University of Lisbon, Lisbon

Vasic D, Alibert G, Skoric D (2001) Protocol for efficient repetitive and secondary somatic embryogenesis in *Helianthus maximiliani* (Schrader). Plant Cell Rep 20:121–125

Von Arnold S, Sabala I, Bozhkovi P, Dyachok J, Filanova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tiss Organ Cult 69: 233-249

Watt MP, Berjak P, Aneliswa M, Blakway F (2003) In vitro field collection techniques for *Eucalyptus* micropropagation. Plant Cell Tiss Organ Cult 75: 233-140

Watt MP, Blakeway FC, MEO Mekotedi MEO, Jain SM (2003) Micropropagation of *Eucalyptus*. In: SM Jain and K Ishii (Eds.), Micropropagation of woody trees and fruits, Kluwer Academic Publishers, Dordrecht, pp 217- 244

Watt MP, Blakway F, Cresswell CF, Herman B (1991) somatic embryogenesis in *Eucalyptus grandis*. South African Forestry Journal 157:59- 65

Watt MP, Blakway FC, Termignoni R, Jain SM (1995) Somatic embryogenesis in *Eucalyptus grandis* and *E. dunnii*. In: SM Jain, Gupta PK and Newton RJ (Eds.) Somatic embryogenesis in woody plants, vol 5, Kluwer Academic Publishers, Dordrecht, pp1- 16

Whitehouse AB, Timothy RM, Glyn AE (2002) Control of hyperhydricity in *Eucalyptus* axillary shoots cultures grown in liquid medium. Plant Cell Tiss Organ Cult 71 (3) 245- 252

Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Ann Bot 57: 443-462

Williams JE, Woinarski JCZ (1997) Eucalypt Ecology: Individuals to Ecosystems. Cambridge University Press, Cambridge, UK

Yeung EC (1995) Structural and developmental patterns in somatic embryogenesis. In: Thorpe TA (ed) In vitro embyogensis in plants. Kluwer Academic Publishers, Dordrecht pp 205-247

Chapter II

Primary somatic embryogenesis

II 1: Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill.

Chapter published as an original paper in a SCI journal:

Pinto G, Santos C, Neves L, Araújo, C (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Reports 21: 208- 213

Abstract

Somatic embryogenesis was induced from juvenile explants of *Eucalyptus globulus* Labill. Mature zygotic embryos, isolated cotyledons, hypocotyls, leaves and stems were cultivated at 24°C in darkness on Murashige and Skoog medium supplemented with 3% (w/v) sucrose and different growth regulator combinations. Callus was formed at the surface of the explant in all tested media containing sucrose but not in those containing mannitol. Calli were transferred to the same medium without growth regulators (MS_{WH}) after 25 days. Somatic embryogenesis was observed in callus derived from cotyledon explants and from entire mature zygotic embryos in the presence of 3–15 mg l^{-1} α-naphthalene acetic acid (NAA) alone or in the presence of 1 mg l^{-1} NAA combined with 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Best embryogenic rates were obtained in the presence of 3–5 mg l^{-1} NAA, as approximately 30% of callus formed on these media produced somatic embryos. Exposure, for >1 week, to the highest NAA concentrations tested (15 mg l^{-1}) failed to induce somatic embryogenesis. Addition of 500 mg l^{-1} casein hydrolysate and 500 mg l^{-1} glutamine to the induction medium increased the number of abnormal somatic embryos. Conversion of somatic embryos to plantlets (21%) was obtained when they were transferred to medium free of growth regulators.

Keywords: embryogenic callus, *Eucalyptus,Eucalyptus globulus,* plant regeneration, somatic embryogenesis

Introduction

The genus *Eucalyptus* comprises approximately 700 species and varieties (Watt *et al.* 1999) although only 1% of them are used for industrial purposes. Although exact figures for the total plantation area are difficult to obtain, there is an estimated 10 million ha of *Eucalyptus* plantations (Kellison 2001) and the *Eucalyptus* kraft pulp industry is based largely on two species, namely *E. globulus* and *E. grandis* hybrids.

The natural genetic diversity within and between *Eucalyptus* species is enormous and can be further enhanced by interspecific hybridisation making it an attractive genus for breeding (Eldridge *et al.* 1993). Besides the production of pulpwood, *Eucalyptus* is also used for timber, veneer, firewood, shelter, ornamentals and essential oil production (Watt *et al.* 1999). *E. globulus* is nowadays grown worldwide (e.g. Australia, South America, South Africa, Portugal, Spain, USA) due to its versatility, fast growth and fibre characteristics.

E. globulus was introduced to Portugal 150 years ago and nowadays it represents the third forest species in Portugal, covering approximately 672,149 ha of forest (Direcção Geral Florestas 2001). Propagation of this species has been carried out mainly from seed. However, *E. globulus* is an outcrossing species and a high level of heterozygosity is found in seeds. Therefore, clonal propagation offers the possibility to capture both additive and non-additive variation created by conventional breeding as well as improving uniformity for cost-effective mechanical harvesting. However, *E. globulus* has a very irregular adventitious rooting behaviour (5–64%) (Marques *et al.* 1999) that hampers vegetative propagation of some desired genotypes. Somatic embryogenesis has the advantage that both a root and a shoot meristem are present simultaneously in somatic embryos. Furthermore, somatic embryogenesis largely simplifies the conservation methods by using a limited space for a large number of genotypes while they are being field tested. In fact, as in other forest species, genotype×environment interactions are particularly important in eucalypt species (Zobel 1993).

Somatic embryogenesis in the *Eucalyptus* genus has been described for *E. citriodora* (Muralidharan and Mascarenhas 1987, 1995, Muralidharan *et al.* 1989), *E. dunnii* (Termignoni *et al.* 1996, Watt *et al.* 1999), *E. grandis* (Watt *et al.* 1991, 1999) and for *E. nitens* (Bandyopadhyay *et al.* 1999, Bandyopadhyay and Hamill 2000). However, *E. globulus* has previously been reported as being extremely recalcitrant to regeneration

through somatic embryogenesis, and in vitro plant regeneration was only obtained through organogenesis (Serrano *et al.* 1996, Bandyopadhyay *et al.* 1999). Recently, Bandyopadhyay *et al.* (1999) reported the appearance of embryogenic structures in *E. globulus* from seedling explants. Also Nugent *et al.* (2001) reported somatic embryogenesis from cotyledons and hypocotyls but with no plantlet development.

We herewith describe, for the first time, a reproducible protocol for somatic embryogenesis in *E. globulus* from mature zygotic embryos.

Materials and methods

Plant material and disinfection:

Half-sib seeds of *Eucalyptus globulus* Labill (Stora-Enso Celbi, Leirosa, Portugal) were imbibed and surface-sterilised by immersion in 50% absolute ethanol (v/v) for 15 min and rinsed in three changes of sterile distilled water (15–20 min per wash), then with 0.1% (w/v) Benlate (Rhône-Poulenc) and finally extensively rinsed in sterile distilled water and left to imbibe for 16 h. Germination was carried out aseptically on Murashige and Skoog (1962) medium (MS) supplemented with 2% sucrose (w/v) and 0.3% gelrite®. All media were autoclaved at 121°C for 15 min. Cultures were maintained at 24±1 ºC either in darkness or under a 16-h photoperiod at a photon flux of 98±2 µmol m^{-2} s^{-1} at plant level. All compounds used in this work were purchased from Duchefa (Haarlem, Netherlands). Data refer to three independent experiments with *n*>7 each.

Induction of somatic embryogenesis:

Cultures were initiated from cotyledons and hypocotyls of 3-day old seedlings, and from leaves and stems of 2-month-old plants. Explants (*n*>40) were grown on MS medium supplemented with 0.3% (w/v) gelrite and the pH adjusted to 5.8. Different carbon sources (sucrose and mannitol) and combinations of growth regulators were tested (E1–E15 media; Table 1). Callus induction always took place in darkness at 24±1 C. Explants were transferred 25 days after explant inoculation to MS medium without growth regulators (MS_{WH}). Cultures were sub-cultured onto fresh medium every 4 weeks and maintained for more than one year. When entire mature zygotic embryos were used, the seed coat was

removed and they were transferred to MS medium supplemented with 3% (w/v) sucrose, and with different α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) concentrations (E6, E12, E13, E14 and E15; Table 1) to induce somatic embryogenesis. After 25 days on callus induction media, explants from E12 (n=20), E13 (n=14) and E14 (n=14) media were transferred to MS_{WH}. Explants growing on E15 medium (15 mg l^{-1} NAA) were divided into three groups (n=7) that were transferred to MS_{WH} medium 8, 15 and 25 days after callus induction, respectively (Table 2).

Development of somatic embryos

After being transferred to dim light for 2 weeks on MS_{WH}, somatic embryos were kept under a 16-h photoperiod (98 µmol m^{-2} s^{-1}) at 24±1°C. Cotyledon-derived somatic embryos were isolated and transferred to fresh MS_{WH} medium and incubated under the conditions described above for somatic embryo development.

Results

Callus proliferation was observed in all tested media containing sucrose, while mannitol (36.44 g l^{-1}) did not promote callus formation (Table 1) although explants remained green. Increasing sucrose levels (E8 and E9 media) decreased callus formation (Table 1) and increased phenolic production (data not shown). Somatic embryogenesis induction only occurred in the presence of NAA, either alone or in combination with 2,4-D (Tables 1, 2). Embryogenic calli emerged mainly from cotyledons of entire zygotic embryos in the presence of NAA (Fig. 1A). Germination of entire mature zygotic embryos was observed in all media tested.

Two weeks after inoculation, the germination process stopped and callus production was initiated mainly in cotyledons. Embryogenic calli were compact, white-brownish and were composed mostly of small isodiametric cells. At this stage, globular structures could already be observed in the callus formed from cotyledons (Fig. 1A, B).

Somatic embryos formed indirectly from embryogenic calli (Fig. 1A), but occasionally direct somatic embryogenesis also occurred from the upper surface of hypocotyls (Fig. 1C). Somatic embryogenesis was observed for all concentrations of NAA tested (3–15 mg l^{-1}), but best results were obtained with the lower NAA concentrations. On

the other hand, exposure for >1 week to the highest concentration of NAA (15 mg l^{-1}) inhibited embryogenic capacity.

Table 1: Embryogenic potential of *Eucalyptus globulus* explants. *E. globulus* callus production, rhizogenesis and embryogenic potential obtained on Murashige and Skoog medium without growth regulators (MS_{WH}) (after 8 weeks) for different explant sources (leaf, stem, cotyledon, hypocotyl and zygotic embryos) grown for the first 25 days on different induction media (E1–E15). All media had the basal MS composition supplemented with different growth regulators (E1–E15 media) and different carbon sources. Non-destructive visual rating of callus production and rhizogenesis based on quantity produce: + poor, ++ good, +++ best. BAP 6-benzylaminopurine; 2,4-D 2,4-dichlorophenoxyacetic acid, NAA α-naphthalene acetic acid, NT not tested

Treat-ment	Additives	Explant response	Leaf	Stem	Cotyledon	Hypocotyl	Zygotic embryo
E1	1 mg l^{-1} 2,4-D + 0.01 mg l^{-1} BAP (30 g l^{-1} Sucrose)	Callus production	+	+	+	+	NT
		Rhizogenesis	+	+	++	++	
		Somatic embryo formation	No	No	No	No	
E2	1 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP (30 g l^{-1} Sucrose)	Callus production	++	+	+	+	NT
		Rhizogenesis	+	+	++	++	
		Somatic embryo formation	No	No	No	No	
E3	1 mg l^{-1} 2,4-D+ 2 mg l^{-1} Zeatin (30 g l^{-1} Sucrose)	Callus production	++	+	++	++	NT
		Rhizogenesis	+	+	++	++	
		Somatic embryo formation	No	No	No	No	
E4	0.5 mg l^{-1} Dicamba (30 g l^{-1} Sucrose)	Callus production	+++	++	+++	+++	NT
		Rhizogenesis	+	+	+	+	
		Somatic embryo formation	No	No	No	No	
E5	1 mg l^{-1} 2,4-D (30 g l^{-1} Sucrose)	Callus production	NT	NT	++	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E6	1 mg l^{-1} 2,4-D+ 1 mg l^{-1} NAA (30 g l^{-1} Sucrose)	Callus production	NT	NT	++	NT	+
		Rhizogenesis			++		++
		Somatic embryo formation			**Yes**		**Yes**
E7	0.5 mg l^{-1} Dicamba + 1 mg l^{-1} Zeatin (30 g l^{-1} Sucrose)	Callus production	NT	NT	+	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E8	1 mg l^{-1} 2,4-D (60 g l^{-1} Sucrose)	Callus production	NT	NT	+	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E9	1 mg l^{-1} 2,4-D (90 g l^{-1} Sucrose)	Callus production	NT	NT	+	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E10	1 mg l^{-1} 2,4-D (36.44 l^{-1} Mannitol)	Callus production	NT	NT	0	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E11	0.5 mg l^{-1} Dicamba (36.44 l^{-1} Mannitol)	Callus production	NT	NT	0	NT	NT
		Rhizogenesis			0		
		Somatic embryo formation			No		
E12	3 mg l^{-1} NAA (30 g l^{-1} Sucrose)	Callus production	NT	NT	++	NT	++
		Rhizogenesis			++		++
		Somatic embryo formation			**Yes**		**Yes**
E13	5 mg l^{-1} NAA (30 g l^{-1} Sucrose)	Callus production	NT	NT	++	NT	+++
		Rhizogenesis			+++		+++
		Somatic embryo formation			**Yes**		Yes
E14	5 mg l^{-1} NAA + 500 mg l^{-1}casein hydrolysade+ 500 mg l^{-1} glutamine (30 g l^{-1} Sucrose)	Callus production	NT	NT	++	NT	++
		Rhizogenesis			+++		+++
		Somatic embryo formation			**Yes**		Yes
E15	15 mg l^{-1} NAA (30 g l^{-1} Sucrose)	Callus production	NT	NT	NT	NT	++
		Rhizogenesis					+++
		Somatic embryo formation					**Yes**/No[a]

[a] Somatic embryogenesis was only obtained in callus that were grown for 1 week on induction medium. Yes: presence of somatic embryos; No: absence of somatic embryos

Embryogenic calli, with globular structures (Fig. 1B), were transferred to MS_{WH}. Two weeks after transfer to this medium, callus browning was intense but it was possible to detect yellowish clusters of embryos (Fig. 1D) at different stages of development (Fig. 1E). However, due to the abundant root formation (Fig. 1A), somatic embryos were not always evident in the embryogenic callus. Some of these embryos (after transfer to light conditions) developed a greenish colour and, rarely, some red pigmentation (anthocyanins) was observed. A few days later it was possible to isolate complete torpedo (Fig. 1F) and cotyledon-phase somatic embryos (Fig. 1G, 1H) that subsequently converted to plants (Fig. 1I, J). Conversion of somatic embryos to plantlets (21%) was obtained and acclimatization is underway. The addition of organic nitrogen supplements (500 mg l^{-1} casein hydrolysate and 500 mg l^{-1} glutamine) stimulated root formation and callus growth but a higher proportion of abnormal somatic embryos was observed (Table 2).

Table 2: Average number of roots and somatic embryos formed on MS_{WH} medium in *E. globulus* callus produced in the cotyledon region of zygotic embryos that grew on E12, E13, E14 and E15 induction media

Treatment	Induction period (days)	No.of explants testeed	Responsive explants (%)	Roots formend per explant (range)	Embryogenic callus	Total embryos indirectly formed in all explants	Abnormal embryos
E12	25	20	30	0-9	**Yes**	13	61
E13	25	14	28.5	2-11	**Yes**	19	63
E14	25	14	21.4	0-17	**Yes**	9	100
E15	8	7	14.2	0-14	**Yes**	9	90
E15	15	7	-	0-12	No	-	-
E15	25	7	-	0-15	No	-	-

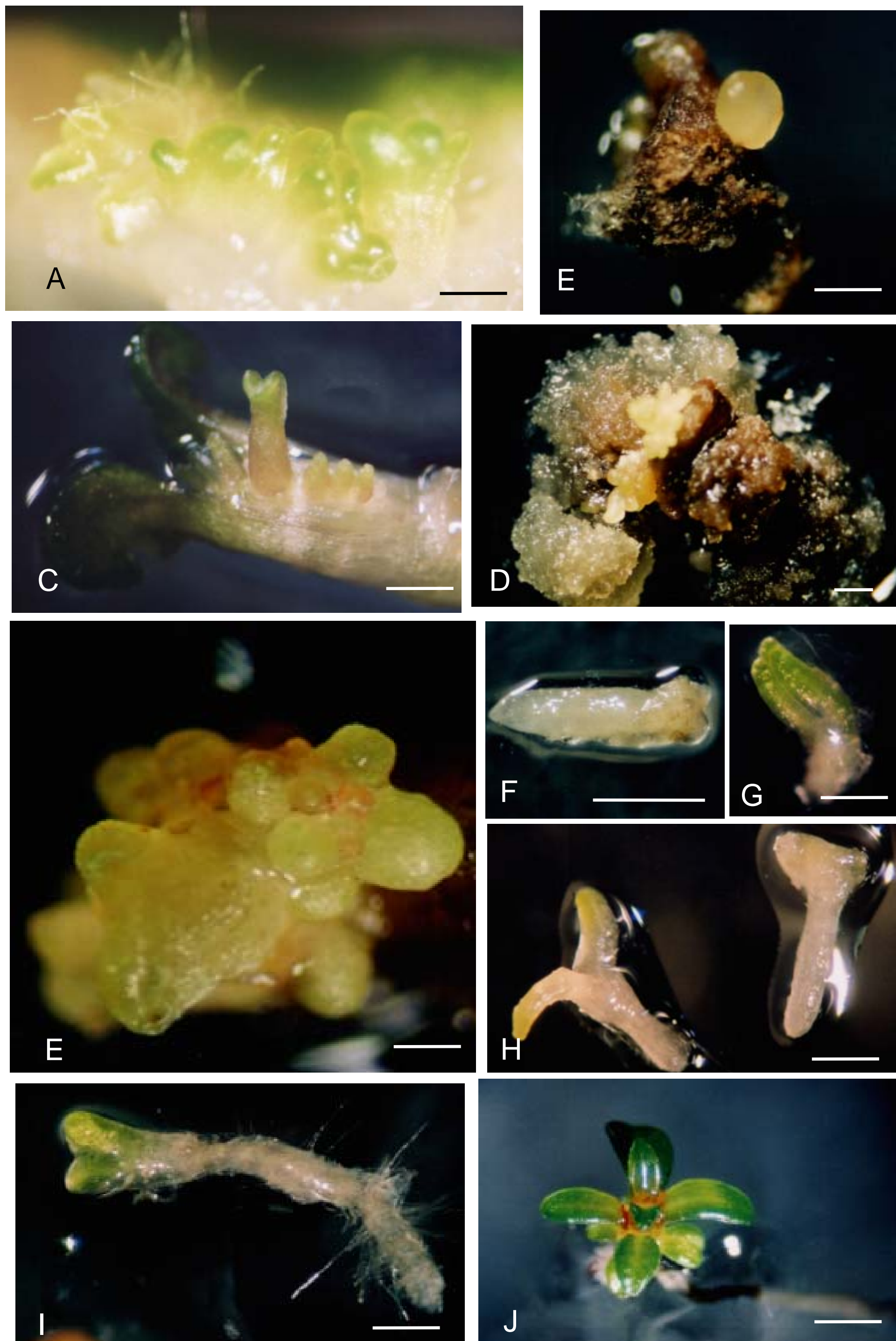


Figure 1A–J Somatic embryogenesis and plant regeneration in *Eucalyptus globulus.* **A)** Embryogenic callus with somatic embryos and abundant roots produced on a cotyledon of a zygotic embryo explant (*bar*=1 mm). **B)** Globular somatic embryo (*bar*=1 mm). **C)** Direct somatic embryogenesis from the upper surface of a hypocotyl of a zygotic embryo explant (*bar*=1 mm). **D)** Cluster of somatic embryos surrounded by tissue with accumulated phenolic compounds (*bar*=1 mm). **E)** Cluster of somatic embryos (*bar*=1 mm). **F)** Torpedo stage somatic embryo (*bar*=0.5 mm). **G)** Cotyledon- stage somatic embryo (*bar*=0.5 mm). **H)** Cotyledon stage somatic embryo 6 weeks after culture initiation (*bar*=0.5 mm). **I)** Conversion of a somatic embryo on MS_{WH} (*bar*=1 mm). **J)** Plantlet from somatic embryo conversion on MS_{WH}, 10 weeks after induction (*bar*=3 mm)

Discussion

The results reported here showed that regeneration through somatic embryogenesis was obtained in *E. globulus* juvenile explants grown on MS medium supplemented with NAA. The use of NAA to induce somatic embryogenesis has already been reported for other *Eucalyptus* species such as *E. citriodora* (Muralidharan and Mascarenhas 1987, Muralidharan *et al.* 1989) and *E. dunnii* (Termignoni *et al.* 1996). Treatments with 2,4-D (1.0–2.0 mg l^{-1}), either alone or in combination with the cytokinin 6-benzylaminopurine (6-BAP) (0.01–0.5 mg l^{-1}) or zeatin (2 mg l^{-1}), failed to induce somatic embryos. Dicamba (0.5 mg l^{-1}) induced a highly friable callus but only root regeneration was observed. The lack of embryo formation on calli induced by Dicamba has previously been described for this species (Trindade 1996). Regeneration through somatic embryogenesis has been described only for a few species of *Eucalyptus* (*E. citriodora* (Muralidharan and Mascarenhas 1987, Muralidharan *et al.* 1989), *E. dunnii* (Termignoni *et al.* 1996) and *E. grandis* (Watt *et al.* 1991)). More recently somatic embryogenesis was also reported for *E. nitens* (Bandyopadhyay *et al.* 1999, Bandyopadhyay and Hamill 2000) and *E. globulus* (Bandyopadhyay *et al.* 1999, Nugent *et al.* 2001) but plantlet regeneration was not achieved.

One of the main reported problems for establishing embryogenic cultures in *Eucalyptus* species is phenolic accumulation (Nugent *et al.* 2001). In fact, phenolic accumulation was also evident in the embryogenic callus of *E. globulus* after transfer to MS_{WH}. Darkness was reported to decrease phenolic accumulation in *E. citriodora* (Muralidharan *et al.* 1989) however, light is also an important signal for somatic embryogenesis since *E. dunnii* responded positively to light with somatic embryogenesis occurring under a 16-h photoperiod of approximately 40 µmol m^{-2} s^{-1} (Termignoni *et al.* 1996). The accumulation of phenolic compounds is probably one of the causes involved in the low induction frequencies observed in this work. Nevertheless, the induction frequencies that were obtained in this study are higher than those reported previously for the same species (Bandyopadhyay *et al.* 1999, Nugent *et al.* 2001) and highly reproducible for the open-pollination family used. The extrapolation of this methodology to other families and genotypes is underway.

Although casein hydrolysate and glutamine were reported to improve somatic embryogenesis in *E. citriodora* (Muralidharan *et al.* 1989), in our experiments addition of

casein hydrolysate (500 mg l^{-1}) and glutamine (500 mg l^{-1}) did not improve somatic embryo production and a higher number of abnormal somatic embryos were observed.

Somatic embryo development in *E. globulus* was asynchronous and all phases up to the cotyledon phase could be observed simultaneously in the same embryogenic callus, as previously described for *E. citriodora* (Muralidharan *et al.* 1989). In fact, asynchrony and the high frequency of abnormal embryos still hampers the industrial application of this regeneration process for plant production and further studies have to be carried out to overcome these problems.

The data reported demonstrated for the first time the regeneration of somatic embryos from juvenile explants of *E. globulus*. The production of somatic embryos in *E. globulus* opens up a new way to overcome rooting difficulties in traditional cloning techniques, as somatic embryos are bipolar structures carrying both a root and shoot meristem. Effective somatic embryogenesis techniques offer the possibility to mass multiply material that has been genetically improved by breeding and preserve a large number of genotypes in a confined space while they are being field tested (breeding-cloning strategy). Although somatic embryogenesis in eucalypt species is not yet ready to be used commercially, the fact that the process is amenable to automation can mean that it will eventually become cheaper than other clonal propagation techniques in use. In fact, vegetative propagation has an important role in progeny testing since the use of clonal replicates allows the estimation of additive and non-additive genetic variance. Furthermore, the correct ranking of individuals within families is largely improved and therefore the cumulative genetic gain obtained during each cycle of breeding increased (Mullin and Park 1992).

References

Bandyopadhyay S, Hamill J (2000) Ultrastructural studies of somatic embryos of *Eucalyptus nitens* and Comparisons with zygotic embryos found in mature seeds. Ann Bot 86:237– 244

Bandyopadhyay S, Cane K, Rasmussen G, Hamill J (1999) Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species- *Eucalyptus nitens* and *E. globulus*. Plant Sci 140(2):189–198

Direcção Geral de Florestas (2001) Inventário Florestal Nacional. 3rd edn. Lisbon pp 3–162

Eldridge K, Davidson J, Harwood C, Van Wyk G (1993) Eucalypt domestication and breeding. Clarendon Press, Oxford

Kellison R (2001) Present and future use of eucalypts wood in the world. In: Developing the eucalypt of the future. IUFRO International Symposium, Valdívia, Chile. pp 10–15

Marques CM, Vasquez-Kool J, Carocha V, Ferreira JC, O'Malley DM, Liu BH, Sederoff R (1999) Genetic dissection of vegetative propagation traits in *Eucalyptus tereticornis* and *E. globulus*. Theor Appl Genet 99:936–946

Mullin TJ, Park YS (1992) Estimating genetic gains from alternative breeding strategies for clonal forestry. Can J For Res 21:14–23

Muralidharan EM, Mascarenhas AF (1987) In vitro plantlet formation by organogenesis in *E. camaldulensis* and by somatic embryogenesis in *Eucalyptus citriodora*. Plant Cell Rep 6:256–259

Muralidharan EM, Mascarenhas AF (1995) Somatic embryogenesis in *Eucalyptus*. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants, vol. 2. Kluwer, Dordrecht, pp 23–40

Muralidharan EM, Gupta PK, Mascarenhas AF (1989) Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus citridora.* Plant Cell Rep 8:41–43

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Nugent G, Chandler S, Whiteman P, Stevenson T (2001) Somatic embryogenesis in *Eucalyptus globulus* Plant Cell Tiss Organ Cult 67:85–88

Serrano L, Rochange F, Semblat JP, Marque C, Teulieres C, Boudet AM (1996) Genetic transformation of *Eucalyptus globulus* through biobalistics: complementary development of procedures for organogenesis from zygotic embryos and stable transformation of corresponding proliferating tissue. J Exp Bot 47:285–290

Termignoni R, Wang PJ, Hu CY (1996) Somatic embryo induction in *Eucalyptus dunnii*. Plant Cell Tiss Organ Cult 45:129– 132

Trindade H (1996) *Eucalyptus globulus* Labill: systems for in vitro regeneration, PhD thesis. University of Lisbon, Lisbon

Watt MP, Blakeway F, Cresswell CF, Harman B (1991) Somatic embryogenesis in *Eucalyptus grandis*. S Afr Bosbout 157:59–65

Watt MP, Blakeway FC, Termignoni R, Jain SM (1999) Somatic embryogenesis in *Eucalyptus grandis* and *E. dunnii*. In:Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 5. Kluwer, UK, pp 63–78

Zobel BJ (1993) Clonal forestry in the *Eucalyptus*. In: Ahuja MR, Libby WJ (eds) Clonal forestry. II. Conservation and application. Springer, Berlin Heidelberg New York, pp 139–148

II 2: Factors influencing somatic embryogenesis induction in *Eucalyptus globulus* Labill.: basal medium and antioxidants

Chapter submitted as an original paper to a SCI journal:

Pinto G, Silva S, Araújo C, Neves L, Park Y-S, Santos C (2007) Factors influencing somatic embryogenesis induction in *Eucalyptus globulus* Labill.: basal medium and antioxidants. Plant Cell Tissue and Organ Culture (submitted)

Abstract

In vitro morphogenesis, and in particular somatic embryogenesis (SE), is highly correlated with mineral composition of the media used during the induction and expression stages. *Eucalyptus globulus* SE process is already well defined, but low rates of induction still hamper its scale up for commercialization. As for other woody species, most published protocols for SE induction/expression in *Eucalyptus* genus are based on one or two predefined media such as B5 and MS. In this paper we analyzed the effectiveness of several media (MS, ½ MS, B5, WPM, DKW and JADS) during the early stages of the SE process: induction and expression. MS, followed by B5 and ½ MS, confirmed to be the best medium for SE induction. In general, MS was the best medium for expression, independently of the medium previously used during induction, showing that mineral composition during this second SE stage conditions important signaling processes overcoming putative deficient induction conditions.

Tissue oxidation/browning during the expression phase is a recurrent problem in this species. Several antioxidants (ascorbic acid, charcoal, DTE, DTT, PVP, PVPP and silver nitrate) were added to the expression medium (MS) in order to reduce phenolisation and thus improve the SE process. All antioxidants reduced the SE potential and only DTE, charcoal and silver nitrate reduced explant oxidation. When added only to the induction period, antioxidants reduced phenolization but also severely reduced SE potential and continuous exposure completely inhibited the SE response. The negative impact of antioxidants on SE potential raises a question on the role of phenolization on the SE signaling process, and suggests that other alternative strategies must be found to overcome this phenomenon during *Eucalyptus* SE process.

Keywords: *Eucalyptus*, medium composition, phenolization, primary somatic embryos, woody species

Introduction

The tremendous potential of *Eucalyptus* species to produce timber and fiber for pulp and paper industries led to the rising commercial dominance of this genus that comprises more than 700 species. With such an economical value this genus became an obvious target for in vitro propagation and genetic manipulation (Merkle and Nairn 2005, Poke *et al.* 2005). Somatic embryogenesis (SE) offers several advantages, including the simultaneous development of two meristems and the possibility to mass multiply material that has been genetically improved by breeding (Park *et al.* 1993). Other important advantage of SE is that embryogenic cultures may be maintained through cryopreservation while clonal lines are tested in the field (Park *et al.* 1998). In fact, the protocol for *Eucalyptus globulus* SE recently reported by Pinto *et al.* (2002, 2004) opens perspectives to use this technique in *Eucalyptus* breeding programs if the induction rates are acceptable for a large number of genotypes (MacKay *et al.* 2006).

. A better understanding of the factors controlling SE induction/expression, and how they condition SE response, will help increase the efficiency of protocols. It is well known that, in general, in vitro development of cells and tissues depends on the combined interaction of internal factors (e.g. genotype, age, type of explant, physiological conditions) and external ones (e.g. culture medium composition, growth regulators, light, temperature, presence of antioxidants). The importance of plant growth regulators (PGR) in the SE process is well documented for several species in the last decades (Jimenez 2005), while little attention was given to the medium composition and to phenolic compounds. However, the success of in vitro tissue culture is strongly dependent on the chemical composition of the culture medium (Ružić *et al.* 2004). In fact, the selection of the best induction/expression medium is particularly important when dealing with recalcitrant species as is the case for *Eucalyptus*.

In the *Eucalyptus* genus, somatic embryogenesis was described for *E. citriodora* (Muralidharan and Mascarenhas 1987; Muralidharan *et al.* 1989) using B5 medium, *E. dunnii* (Termignoni *et al.* 1996; Watt *et al.* 1999) with B5 and MS media, *E. grandis* (Watt *et al.* 1991, 1999) using MS medium and for *E. nitens* (Bandyopadhyay *et al.* 1999) using MS medium. Nugent *et al.* (2001) and Pinto *et al.* (2002, 2004) induced SE in *E. globulus* using MS medium. More recently, Prakash and Gurumurthi (2005) reported somatic embryogenesis in *Eucalyptus tereticornis* using MS and B5 media.

Although most media formulations have been developed by a time consuming process of trial and error, it is possible to decrease this empirical approach. In fact, the mineral content of MS medium is based on an analysis of the ash of incenerated tobacco tissue (see Bonga and von Aderkas 1992). More recent studies with other species such *Cydonia oblonga* (Fisichela *et al.* 2000) also compared the macronutrient composition on SE production. Other authors performed (Ruggini *et al.* 1984) or suggested (e.g. Nas and Read 2004) a comparison between the analyses of mineral elements in the explants (e.g. zygotic embryos) in order to find a possible correlation between the mineral content of tissue and the media protocols used in micropropagation. Unfortunately, few works have addressed this area, and only one is reported in the *Eucalyptus* genus for the hybrid *E. uropphylla* x *E. grandis* (Gribble *et al.* 2002).

Tissue death caused by browning (phenolic oxidation), and the accumulation of phenolic compounds during the SE process was often reported, and some authors suggested that the accumulation of phenolics may be one of the causes of vitro recalcitrance (e.g. Pinto *et al.* 2002, Alemanno *et al.* 2003). However, little attention was given to prevent deleterious effects of phenols on SE process using antioxidants (Malabadi and Staden 2005). *Eucalyptus* is a rich source of phenolic constituents (Close *et al.* 2001) and the oxidation of polyphenols may become a limiting factor that prevents proper tissue multiplication and maintenance.

This work focused on the conditions affecting the induction of somatic embryogenesis in mature zygotic embryos of *Eucalyptus globulus* Labill. The role of media composition and the presence of antioxidants in the SE induction and expression stages are studied. By comparing several basal media during the induction process, we were able to select the best culture medium for SE induction and, improve the process beyond these two stages, i.e., germination and conversion stages.

Materials and Methods

Plant material and disinfection: Half- sib seeds of *Eucalyptus globulus* ssp. *globulus* Labill. produced in 2002 from the breeding program of Celbi, Leirosa, Portugal were used. Seeds were surface-sterilized with a mixture of 1:1 absolute ethanol and 30% of hydrogen peroxide (v/v) for 15 min, washed twice in sterile distilled water (10 min), and

then rinsed with 0.1% (w/v) Benlate (Benomyl, Rhône-Poulenc) for 15 min. Seeds were then left to imbibe over night in sterile distilled water until processing.

Experiment 1 - Culture media effect

To assess the influence of the basal medium during SE induction and expression stages, cultures were initiated using entire mature zygotic embryo explants after seedcoat had been removed. Six groups of 100 explnats (10 embryos per Petri dish) were inoculated and cultured for three weeks, in one of the following induction media:

MS (Murashige and Skoog 1962),
1/2MS (half strength MS medium),
B5 (Gamborg medium; Gamborg *et al.* 1968),
DKW (Driver Kuniyuki Walnut medium; Driver and Kuniyuki 1984),
WPM (Woody Plant Medium; Lloyd and Mc-Cown 1980),
JADS (Correia *et al.* 1995).

For a comparison of ion and element levels in the various media used to assess somatic embryogenic potential in *E. globulus* see Table 1. All media were supplemented with 3 mg l^{-1} NAA (16.1 µM, α-naphthalene acetic acid, 3NAA).

Table 1: Comparison of ion and element levels in the various media used to assess somatic embryogenic potential in *E globulus*.

Macro and Microelement combinations (mM)

Ions	**MS**	**B5**	**DKW**	**WPM**	**JADS**
NO_3^-	39.31000	24.73000	34.20000	9.70000	22.00000
$H_2PO_4^-$	1.25000	1.09000	1.95000	1.24000	3.00000
SO_4^{2-}	1.62000	2.08600	12.22000	7.30000	0.30000
Cl^-	5.98020	2.04000	2.02000	1.30000	0.00022
K^+	19.95500	24.73452	19.85000	12.61500	11.00000
Ca^{2+}	2.99900	1.02000	9.31000	3.00000	5.00000
Na^+	0.10200	1.19000	0.12320	0.10200	0.40124
Mg^{2+}	1.50000	1.01000	3.00000	1.50000	3.00000
NH^{4+}	20.61000	2.02000	17.60000	5.00000	4.00000
Total N	59.92000	26.75000	51.80000	14.70000	26.00000
NH^{4+} / NO_3^-	0.52420	0.08160	0.51460	0.51500	0.18000

After the induction period, explants were transferred every four weeks during three months to expression conditions: one group of 50 explants from each medium was transferred to MS without growth regulators (MS_{WH}). As a control, another group of 50 explants from each induction medium was transferred to the same basal medium used during induction (i.e., MS, 1/2MS, B5, DKW, WPM, JADS) but without growth regulators, for expression.

Both induction and expression stages took place in the dark at 24±1ºC in 90 mm diameter petri dishes (Sarstedt, Germany).

Media preparation: All media were supplemented with 30 g l^{-1} sucrose and 2.5g l^{-1} gelrite® (Duchefa, Netherlands), pH was adjusted to 5.8 and the media were autoclaved at 121ºC for 20 min. The JADS medium was made using stock solutions of chemicals purchased to Sigma (USA) and the MS vitamins from Duchefa (Haarlem, Netherlands). All other culture media, sucrose, gelrite® and NAA (α-naphthalene acetic acid) were purchased to Duchefa (Haarlem, Netherlands).

Analyses of explant responses: Each parameter observation was performed with a minimum of 50 explants for each treatment. Explants were examined after the induction period (3 weeks) and every month after transfer to expression medium. *Embryogenic potential* was analyzed in a magnifying lens (Olympus SZ60) and was expressed as the percentage of explants showing somatic embryos after 4, 8 and 12 weeks.

After 12 weeks on expression conditions, explants were scored for callus, phenolization, and somatic embryo production. *Embryogenic response* was expressed as percentage of induction. Embryogenic structures were characterized as the total number of globular or cotyledonary somatic embryos and the total number of somatic plantlets. For callus production and explant browning, five different ranks were established: rank 0: 0%; rank 1: 0-25%; rank 2: 25-75%; rank 3: 75-100%; rank 4: 100%. Callus production and phenolization were expressed as the total number of explants classified into each rank.

Experiment 2 – Antioxidant effects

Due to the high phenolization that recurrently occurs during the SE expression stage, we assessed the influence of antioxidants on the phenolization and on the SE capacity. In a

first assay (*Condition 1*), explants were induced for three weeks in the dark in MS supplemented with 3 mg l^{-1} NAA and then transferred to MS_{WH} supplemented with different antioxidants in the dark for 5 weeks, for expression . The following antioxidants were used: ascorbic acid (100 mg l^{-1}), activated charcoal (0.01% w/v), dithiothreitol (DTT, 0.5 mg l^{-1}), dithioerythritol (DTE, 0.5 mg l^{-1}), polivinylpirrolidone (PVP, 1% w/v), polivinylpolypirrolidone (PVPP, 1%, w/v) and silver nitrate (10 mg l^{-1}) separately.

The antioxidants that gave better results during expression were then selected for another assay (*Condition 2*): explants were exposed during induction to MS supplemented with one of the antioxidants, and then transferred to MS_{WH} devoid of antioxidants, for expression. As a control, explants were grown during induction and expression on MS medium in absence of the antioxidant. Explants were also grown during induction and expression in the continuous presence of the antioxidant (*Condition* 3). DTE (0.5 mg l^{-1}), activated charcoal 0.01% (w/v), 0.025% (w/v) and 0.25% (w/v) and silver nitrate (10 mg l^{-1}) were added, separately, to the induction medium MS supplemented with 3 mg l^{-1} NAA.

Each experiment was performed with a minimum of 30 explants for each treatment. Data was taken five weeks after transfer to expression medium. The phenolization (expressed as total of explants classified into each rank, as describe above) and the embryogenic potential (expressed as the percentage of explants showing somatic embryos) were assessed in these explants).

All treatments were incubated in the dark at 24±1ºC. All media were supplemented with 30g l^{-1} sucrose and 2.5g l^{-1} gelrite, pH was adjusted to 5.8 and media were autoclaved at 121ºC for 20 min. Antioxidants were purchased from Sigma (USA).

Statistical analyses

After 12 weeks in culture, combined media effects of six media and two conditions (one experiment and one control) were analysed for SE expression using ANOVA procedure (SAS Institute). A Duncan´s test was applied to determine which groups were different.

Results

High rates of germination (>90%) of entire mature zygotic embryos were observed in all media tested. After two weeks on induction media, the germination process stopped and callus production started, mainly in cotyledons. After three weeks (immediately before

transferring to expression media), a combination of whitish-friable (Plate 1.A) and whitish-compact (Plate 1.B) calluses could be observed in all media tested and no phenolization occurred (Plate 1.C).

Experiment 1 - Culture media effect

Response after 4 weeks: Concerning the expression stage, four weeks after transfer to MS_{WH} medium, embryogenic structures were observed in explants previously grown on JADS, B5, MS and ½ MS , with SE response rates of, respectively, 2.0%, 6.0%, 6.0% and 8.0% (Figure 1a).

In the control experiment (explants transferred, respectively, to $JADS_{WH}$, $B5_{WH}$, MS_{WH}, ½ MS_{WH}, WPM_{WH} and DKW_{WH}) somatic embryos formed only in MS_{WH} (6.0%) followed by $JADS_{WH}$ (4.0%). By this time, the first symptoms of phenolization could already be observed in all conditions, after this period (Plate 1.D).

Response after 8 weeks: Explants previously induced on DKW and WPM presented a low percentage of embryogenic structures (2.0%) when transferred to MS_{WH}. Higher rates of SE expression were obtained for explants induced on MS (20.0%), B5 (16.0%), ½ MS (14.0%) and JADS (12.0%) (Figure 1a).

The highest frequencies of SE were observed, respectively, in MS-induced explants transferred to MS_{WH} (20.0%) and in B5-induced explants transferred to $B5_{WH}$ (16.0%) (Figure 1b). At this time, it was evident that there was higher SE potential in explants transferred to MS_{WH}, when compared to those explants transferred to other expression media. The number of newly formed roots increased dramatically during this period, together with an increase of phenolization (Plate 1.E).

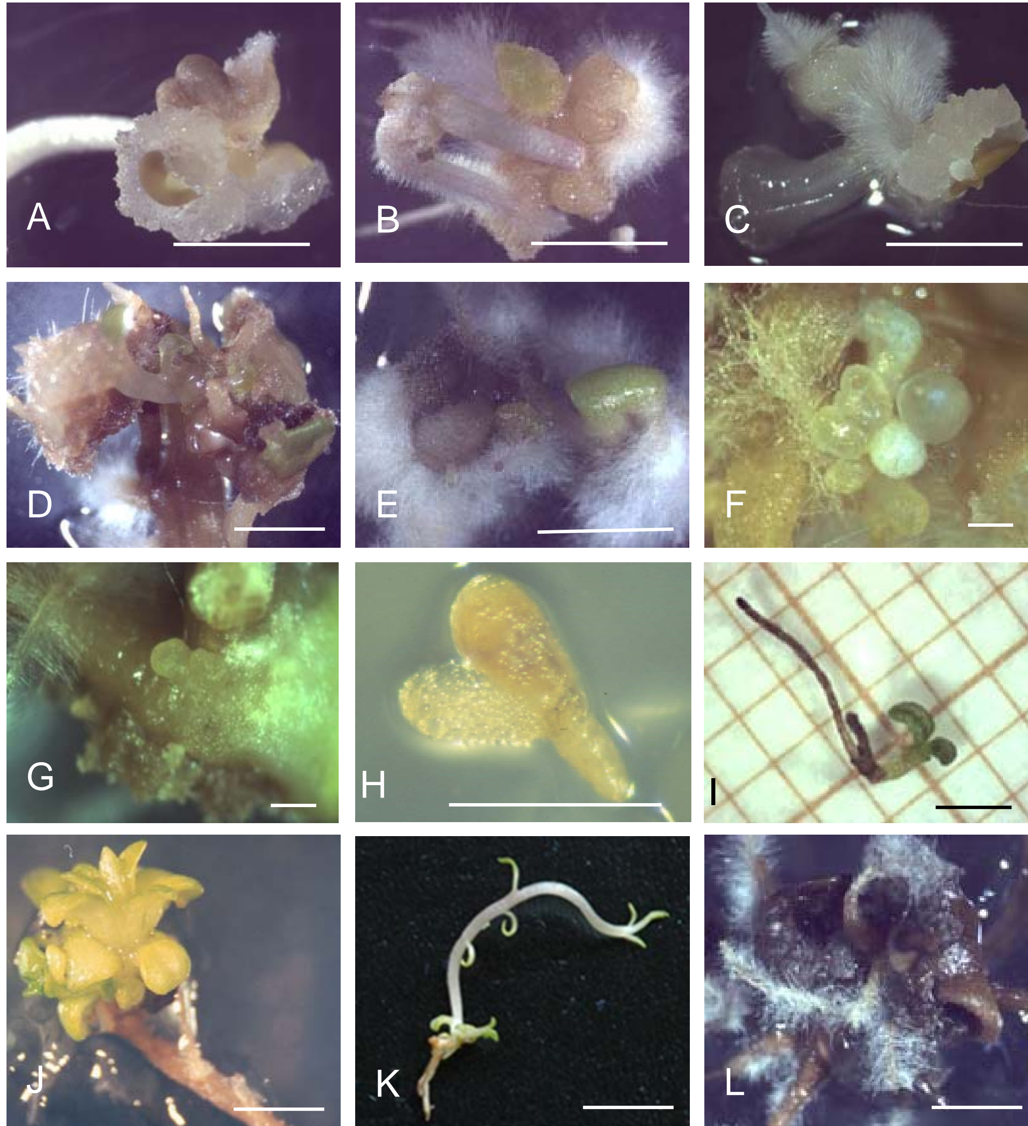


Plate 1 Different aspects of *E. globulus* SE induction and expression stages: **A**) Friable callus (developed for three weeks on JADS induction medium) (*bar*=0.6cm). **B**) Compact callus (developed for three weeks on JADS) (*bar*=1cm). **C**) Callus with no phenolization (after three weeks of induction, developed on MS) (*bar*=0.6cm). **D**) First signals of phenolization in a callus four weeks after transfer to expression medium (induction on MS, and expression on MS_{WH}) (*bar*= 1cm). **E**) Browning increase in eight-weeks-old callus (grown on ½ MS_{WH}) and abundant rooting (*bar*= 1cm). **F** and **G**) Globular somatic embryos after eight weeks on expression medium on MS_{WH} medium (*bars*= 1mm). **H**) Cotyledonar somatic embryo, twelve weeks after expression (*bar*= 1mm). **I**) Somatic embryo germination (*bar*=1mm). **J**) Plantlet conversion (*bar*= 1cm). **K**) Plantlet conversion (*bar*= 0.5cm). **L**) Example of an explant showing generalised phenolization after twelve weeks on MS medium (*bar*= 0.8cm).

Response after 12 weeks: Analysis of variance of SE after 12 weeks of culture indicated that there were significant differences among the media but the difference between the experiment and control was not significantly different (Table 2). Mean initiation percentages among media ranged from 2 to 30% with MS being the best (Table 3).

Figure 1. Somatic embryogenic response during 12 weeks on expression media: **a**) SE response of explants induced on different media and transferred to MSWH for expression; **b**) SE response of explants induced on different media and transferred to the same medium devoided of growth regulators (control). Explants were subcultured every month to fresh medium. (values are means and verticals bars are standard error).

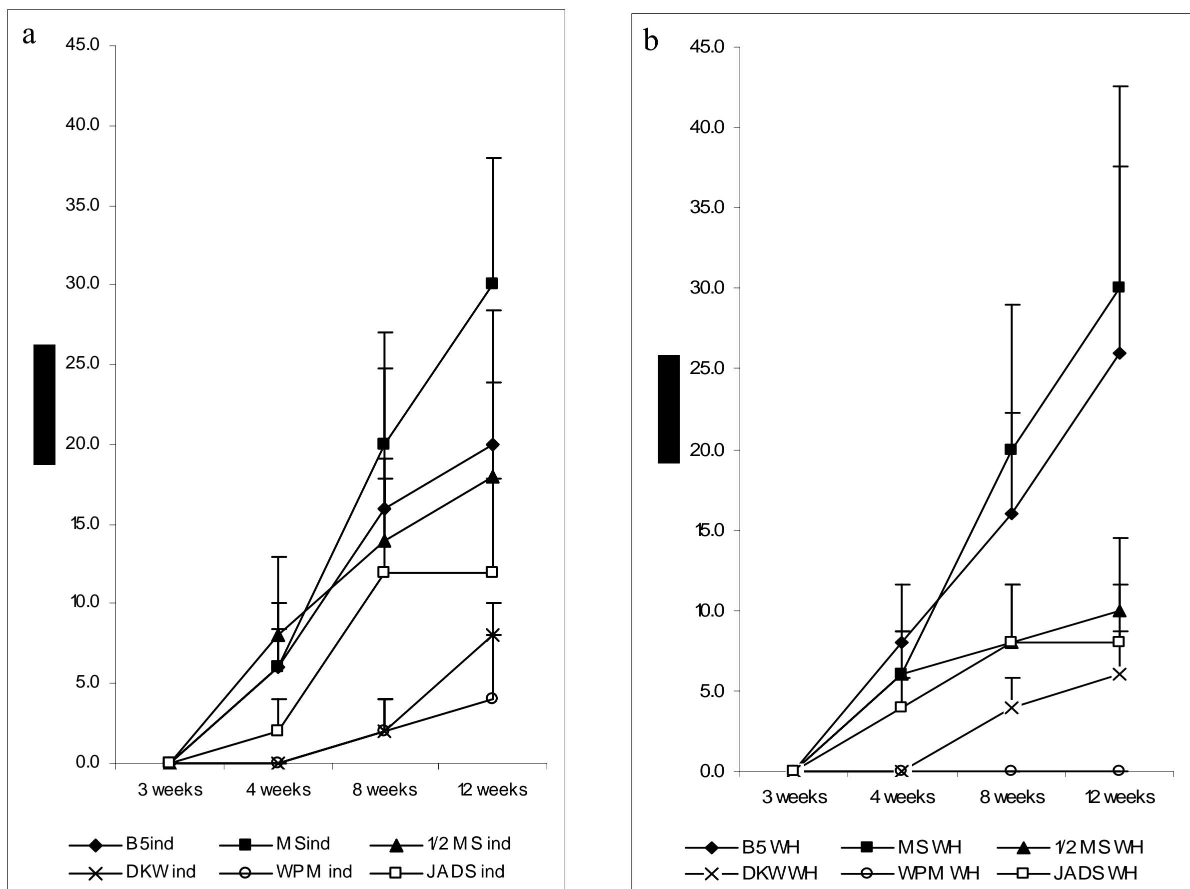


Table 2. Analysis of variance for media and conditions within each medium for SE initiation after 12 weeks.

Source	df	Maen Squares	F-ratio	P-value
Media	5	1091.17	6.83	0.0000
Condition	6	56.40	0.35	0.9047
Error	48	159.76		

Table 3. Effects of media and conditions within each medium on initiation (%) of SE after 12 weeks. Means followed by the same letter are not significantly different at P≤0.05 according to Duncan´s test

	Media					
Conditions	MS	B5	½ MS	JADS	WPM	DKW
MS_{WH}	30.0	20.2	20.0	12.0	4.0	8.4
Control	30.0	26.0	12.0	8.0	0.0	6.0
Mean	30.0a	23.1a	16.0bc	10.0cd	2.0d	7.2cd

After 12 weeks expression on MS_{WH} medium, the SE expression of JADS-induced explants stabilized, while the proliferation of undifferentiated friable tissue increased. In all other treatments, an increase of SE expression occurred. However, the lowest SE induction rates were observed in explants previously grown on DKW (8 % of explants showed SE, with a total of 6 somatic embryos) and on WPM (4 %, a total of 16 somatic embryos) (Table 4a). The higher SE rates were observed for explants previously grown on MS (30 %, a total of 67 somatic embryos), B5 (20 %, a total of 45 somatic embryos), ½ MS (18%, a total of 29 somatic embryos) and JADS (12%, a total of 32 somatic embryos).

Assynchronism was observed for explants previously grown on MS, ½ MS, B5 and JADS, as globular and cotyledonar somatic embryos coexisted, although the first ones were the most abundant (Plate 1.F-G). This was particularly evident for MS-induced explants (Table 4a), which also showed the highest number of cotyledonary embryos (Plate1.H-I) and in some of these explants plantlets could be observed (Plate 1.J-K). Most of the globular embryos remained at this stage and did not develop further. Cotyledonary somatic embryos were generally small (2 mm long on average) independent of the treatments and had with two cotyledons with both root and shoot poles.

Rhizogenesis occurred in all media, and the roots formed were in general fragile. Long hairy roots were particularly abundant on explants induced on ½ MS (Plate 1.L), making somatic embryos difficult to quantify and isolate. This suggests that the induction medium plays also a central role in rhizogenesis signaling.

Table 4 Characterization of embryogenic response in *Eucalyptus globulus*. **a)** Characterization of embryogenic response after 12 weeks on expression medium (MS_{WH}) after induction on the six media tested. **b)** Characterization of embryogenic response after 12 weeks on expression medium under control conditions (induction and expression in the same medium).(G- globular, C- cotiledonar, P- plant). For callus production and explant browning, five different ranks were established: rank ≤ 1: 0-25% rank 2: 25 -75%; rank 3: 75-100%; rank 4: 100%. Callus production and phenolization were expressed as the total number of explants classified into each rank.

(a)		% of Induction (n=50)			Total Nº. Somatic embryos	Somatic embryo type			Phenolization				Callus production		
Induction medium	Expression medium	**4weeks**	**8weeks**	**12weeks**		**G**	**C**	**P**	**≤1**	**2**	**3**	**4**	**≤1**	**2**	**≥3**
MS	MS_{WH}	6	20	30	67	39	28	8	0	8	26	9	13	26	4
½ MS	MS_{WH}	8	14	20	29	25	4	0	0	8	14	28	48	2	0
B5	MS_{WH}	6	16	20	45	39	6	0	0	25	22	0	49	0	0
DKW	MS_{WH}	0	2	8	6	6	0	0	1	27	18	2	38	8	2
WPM	MS_{WH}	0	2	4	16	16	0	0	3	27	9	7	3	44	0
JADS	MS_{WH}	2	12	12	32	30	2	0	1	24	21	1	27	18	2

(b)		% of Induction (n=50)			Total Nº. Somatic embryos	Somatic embryo type			Phenolization				Callus production		
Induction medium	Expression medium	**4weeks**	**8weeks**	**12weeks**		**G**	**C**	**P**	**≤1**	**2**	**≥3**	**4**	**≤1**	**2**	**≥3**
MS	MS_{WH}	6	20	30	67	39	28	8	0	8	26	9	13	26	4
½ MS	½ MS_{WH}	6	8	12	8	3	2	3	0	3	27	17	2	43	2
B5	$B5_{WH}$	8	16	26	44	22	20	2	15	30	3	0	15	30	3
DKW	DKW_{WH}	0	4	6	6	0	5	1	8	39	1	0	6	40	1
WPM	WPM_{WH}	0	0	0	0	0	0	0	0	18	24	0	8	39	1
JADS	$JADS_{WH}$	4	8	8	6	3	2	1	4	17	15	10	21	24	1

In the explants used as control (induction and expression on the same medium) SE potential ranged from 0.0 % (on WPM_{WH}) to 30.0% (on MS_{WH}) medium (Table 4b). All explants induced on ½ MS, DKW, WPM and JADS showed a lower SE potential, and produced a lower number of somatic embryos when transferred to the same medium devoid of growth regulators (control), then when transferred to MS_{WH}. These results showed that this medium had a positive influence in SE expression.

When both induction and expression occurred in the same medium, somatic embryos showed a more marked assynchronism than those transferred to MS_{WH} only for expression (Tables 4a and b). Conversion of somatic embryos to plantlets was observed in

all media except in WPM. Nonetheless, higher rates of cotyledonary embryos and plantlets were obtained in MS_{WH}.

Phenolization increased along the expression period and it was in general more abundant when ½ MS_{WH} and MS_{WH} (the most SE-responsive medium) were present on the expression stage (Table 4a). This fact leads to an interesting question of studying if the addition of antioxidants may reduce phenolization and simultaneously improve the quality of the SE process. To answer this question a second experiment was performed testing the effects of antioxidants during induction and/or expression stage(s), for phenolization and SE response.

Experiment 2: Effect of antioxidants on the embryogenic response

During the induction period (3 weeks) little or no oxidation was observed in the zygotic embryo explants. The first symptoms of oxidation occurred, for all media, mostly after explants were transferred to new media (MS_{WH}) and increased thereafter.

Table 5: Embryogenic potential and antioxidant effects after 5 weeks in expression medium for each condition. Rank ≥ 3correspond to: 75-100% of explant browning.

	Condition 1 *antioxidants in expression medium*		***Condition 2*** *antioxidants in induction medium*		***Condition 3*** *Continuos exposure to antioxidants*	
	% induction	% explants in Rank ≥ 3	% induction	% explants in Rank ≥ 3	% induction	% explants in Rank ≥ 3
No antioxidants (**control**)	20.0	73.3	-	-	-	-
PVP	10.0	90.0	-	-	-	-
PVPP	10.0	83.3	-	-	-	-
DTT	10.0	80.0	-	-	-	-
Ascorbic acid	6.7	83.3	-	-	-	-
A. charcoal (0.01%)	13.3	73.3	0.0	3.3	0.0	20
DTE	10.0	70.0	14.3	60.0	0.0	13.3
Silver nitrate	6.7	66.6	4.8	30.0	0.0	16.6
A. charcoal (0.0 25%)	-	-	0.0	10.0	0.0	33.3
A. charcoal (0.25%)	-	-	0.0	16.6	0.0	20.0

The addition of DTE and silver nitrate to the expression medium reduced explant oxidation, when compared to the control (explants without antioxidants) (Table 5). However, these two antioxidants affected negatively SE potential (respectively, 6.7% and 10.0% of explants showed SE) when compared to the explants growing in the absence of antioxidants (control, with 20% of SE induction rate). The addition of charcoal led to the elongation of the explant and little production of callus and showed the same degree of phenolization as control. On the other hand, DTT, PVP, PVPP and ascorbic acid did not reduce oxidation and had negative effects on the SE induction rate (Table 5).

The few antioxidants that reduced or did not affect oxidation with respect to control in *Condition 1* (DTE, silver nitrate and charcoal) were used in *Condition 2* (during induction period) to assess the impact of antioxidant addition in this first stage of SE. The concentration of charcoal was increased (up to 0.25%) to evaluate if higher concentrations of this antioxidant were more efficient in reducing oxidation. Silver nitrate and charcoal (in concentrations higher than 0.01%) when added to induction media (*condition 2*) decreased phenolization as previously observed in *condition 1* (Table 5). Despite this decrease of phenolization, SE potential was also severely repressed. Contrarily, DTE reduced phenolization and had a slight increase on SE potential, respectively to its use in *condition 1* (expression media). Nevertheless, the SE potential in the presence of antioxidants was always lower than SE potential in control.

In the absence of antioxidants, explants showed higher oxidation than those exposed continuously to the antioxidants, but continuous exposure to antioxidants completely inhibited the SE response (Table 5).

Discussion

Culture medium effect

The best SE induction rates were obtained with MS medium, followed by B5. Prakash and Gurumurthi (2005) who found that callus induction and SE were higher on MS medium compared to B5 reported similar results. Muralidharan *et al.* (1989) succeeded *Eucalyptus citriodora* plant regeneration through high frequency SE, using B5 medium. More recently, Pinto *et al.* (2002) reported a protocol for SE-plant regeneration using MS. From literature, it can therefore be concluded that all the protocols reporting plant regeneration by SE in

Eucalyptus genus used B5 (e.g. Muralidharan *et al.* 1989) or MS media (e.g. Pinto *et al.* 2002, 2004, Prakash and Gurumurthi 2005) with no preliminary validation of this choice

The importance of tissue culture mineral nutrients has been demonstrated previously (e.g. Fisichella *et al.* 2000, Ramage and Williams 2002, Chauhan and Kothari 2004, Gaj 2004) to affect morphogenesis. Therefore, it is crucial to ensure the adequacy of the culture medium, when one intends to develop protocols for large scale plant production by somatic embryogenesis. Considering the low induction rates observed for primary SE together with the need to carefully control all SE steps, we compared several media (MS, 1/2MS, B5, WPM, DKW and JADS) on both induction and expression steps. In this study, MS was used because this medium was already used with success in this species, leading to conversion of somatic embryos to plantlets (Pinto *et al.* 2002, 2004) and because it is the most popular medium for hardwood tree species, and in particular for *Eucalyptus* genus (e.g. see Prakash and Gurumurthi 2005). However, MS is often reported as a very rich and saline medium and can be too salty to some species. For this reason, we reduced the macronutrient concentration and used half strength MS medium (1/2MS). WPM and DKW were developed for micropropagation of, respectively, mountain laurel and *Juglans hindsii* x *J. regia* and are used in morphogenesis and SE of woody species such as chestnut (Carraway and Merkle 1997) and *Juglans nigra* (Steger and Preece 2003). B5 medium is also largely used in *Eucalyptus* SE; for example, it was already tested with success in SE of *E. tereticornis* (Prakash and Gurumurthi 2005). JADS medium was formulated for micropropagation of *E. grandis* x *E. urophilla* (Correia *et al.* 1995). Except for WPM, plant regeneration was achieved when the same basal medium was used for both induction and expression with best results to MS followed by B5. Besides, no statistical differences were found between MS and B5, MS gave best results concerning total number of cotyledonary somatic embryos and plant conversion.

Transferring explants previously grown on several media during induction to MS_{WH} for expression recovered the SE-potential of explants. These SE potential recovering strongly suggest that salt composition conditions SE, in particular during the expression. Our data also suggest that signalling pathways induced by saline media during the first weeks may be reversed during expression.

Further investigation to clarify these results must be encouraged. In fact, a comparison between mineral composition in each medium does not lead to any definitive

conclusion (Table 1). Also, the minimal differences in the micronutrient and vitamins among the media may not be enough to explain differences in SE induction among the media.

Concerning macronutrients, DKW has high levels of sulphate and calcium, supporting a putative toxicity that must, however be investigated (Table 1). For nitrogen, while WPM has the lowest levels of this nutrient (suggesting that the lack of response may be due to deficiency of N), MS has the highest levels of nitrate. The ranges of "optimal" NO_3^-/ NH_4^+ ratios for SE seem highly dependent on the species, and several studies found that a proper ratio stimulates morphogenesis and embryogenesis (Ramage and Williams 2002). For example, some species such as carrot (Tazawa and Reinert 1969) or *Medicago sativa* (Meijer and Brown 1987) require certain levels of ammonium for SE to occur, while other species showed SE with nitrate as the only inorganic nitrogen source. Besides indirect effects induced by different NO_3^-/ NH_4^+ levels must not be excluded as it was shown for tobacco that NO_3^-/ NH_4^+ ratio strongly affect pH changes during culture influencing absorption of other cations and affecting morphogenesis (Cousson and Tran Thanh Van 1993). Besides, the most conditioning factor in these media may not be the absolute concentrations of the elements, but their proportion in each medium. When analyzing the mineral composition of somatic and zygotic embryos and some culture media, Pinto *et al.* (2006) found that MS, ½ MS, JADS, B5, WPM and DKW media had highly different mineral compositions and ion proportion, compared to both zygotic and secondary somatic embryos while the MS mineral proportion was the most similar to tissue mineral content. The same authors also found different levels of most elements between zygotic and somatic embryos (e.g. zygotic embryos had lower levels of K), suggesting that adjustments of induction/expression media based on tissue mineral composition could enhance development and subsequent plant yield (Pinto *et al.* 2006). McCown and Sellmer (1987) stressed that some of the factors conditioning the medium adequacy for in vitro response of a particular species/explant are ionic strength, total nitrogen, ammonium/nitrate ratio, calcium levels and chloride sensivity. Also, earlier experiments in tissue culture showed the importance of nitrogen, potassiun, calcium, phosphorus, magnesium and sulfur (George and Sherrington 1988 see Bonga and von Aderkas 1992).

Despite these general effects of macronutrients in SE process, predicting their specific role and importance in SE is highly complex, and extrapolation for a specific

species, such as *E. globulus* must be regarded carefully, interfering with the concept of recalcitrance (Benson 2000) traditionally associated with this species. For example, in *Cocos nucifera,* $Ca^{2+,}$ $NH_4^{+,}$ and Mg^{2+}, were correlated with somatic embryogenesis induction while other nutrients such as nitrate, sulphate, phosphate, cloride and potassium were not distinguishable between SE-callus and undifferentiated callus (Magnaval *et al.* 1997).

The best SE results observed in this report with MS and B5 for *E. globulus* confirm the empiric reports described for the *Eucalyptus* genus, where only these media were used to induce SE (Muralidharan and Mascarenhas 1987; Muralidharan *et al*. 1989, Termignoni *et al*.1996, Watt *et al*. 1991, 1999, Bandyopadhyay *et al*. 1999, Nugent *et al*. 2001, Pinto *et al*. 2002, 2004, Prakash and Gurumurthi 2005).

Effect of antioxidants on the embryogenic response

We showed that addition of antioxidants to the medium reduced SE potential. Despite being often regarded as a negative phenomenon, the influence of phenolisation during the early stages of SE must be re-evaluated carefully, as in some woody species phenolization often precedes somatic embryo formation. Canhoto *et al.* (1999), working with a Myrtaceae species, suggested a possible correlation between phenolization and SE development. The enrichment of phenolics containing cells, mostly those surrounding the embryos, suggest that phenolics may, in some way, provide a barrier for somatic embryos isolation.

Browning of cultures is often a problem during morphogenesis, and this phenomenon can occur in response to excision or later in culture. Explant browning and its subsequent necrosis, generally attributed to phenolic compounds, is a major unsolved problem in the initiation of tissue cultures, especially for woody plants (Thomas and Ravindra 1997). It is generally accepted that this phenomena leads to a progressive decline in culture competence with eventual loss of totipotency (Benson 2000). This approach led to an investment of several groups (e.g. Pan and Staden 1998, Anthony *et al.* 2004, Malabadi and Staden 2005) on the effect of antioxidants in the morphogenic process. In *Musa* ssp. several methods were tested for alleviating shoot necrosis, including shortening the culture period, altering the media salt strength, use of various plant growth regulators, different levels of sucrose, fructose, silver nitrate, and increasing the concentration of

calcium chloride. Only the addition of calcium chloride proved effective in reducing shoot necrosis (Martin *et al.* 2007). As we reported above, the first signals of oxidation and tissue browning started after transfer the explant to the expression medium and expanded over time. In in vitro cultures of *Eucalyptus* species the occurrence of oxidation has been well described (e.g. Nugent 2001, Pinto *et al.* 2002). It is however rare the use of antioxidants as a strategy for preventing oxidation. Termignoni *et al.* (1996) report the occurrence of brownish calli in *Eucalyptus grandis.* Gupta *et al.* (1983) also report that the use of 0.25% charcoal in *E. torelliana* and *E. camaldulensis* after an induction period with auxins. The same authors did not report if charcoal affected SE process.

Although the positive effects that adding these kinds of compounds may have on preventing tissue browning and eventual necrosis, one should consider the impact that they may have on the SE potential. Some works on other species suggested that silver nitrate has no negative impact on SE. For example, Santos *et al.* (1997) saw that in soybean the addition of silver nitrate or aminoethoxyvinylglycine had no effect on somatic embryos induction, although the ethylene synthesis is inhibited by silver nitrate. The same authors also concluded that histodifferentiation and embryo conversion were stimulated by this compound. Kong and Yeung (1995) also found that silver nitrate stimulated embryo maturation on white spruce. These data are contradictory to our findings for *Eucalyptus globulus* where silver nitrate negatively affected somatic embryogenesis independent of the SE stage when it was added to culture. Also, in a general way, the use of antioxidants in the induction medium is counterproductive to the SE process in this species. Interestingly, the continuous presence of antioxidants (in both induction and expression media) slightly reduced oxidation but completely inhibited somatic embryo formation. Malabadi and Staden (2005) found that, apart from DTT, pretreatment of explants, or incorporation of antioxidants in the basal nutrient medium, had a negative effect on the initiation of embryogenic cultures, somatic embryo production, and plantlet recovery. The negative effect of high concentrations of activated charcoal in both callus production and SE potential may be due to an excessive adsorption of either auxin and/or nutrients. Though charcoal is commonly used in tissue culture media, it may have either beneficial or harmful effects, especially on organogenesis and embryogenesis (Pan and Staden 1998). Activated charcoal had no beneficial effects on maturation of chicory somatic embryos or on adventitious shoot growth of *Picea abies* (Von Arnold 1982). Besides, charcoal did not

significantly improve somatic embryo yield or germination in larch though later, it improved plantlet development (Von Aderkas *et al.* 2002).

Conclusions

As previously reported for other species (e.g. Park *et al.* 1998), to capture the large genetic gains from *E. globulus* breeding programs, clonal propagation methods by SE must work on a wide range of genotypes producing large numbers of vigorous somatic seedlings. Therefore, all the steps (from induction to acclimatization) of *E. globulus* SE protocol must be studied and controlled in order to optimize the process.

Our results showed that MS, followed by B5, is the best medium for inducing SE in this species. However, these results are insufficient to correlate mineral composition and SE potential in *E. globulus*. Based on these results and those previuosly described by Pinto *et al.* (2006) on the mineral composition of *E. globulus* zygotic embryos, we are presently clarifying the relationship between mineral uptake and *E. globulus* SE, by developing different media based on the adjustment of the mineral composition of MS medium for evaluation of their efficiency in the improvement of *E. globulus* SE induction and expression, and somatic embryos nutrient contents. Also, these results showed that the inclusion of antioxidants in the protocol currently in use for this species have negative effect on SE and therefore their addition is not recommended.

References

Aderkas PV, Label P, Lelu M-A (2002) Charcoal affects early development and hormonal concentrations of somatic embryos of hybrid larch. Tree Physiol 22:431–434

Alemanno L, Ramos T, Gargadenec A, Andary C, Ferriere N (2003) Localization and Identification of Phenolic Compounds in *Theobrama cacao* L. Somatic Embryogenesis. Ann Bot 92:613-623

Anthony JM, Senaratnal T, Dixon KW, Sivasithamparam K (2004) The role of antioxidants for initiation of somatic embryos with *Conostephium pendulum* (Ericaceae). Plant Cell Tiss Organ Cult 78: 247–252

Bandyopadhyay S, Cane K, Rasmussen G and Hamill JD (1999) Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species- *Eucalyptus nitens* and *E. globulus*. Plant Sci 140 (2):189-198

Benson EE (2000) Special Symposium: In vitro plant recalcitrance. Do free radicals have a role in plant tissue culture recalcitrance? In Vitro Cell Dev Biol Plant 36:163–170

Bonga JM, Von Aderkas PM (1992) In vitro culture of trees. Kluwer Academic Publishers, Dordrecht

Brown S, Wetherell DF, Dougall DK (1976) The potassium requirement for growth and embryogenesis in wild carrot suspension cultures. Physiol Plant 37:73-79

Canhoto JM, Lopes ML, Cruz GS (1999) Somatic embryogenesis and plant regeneration in myrtle (Myrtaceae). Plant Cell Tiss Organ Cult 57:13-21

Carraway DT, Merkle SA (1997) Plantlet regeneration from somatic embryos of American chestnut. Can J For Res 27(11):1805-1812

Chauhan M, Kothar SL (2004) Optimization of nutrient levels in the medium increases the efficiency of callus induction and plant regeneration in recalcitrant indian barley (*Hordeum vulgare L.*) in vitro. In Vitro Cell Dev Biol Plant 40 (5):520–527

Close DC, Davies NW, Beadle CL (2001) Temporal variation of tannins (galloylglucoses), flavonols and anthocyanins in leaves of *Eucalyptus nitens* seedlings: implications for light attenuation and antioxidant activities. Aust J Plant Physiol 28 (4):269 – 278)

Correia D, Gonçalves NA, Zarate do Couto HT, Ribeiro MC (1995) Efeito do meio de cultura líquido e sólido no crescimento e desenvolvimento de gemas de *Eucalyptus grandis* x *Eucalyptus urophylla* na multiplicaçao in vitro. IPEF 48/49:107-116

Cousson A, Tran Thanh Van K (1993) Influence of ionic composition of the culture medium on *de novo* flower formation in tobacco thin cell layers. Can J Bot 71:506- 511

Driver JA, Kuniyuki AH (1984) In vitro propagation of paradox walnut rootstock. HortSci 19:507–509

Fisichella M, Silvi E, Morini S (2000) Regeneration of somatic embryos and roots from quince leaves cultured on media with different macroelement composition. Plant Cell Tiss Organ Cult 63:101–107

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh Plant Growth Regul 43 (1):27-47

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gribble K, Conroy J, Holford P, Milham P (2002) In vitro uptake of minerals by *Gypsophila paniculata* L. and hybrid eucalypts and relevance to media mineral formulation. Aust J Bot 50:1-11

Gupta PK, Mehta UJ, Mascarenhas AF (1983) A Tissue culture method for rapid clonal propagation of mature trees of *Eucalyptus torelliana* and *Eucalyptus camaldulensis*. Plant Cell Rep 2:296-299

Jiménez VM (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regul 47:91–110

Kong L, Yeung EC (1995) Effects of silver nitrate and polyethylene glycol on white spruce (*Picea glauca*) somatic embryo development: enhancing cotyledonary embryo formation and endogenous ABA content. Physiol Plant 93:298- 304

Lloyd G, McCown BH (1980) Commercially-feasible micropropagation of Mountain Laurel, *Kalmia latifolia*, by shoot tip culture. Int Plant Prop Soc Proc 30:421-427

MacKay JJ, Becwar MR, Park YS, Coederro JP, Pullman GS (2006) Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genetics & Genomes 2:1- 9

Magnaval C, Noirotm M, Verdeil JL, Blattes A, Huet C, Grosdemange F, Beule T, Buffard-Morel J (1997) Specific nutritional requirements of coconut calli (*Cocos nucifera L.*) during somatic embryogenesis induction . J Plant Physiol 150 (6):719- 728

Malabadi RB, Staden JV (2005) Role of antioxidants and amino acids on somatic embryogenesis of *Pinus patula*. In Vitro Cell Dev Biol Plant 41:181–186

Martin KP, Zhang C-L, Slater A, Madassery J (2007) Control of shoot necrosis and plant death during micropropagation of banana and plantains (*Musa* spp.). Plant Cell Tiss Organ Cult 88:51–59

McCown BH, Sellmer JC (1987) General media and vessels suitable for woody plants culture. In: Bonga, J. M.; Durzan, D. J., eds. Cell and tissue culture in forestry, vol. 1. Dordrecht: Martinus Nijhoff Publishers pp 4–17

Meijer EGM, Brown DCW (1987) Role of exogenous reduced nitrogen and sucrose in rapid high frequency somatic embryogenesis in *Medicago sativa*. Plant Cell Tiss Organ Cult 10:11-19

Mekle AS, Nairn CJ (2005) Hardwood tree biotechnology. In Vitro Cell Dev Biol Plant 41:602–619

Muralidharan EM, Gupta PK, Mascarenhas AF (1989) Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus citriodora*. Plant Cell Rep 8:41–43

Muralidharan EM, Mascarenhas AF (1987) In vitro plantlet formation by organogenesis in *E. camaldulensis* and by somatic embryogenesis in *Eucalyptus citriodora*. Plant Cell Rep 6:256–259

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

Nas MN, Read PE (2004) A hypothesis for the development of a defined tissue culture medium of higher plants and micropropagation of hazelnuts. Sci Hort 101 (1-2):189-200

Nugent G, Chandler S, Whiteman P, Stevenson T (2001) Somatic embryogenesis in *Eucalyptus globulus*. Plant Cell Tissue Org Cult 67:85–88

Pan MJ, Staden JV (1998) The use of charcoal in in vitro culture – A review. Plant Growth Reg 26:155–163

Park YS, Barret D, Bonga JM (1998) Application of somatic embryogenesis in high- value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell Dev Biol Plant 34: 231- 239

Park YS, Pond SE, Bonga JM (1993) Initiation of somatic embryogenesis in withe spruce (*Picea gauca*): genetic control, culture treatment effects, and implications for tree breeding. Theor Appl Genet 86: 427- 436

Pinto G, Loureiro J, Lopes T, Santos CV (2004) Analysis of genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry. Theor Appl Genet 109:580- 587

Pinto G, Santos C, Neves L, C Araújo (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21:208-213

Pinto G, Silva S, Coutinho J, Araujo C, Neves L, Santos C. (2006) Importance of media mineral composition on the induction of somatic embryogenesis *in Eucalyptus globulus* Labill. 2º Simposio Iberoamericano de *Eucalyptus globulus*. 17- 20 of October. Pontevedra, Spain

Poke FS, Vaillancourt RE, Potts BM, Reid JB (2005) Genomic research in *Eucalyptus*. Genetica 125: 79- 101

Prakash MG, Gurumurthi K (2005) Somatic embryogenesis and plant regeneration in *Eucalyptus tereticornis* Sm. Cur Sci 88 (8): 1311- 1316

Ramage CM, Williams RR (2002) Mineral nutrition and plant morphogenesis. In Vitro Cell Dev Biol Plant 38: 116- 124

Ruggini E (1984) In vitro-propagation of some olive (*Olea europaea sativa* L.) cultivars with different root-ability, and medium development using analytical data from developing shoots and embryos. Sci Hort 24:123-134

Ružić D, Sarić M, Cerović R, Ćulafić Lj (2004) Contents of macronutrients and growth of sweet cherry rootstock. In Vitro Cell Dev Biol Plant 47:463–465

Santos KGB, Mundstock E, Bodanese-Zanetti MH (1997) Genotype- specific normalization of soybean somatic embryogenesis through the use of an ethylene inhibitor. Plant Cell Rep 16 (12):859-864

Steger MM, Preece JE (2003) The influence of source tree on somatic embryogenesis from eastern black walnut (*Juglans nigra*) immature cotyledons. Acta Hort (ISHS) 625:249-253

Tazawa M, Reinert J (1969) Extracellular and intracellular chemical environments in relation to embryogenesis in vitro. Protoplasma 68: 157- 173

Termignoni R, Wang PJ, Hu CY (1996) Somatic embryo induction in *Eucalyptus dunnii*. Plant Cell Tiss Organ Cult 45:129– 132

Thomas P, Ravindra MB (1997) Shoot-tip culture in mango: influence of medium, genotype, explant factors, season and decontamination treatments on phenolic exudation, explant survival and axenic culture establishment. J. Hort Sci 72:713–722

Von Arnold S (1982) Factors influencing formation, development and rooting of adventitious shoots from embryos of *Picea abies* L. Karst. Plant Sci Lett 27: 275–287

Watt MP, Blakeway F, Cresswell CF, Harman B (1991) Somatic embryogenesis in *Eucalyptus grandis*. S Afr Bosbout 157:59–65

Watt MP, Blakeway FC, Termignoni R, Jain SM (1999) Somatic embryogenesis in *Eucalyptus grandis* and *E. dunnii*. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 5. Kluwer, UK, pp 63–78

II 3: Importance of media mineral composition on the induction of somatic embryogenesis in *Eucalyptus globulus* Labill.

Chapter published as proceeding in an international meeting

Pinto G, Silva S, Coutinho J, Araujo C, Neves L, Santos C. (2006) Importance of media mineral composition on the induction of somatic embryogenesis *in Eucalyptus globulus* Labill. 2º Simposio Iberoamericano de *Eucalyptus globulus*. 7- 20 of October. Pontevedra, Spain (Oral communication)

Abstract

Eucalyptus globulus is one of the main species for the pulp industry and it is the third most important forest species in Portugal. As other *Eucalyptus* species, it is an obvious target for in vitro propagation (*e.g*. somatic embryogenesis) and genetic manipulation.

We have compared several published media (MS, 1/2MS, B5, WPM, DKW and JADS) both on the somatic embryogenesis induction and expression steps. Different media induced heterogeneous responses, and MS, followed by B5, was the best medium to induce primary somatic embryos. Therefore, we compared its mineral composition with the mineral content of zygotic embryos (explants). Simultaneously, and as the secondary somatic embryos are routinely maintained on MS medium and used to regenerate plants, we also compared the mineral composition of zygotic and secondary somatic (grown in MS media) embryos to MS. Fresh samples were digested with $HClO_4$ for Mg, Ca, Cu, Zn, Fe and Mn, and with H_2SO_4 for P, N and K analyses.

Preliminary results showed that zygotic embryos have different mineral proportions when compared to all basal media used, including the MS medium. Also, when compared to secondary somatic embryos, zygotic embryos showed higher levels of Mg (7.6:1), Cu (11.4:1), Zn (3.6:1), Fe (1.7:1), Mn (17.9:1), N (1.8:1), P (4.0:1) but less K (0.7:1), showing that SE induction conditions lead to changes in tissue mineral content relatively to zygotic embryos.

Key words: mineral media composition, somatic embryos, tissue mineral composition, zygotic embryos

Introduction

Somatic embryogenesis (SE) in *Eucalyptus globulus* is a recent advance in vegetative propagation that could have a great impact on tree breeding and commercial plantation forestry. Besides all the advantages of this technique, the most promising application of somatic embryogenesis is in high-value clonal forestry. The commercial use of somatic embryo-derived plants is already a reality for conifers, but concerning *Eucalyptus* genus practical applications of this technique are far from those published for conifers.

Recently, we described a true-to-type plant regeneration protocol from secondary somatic embryogenesis and studied the influence of growth regulators, induction period, explant type and carbohydrate source on the SE primary induction (Pinto *et al.* 2002, Pinto *et al.* 2004a). We have also proved that the methodology used did not induce major genetic changes in the somatic embryos as evaluated by flow cytometry, and that our primary goal of "true-to-type" propagation was attained (Pinto *et al.* 2004b).

Our studies showed that induction of somatic embryos is a highly sensitive step, and its full control is of crucial importance to develop a robust protocol applicable to several genotypes. It is necessary to maintain a broad genetic base for clonal selection and management of genetic diversity (Park *et al.* 1998). Therefore, for the development of superior clonal varieties and development in high-value clonal forestry, it is important to have a high rate of SE induction.

The selection of a proper medium formulation plays a crucial role on the establishment of an efficient tissue culture system for plant regeneration (Kothari *et al.* 2004) but usually this selection is based on empirical approaches. Many researchers use the MS medium for several plant species, without previously screening other media, under a general belief that plants respond well enough to MS salts. Nevertheless, it is crucial to ensure the adequacy of the culture medium, when one intends to develop protocols for large scale plant production. Unfortunately, few works have addressed this area, and only one is reported to the *Eucalyptus* genus for the hybrid *E. uropphylla* x *E. grandis.* (Gribble *et al.* 2002). One of the strategies for defining the best medium is to analyze the mineral content of the plant tissue (e.g. explant) to model the mineral balance definition of the culture medium.

This work focuses on the conditions influencing the induction of embryogenic potential in mature zygotic embryos of *Eucalyptus globulus* Labill.. We also present preliminary studies of the mineral content of zygotic embryos and secondary somatic embryos.

Materials and Methods

Plant material and disinfection: Half-sib seeds of *Eucalyptus globulus* Labill. (supplied by Celbi, Leirosa, Portugal) were surface-disinfected with a mixture of absolute ethanol and hydrogen peroxide for 15 min, washed twice in sterile distilled water (10 min each) and rinsed for 15 min with 0.1% (w/v) Benlate (Rhône-Poulenc). Then, seeds were imbibed over night in sterile distilled water.

Zygotic embryo explants were inoculated on different basal media: MS (Murashige an Skoog 1962), ½ MS, B5 (Gamborg 1968), DKW (Driver and Kuniyuki 1984), WPM (Lloyd and McCown 1980), JADS (Correia 1993). All media were supplied with 3 mg l^{-1} NAA (3NAA) according to Pinto *et al*. (2002, see also Chapter II.2). Groups of 50 explants were distributed by five 90 mm diameter Petri dishes (each containing 10 embryos), for each medium tested. Induction of somatic embryos took place in the dark, at 22- 24 ºC for three weeks.

Explants were then transferred to the same medium used during induction but without growth regulators (MS_{WH}, ½ MS_{WH}, $B5_{WH}$, DKW_{WH}, WPM_{WH}, $JADS_{WH}$) for 12 weeks in the dark, for expression. Explants were monthly transferred to fresh medium.

All media were supplemented with 30g l^{-1} sucrose and 2.5g l^{-1} gelrite®, pH was adjusted to 5.8 and the media were autoclaved at 121ºC for 20 min. The JADS medium was made using stock solutions of chemicals purchased to Sigma (USA) and the MS vitamins from Duchefa (Haarlem, Netherlands). All other culture media, except JADS, sucrose, gelrite® and NAA (α-naphthalene acetic acid) were purchased to Duchefa (Haarlem, Netherlands).

Explants expression was analyzed in 50 replicates for each condition (n=50), 12 weeks after transfer to expression medium. The following parameters were analyzed with a magnifying binocular (Olympus SZ60): a) % of explants showing SE response and b) total number of somatic embryos.

Mineral composition determination

Fresh samples of mature zygotic embryos and secondary somatic embryos were digested with a $HClO_4$ and HNO_3 solution for Mg, Ca, Cu, Zn, Fe and Mn (Mills and Jones 1996). Those ions were determined by atomic absorption spectroscopy. A sulphuric acid digestion was carried out for N, P and K content and N and P were determined by molecular absorption spectroscopy and K by flame emission spectroscopy (Walinga *et al.* 1989).

Results

Medium effect on induction and expression

Germination of entire mature zygotic embryos was higher than 90% in all media tested. After two weeks on induction media, the germination process stopped and callus production started, mainly in cotyledons. After three weeks of induction, no visual differences were detected among the six media tested. A combination of whitish friable and compact calluses occurred simultaneously in the same explant while no phenolization was observed.

After four weeks on expression medium, all explants showed browning and first embryogenic responses were observed mostly on cotyledon regions. After this period the number of adventitious roots newly formed, increased dramatically during this period, together with oxidation (Table 1).

After 12 weeks on expression media, explant responses were heterogeneous and highly dependent on medium composition. Higher SE rates were obtained in MS_{WH} (30% of explants showed SE, in a total of 67 somatic embryos per 50 explants evaluated and $B5_{WH}$ (26%, a total of 44 somatic embryos), followed by $1/2MS_{WH}$ (10%, a total of 8 somatic embryos), $JADS_{WH}$ (8%, a total of 6 somatic embryos) and DKW_{WH} (6%, a total of 6 somatic embryos). SE was not observed on WPM_{WH}. MS revealed to be the best medium for both percentage of explants showing somatic embryogenesis response and the total somatic embryos formed per explant (Table 1)

Somatic embryos formed were whitish, compact, and mostly at the globular stage, although other advanced stages could be found, showing some assynchronism of the process. Germination and conversion were observed independently of the medium,

although MS_{WH} continued to give the highest number of cotyledonar embryos and of plantlets (Table 1).

Table 1: Embryogenic response after 12 weeks on the expression medium for the six media tested

Induction medium	Expression medium	Induction (%) (n=50)	Total No. of Somatic embryos	Somatic embryo type		
				Globular	**Cotyledonar**	**Plant**
MS	MS_{WH}	30	67	39	28	8
½ MS	½ MS_{WH}	10	8	3	2	3
B5	$B5_{WH}$	26	44	22	20	2
DKW	DKW_{WH}	6	6	0	5	1
WPM	WPM_{WH}	0	0	0	0	0
JADS	$JADS_{WH}$	8	6	3	2	1

Mineral Composition

The mineral composition of the six tested media differ largely (Table 2), with MS being the richest medium in nitrogen (both nitrate and ammonium), while JADS contains high levels of phosphate and DKW is the richest in sulphate, calcium and magnesium.

Except for Fe supply that differed in JADS, the absence of KI in DKW, WPM and JADS, and the lower concentration of Zn in B5, no major differences were found among micronutrient composition. The same was observed for vitamins, as the most significant changes were the absence of glycine in B5 and of pyridoxine in DKW (Table 2).

Table 2: Mineral composition of plant tissue culture media used.

mM	**MS**	**B5**	**½ MS**	**DKW**	**WPM**	**JADS**
Macro Elements						
$Ca(NO_{3)2}.4H_2O$						5.000
$Ca(NO_3)_2.2H_2O$				8.300	2.350	
$CaCl_2$	2.990	1.020	1.500	1.010	0.650	
KH_2PO_4	1.250		0.630	1.950	1.250	3.000
K_2SO_4				8.950	5.680	
KNO_3	18.700	24.730	9.400			8.000
$MgSO_4$	1.500	1.010	0.730	3.000	1.500	
$MgSO_4.7H_2O$						3.000
$(NH_4)_2SO_4$		1.010				
NaH_2PO_4		1. 0900				
NH_4NO_3	20.610		10.300	17.600	5.000	4.000
Micro Elements						
$CoCl_2.6H_2O$	0.000	0.000	0.000			0.000
$CuSO_4.5H_2O$	0.000	0.000	0.000	0.000	0.000	0.005
$Na_2EDTA.2H_2O$						0.200
$FeSO_4.7H_2O$						0.200
FeNaEDTA	0.100	0.100	0.100	0.120	0.100	
H_3BO_3	0.100	0.048	0.100	0.078	0.100	0.050
KI	0.005	0.005	0.005			
$MnSO_4.H_2O$	0.100	0.059	0.100	0.200	0.130	0.075
$Na_2MoO_4.2H_2O$	0.001	0.001	0.001	0.002	0.001	0.001
$ZnSO_4.7H_2O$	0.030	0.007	0.030	0.072	0.030	0.015
Vitamins						
Glycine	0.0266		0.0266	0.0266	0.0266	0.0266
Myo-Inositol	0.5600	0.5600	0.5600	0.5600	0.5600	0.5600
Nicotinic acid	0.0041	0.0081	0.0041	0.0081	0.0041	0.0041
Pyridoxine HCl	0.0024	0.0049	0.0024		0.0024	0.0024
Thiamine HCl	0.0003	0.0030	0.0003	0.0059	0.0030	0.0003

As to embryo mineral composition, results showed that zygotic embryos have different mineral proportions when compared to all basal media used, including the MS medium. When compared to secondary somatic embryos, zygotic embryos showed higher levels of Mg (7.6:1), Cu (11.4:1), Zn (3.6:1), Fe (1.7:1), Mn (17.9:1), N (1.8:1), P (4.0:1) but less K (0.7:1), showing that SE induces changes in tissue mineral composition ,and that these changes must be clarified (Table 3).

Table 3: Total levels of macro and micronutrients in zygotic embryos and secondary somatic embryos.

mg/Kg fw	Mg	Ca	Zn	Cu	Fe	Mn	N	K	P
Zygotic embryos	1739.4	826.2	40.9	16.0	66.5	315.5	21656.2	2913.3	4139.7
Secondary somatic embryos	228.4	542.5	11.2	1.4	39.5	17.6	11807.7	4379.0	1039.5

Discussion and Conclusions

The combination of minerals necessary for plant development/morphogenesis is dependent on the species, and usually determined by empirical manipulation of one or a combination of existing published formulations (Ramage and Williams 2002).

Concerning the combination of mineral nutrients in SE media, we hypothesized, for *Eucalyptus globulus*, that the medium having a mineral proportion/composition similar or close to the explant mineral proportion/composition will give higher SE induction rates. To test this hypothesis we chosed four media (MS, B5, DKW, WPM) largely used in woody species micropropagation and morphogenesis. Besides, we also used the JADS specifically designed for *E. grandis* micropropagation (Correia 1993) but with no use, up to moment, in *E. globulus* SE studies.

A previous analysis of MS, B5, ½ MS, JADS, DKW and WPM mineral compositions led us also to the hypothesis that explant responses, during induction/expression steps, may be mostly due to macronutrient content differences, as all other experimental conditions such as pH, growth regulators or carbohydrate, were similar and no large differences were found in the micronutrient and vitamin composition. As far as we know, all reports concerning somatic embryogenic response for the *Eucalyptus* genus just used MS and B5 media for induction (Muralidharan and Mascarenhas 1995,

Prakash and Gurumurthi 2005). Although no explanation is given and no reference is made to the use/effectiveness of other media in those works (Muralidharan and Mascarenhas 1995, Prakash and Gurumurthi 2005), results presented here confirm the authors' decisions. In fact, these preliminary results show that: 1) zygotic embryos have different mineral proportions when compared to all basal media used, including the MS medium; 2) this last medium has, however, the mineral proportion most close to the zygotic embryo one.

Furthermore, when compared to secondary somatic embryos, zygotic embryos showed higher levels of Mg, Cu, Zn, Fe, Mn, N, P but less K, showing that the SE protocol used here led to significant differences between tissue mineral composition of the zygotic embryo (explant) and the resulting secondary somatic embryos. This generalized decrease of mineral level in secondary somatic embryos (when compared to zygotic embryos) could be involved in the problems observed during maturation where low germination and conversion were found. Conversion low rates compromise the effectiveness of the SE process once we presently use secondary somatic embryos to regenerate plants. Pullman *et al.* (2003) refer that the nutrional, osmotic and hormonal environments surronding an embryo are well know to control embryo growth. Optimization of these environments is critical for growth and development of high- quality vigours somatic embryos. These authors propose to optimize the nutritional environment to somatic embryos based on the analysis of female gamethophyte and zygotic embryos mineral contents of *Pinus taeda* L.

In conclusion, minerals appear to play an important role in the regulation of plant morphogenesis, and in particularly somatic embryogenesis. Our preliminary results suggest that MS gave the best SE induction results followed by B5. Results also show that, although mineral proportion of all media differs from the mineral proportion found in zygotic embryos, MS has the most close profile. Based on these data we hypothetise that SE induction may be improved if the medium salt composition (e.g. MS) is adjusted to similar mineral proportions to those found in the explant (zygotic embryos), but further studies on the formulation of a newly and optimized medium for SE process in *E globulus* are needed. In addition, there are many challenges in the area of embryo quality and vigor in order to secondary somatic embryogenesis become commercial availed for this specie.

References

Correia D (1993) Crescimento e desenvolvimento de gemas na multiplicação de *Eucalyptus* spp. em meio de cultura líquido e sólido. Piracicaba, ESALQ, 1993. 113p. Master Thesis

Gamborg O L, Miller R A, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151-158

Gribble K, Conroy J, Holford P, Milham P (2002) In vitro uptake of minerals by *Gypsophila paniculata* L. and hybrid eucalypts and relevance to media mineral formulation. Aust J Bot 50:713-723

Kothari SL, Agarwal K, Kumar S (2004) Inorganic nutrient manipulation for highly improved in vitro plant regeneration in finger millet- *Eleusine coracana* (L.) Gaertn. In Vitro Cell Dev Biol Plant 40: 515- 519

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

Mills HA, Jones JB Jr (1996) Plant Analysis Handbook II. MicroMacro Publishing, Inc. Athens, Georgia, USA

Muralidharan EM, Mascarenhas AF (1995) Somatic embryogenesis in *Eucalyptus*. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants, vol. 2. Kluwer, Dordrecht, pp 23–40

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

Park YS, Barret JD, Bonga JM (1998) Application of somatic embryogenesis in high-value clonal forestry: Deployment, genetic control, and stability of cryoprserved clones. In vitro Cell Dev Biol Plant 34:231- 239

Pinto G, Loureiro J, Lopes T, Santos CV (2004a) Analysis of genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry. Theor Appl Genet 109:580-587

Pinto G, Santos C, Neves L, C Araújo (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21:208-213

Pinto G, Silva S, Santos C, Neves L, Araújo C (2004b) Somatic embryogenesis of *Eucalyptus globulus* Labill. and assessment of genetic stability. International IUFRO

Conference of the WP2.08.03 on Silviculture and improvement of *Eucalyptus* "*Eucalyptus* in a changing world", 11 a 16 of October. Aveiro. Portugal

Prakash MG, Gurumurthi K (2005) Somatic embryogenesis and plant regeneration in *Eucalyptus tereticornis* Sm. Curr Sci 88:1311- 1316

Pullman GS, Montello P, Cairney J, Xu N, Feng X (2003) Loblolly pine (*Pinus taeda* L.) somatic embryogenesis: maturation improvements by metal analyses of zygotic and somatic embryos. Plant Sci 164:955-969

Ramage CM, Williams RR (2002) Mineral nutrition and plant morphogenesis. In Vitro Cell Dev Biol Plant 38:116- 124

Walinga I, van Sark W, Houba VJG, van der Lee IJ (1989) Soil and Plant Analysis, part 7, Plant Analysis Procedures. Wageningen Agricultural University, The Netherlands

II 4: Histocytological studies of primary somatic embryogenesis in *Eucalyptus globulus*

Chapter submitted as an original paper to a SCI journal:

Pinto G, Silva S, Araújo C, Neves L, Santos C (2007) Histocytological studies of primary somatic embryogenesis in *Eucalyptus globulus*. Annals of Botany (Submitted)

Abstract

Comparative studies of histocytological and reserve changes during the primary somatic embryogenesis (SE) in *Eucalyptus globulus* give valuable information to understand cell/tissue evolution and the role of reserves during SE process. Zygotic embryos were grown on Murashige and Skoog medium (MS) supplemented with 3 mg l^{-1} α- naphthalene acetic acid (NAA). After three weeks, explants were transferred to MS medium without growth regulators (MS_{WH}), for expression, histodiferentiation and later conversion into plants. Explant cotyledonar regions showed poorly differentiated mesophyll cells rich in lipid and protein bodies. After one week, explants showed protein and lipid body degradation while starch granules increased. Also, ocasional meristematic activity was present in the mesophyll. After three weeks, explants presented mitotically responsive and non responsive regions. Non responding regions followed normal cotyledonar ontogenic evolution with progressive differentiation of the mesophyll and increasing starch levels (mostly near vascular bundles). By this time mitotically responsive regions showed abundant meristematic regions and first phenolised cells were observed, while starch distribution was diffuse.

First globular embryos were seen after 3 weeks on $MS_{WH,}$ had a protoderm surrounding vacuolated cells, and amyloplasts or amylo-etioplasts density increased, but no protein bodies were present. Cotyledonar embryos showed higher predominance of etioplasts while starch granules were in lower amounts. Frequently, mesophyll first differentiation appeared as one layer of palisade cells. In SE derived plants, leaves showed typical histological organization with mesophyll differentiation and chloroplast dominance. Data show that several histocytological changes occur during the primary SE process in *E. globulus*, opening perspectives to a better understanding and control of this process in this recalcitrant species and in *Eucalyptus* in general.

Key words: embryo reserves, *Eucalyptus*, histological differentiation, micropropagation, Myrtaceae, ontogenesis, somatic embryos, ultrastructural studies

Introduction

Somatic embryogenesis in the *Eucalyptus* genus was described in *E. citriodora* (Muralidharan and Mascarenhas 1987, Muralidharan *et al.* 1989), *E. nitens* (Ruaud *et al*. 1997), *E. dunni* (Termignoni *et al*. 1996, Watt *et al.* 1999), *E. grandis* (Watt *et al*. 1999) and *E. tereticornis* Prakash and Gurumurthi 2005). *Eucalyptus globulus* has been considered highly recalcitrant in somatic embryogenesis process and only Pinto *et al.* (2002) reported the regeneration of SE-derived plants, using mature zygotic embryos, although low induction rates were observed.

It is widely accepted that somatic embryogenesis is a process by which somatic cells undergo a development sequence similar to that seen in zygotic embryos, except that they do not became dormant and the integuments and endosperm are not formed (e.g. Williams and Maheswaran 1986, Dodeman *et al.* 1997, Kärkönen 2000). The knowledge of the mechanisms controlling the explants embryogenic responses, and later the somatic embryo histodifferentiation, may turn it possible to improve the efficiency of this process (e.g. to control the induction and later conversion stages).

The accumulation of reserve substances represents a key stage to zygotic embryogenesis, providing compounds that are utilized by the germinating embryo until the development of autotrophy. A comparative study of the accumulation of these substances during somatic embryos development (*vs* zygotic embryos) will provide information on the quality and evolution of somatic embryogenesis. The correct accumulation of reserves in somatic embryos may indicate a high degree of vigor and subsequent germination (Merkle *et al.* 1995).

Cytological and histo-anatomical studies have been performed to understand histodifferentiation in several Dicotyledonous woody species in order to understand: a) the induction and unicellular/ multicellular origin of somatic embryos (e.g. Canhoto and Cruz 1996, Canhoto *et al*. 1999, Quiroz *et al*. 2002), b) to clarify cytological aspects of both competent cells and neighboring cells apparently not involved in somatic embryogenesis (e.g. Canhoto *et al*. 1996), c) to histo-chemically follow somatic embryo development in what concerns, for example, starch mobilization (e.g. by PAS staining), protein and polyphenol contents (Canhoto *et al.* 1996), or d) to compare the evolution of somatic and zygotic embryos (e.g. Dodeman *et al*. 1997).

In Myrtaceae family little attention was given to the cellular changes during SE. In *Eucalyptus* genus, the few available studies report to *E. nitens* ultrastructure evolution in comparison with zygotic embryos. Bandyopadhyay and Hamill (2000) and Arruda *et al.* (2000) demonstrated the effect of calcium at the morphological and histological levels and concluded that an increase of calcium favored the morphogenic route for somatic embryogenesis in *E. urophilla*.

The objective of this work was to present a histological and ultrastructural characterization of *Eucalyptus globulus* somatic embryogenesis, from induction to plant conversion, using the protocol described by Pinto *et al.* (2002) for this species. Such information could provide insight into the main tissues that contribute to embryogenic masses, the main changes associated with the dedifferentiation process of competent and neighboring cells, as well as the evolutionary characteristics that somatic embryos suffer as well as the evolution of reserves accumulation during the whole process.

Material and Methods

Induction of somatic embryogenesis

Half-sib seeds of *Eucalyptus globulus* Labill. (Celbi, Leirosa, Portugal) were collected in the Centre of Portugal from open pollinated families. Seeds were surface disinfected and somatic embryogenenic cultures were initiated from zygotic embryos (ZE) in accordance with the protocol established by Pinto *et al.* (2002, see also Chapter II.2). Briefly, the seed coat was removed from the mature zygotic embryos and they were transferred to Murashige and Skoog (1962) medium (MS) supplemented with 3 % (w/v) sucrose and 3 mg l^{-1} (16.1 μM) α-naphtaleneacetic acid (NAA) to induce somatic embryogenesis. After three weeks on callus induction medium, explants were transferred to MS medium without growth regulators (MS_{WH}). Thereby, they were maintained on this medium in the dark.

Medium culture, NAA, sucrose and gelrite were purchase from Duchefa (Netherlands). All other chemicals used in these experiments (unless otherwise specified) were purchased to Sigma (St. Louis, MO, USA).

Histological and cytological characterization

For histological and cytological characterization, samples were collected from: a) cotyledons of mature zygotic embryos (explant at day 0); b) explants after one week on induction medium; c) non mitotically active regions of explants after three weeks on induction medium; d) mitotically active regions of explants after three weeks on induction medium; e) primary globular somatic embryos; f) primary dicolyledonar somatic embryo; g) leaf of SE-derived plant.

Samples were fixed in 2.5% (v/v) glutaraldehyde in 1.25% (w/v) piperazine-N,N'-bis-(2-ethanesulfonic acid) (PIPES) buffer (pH 7.4) for 3 h and washed in PIPES. Tissues were then fixed in 1% (w/v) osmium tetroxide in PIPES buffer for 1h, rinsed in the same buffer and dehydrated through a graded ethanol series and embedded in a graded low-viscosity epoxi resin (Embed-812). The blocks were polymerised at 60ºC for 48 h. Ultra-thin sections were cut with a LKB ultra-microtome using a diamond knife and collected on uncoated copper grids. The sections were stained with uranyl acetate for 15 min and lead citrate for 10 min and observed with a Elmiskop-101 transmission electron microscope (Siemens AG, Germany) at 80 kV.

For light microscopy, semi-thin sections (app 1.0 μm) from the material embedded for electron microscopy were obtained using glass knife and stained with toluidine blue (0.1 %) (w/v) for general staining, Sudan Black B (0.1%) (w/v) for lipid satining, bromophenol blue as a protein stain or by periodic Acid-Schiff reaction (PAS) for carbohydrate staining.

Samples were analysed using a Nikon eclipse 80i light microscope (Nikon Corporation, Kanagawa, Japan) and digital photographs were taken using a Leica DC 200 digital camera (Leica Microsystems AG, Wetzlar, Germany). PIPES buffer was acquired to Duchefa (Haarlem, The Netherlands), while the remaining chemicals were purchased to Agar Scientific (Essex, U.K.).

Morphometric studies

Microphotographs were used in morphometric studies. Predefined selected areas of samples from the different evolutionary stages were measured using ImageTool for Windows (version 3.00, University of Texas Health Science Center, San Antonio, TX, USA) for the percentage of occupation of the different tissues: epidermis, undifferentiated

parenchyma, palisade parenchyma, spongy parenchyma and vascular strands. In specifically stained sections, comparative measurements were performed for lipid occupation rates and for starch and protein number per cell.

Results

Somatic embryo formation and development

After three weeks on induction medium, explants showed compact and whitish calluses and no phenolisation occurred (Plate 1.A). After transfer to MS_{WH} browning occured and after two to three weeks globular embryos could be observed that evolved to cotyledonary stage and with time became greenwish (Plate 1.B, C). Somatic embryo development was asynchronous and abnormal somatic embryos (with altered number of cotyledons and/or cotyledons of different sizes) were frequent. Cotyledonar embryos germinated (Plate 1.D) and subsequently converted to plants.

Cytological and Histo-anatomical studies

1. Induction period

Week 0 (inoculation)

Cotyledons from imbibed zygotic embryo explants were white, thick and were on average 3 mm long. The mesophyll was still highly undifferentiated. Mesophyll cells (7- 9 cell layers) varying in size and shape and had few and small intercellular spaces (Plate 1.E, F). Nevertheless, one cell layer of palisade parenchyma was present near the adaxial epidermis (23% of total occupied area, Table 1) (Plate 1.E, F). Vascular strands had a low percentage (3.95%) of total occupation (Table 1, Plate 1.F, H).

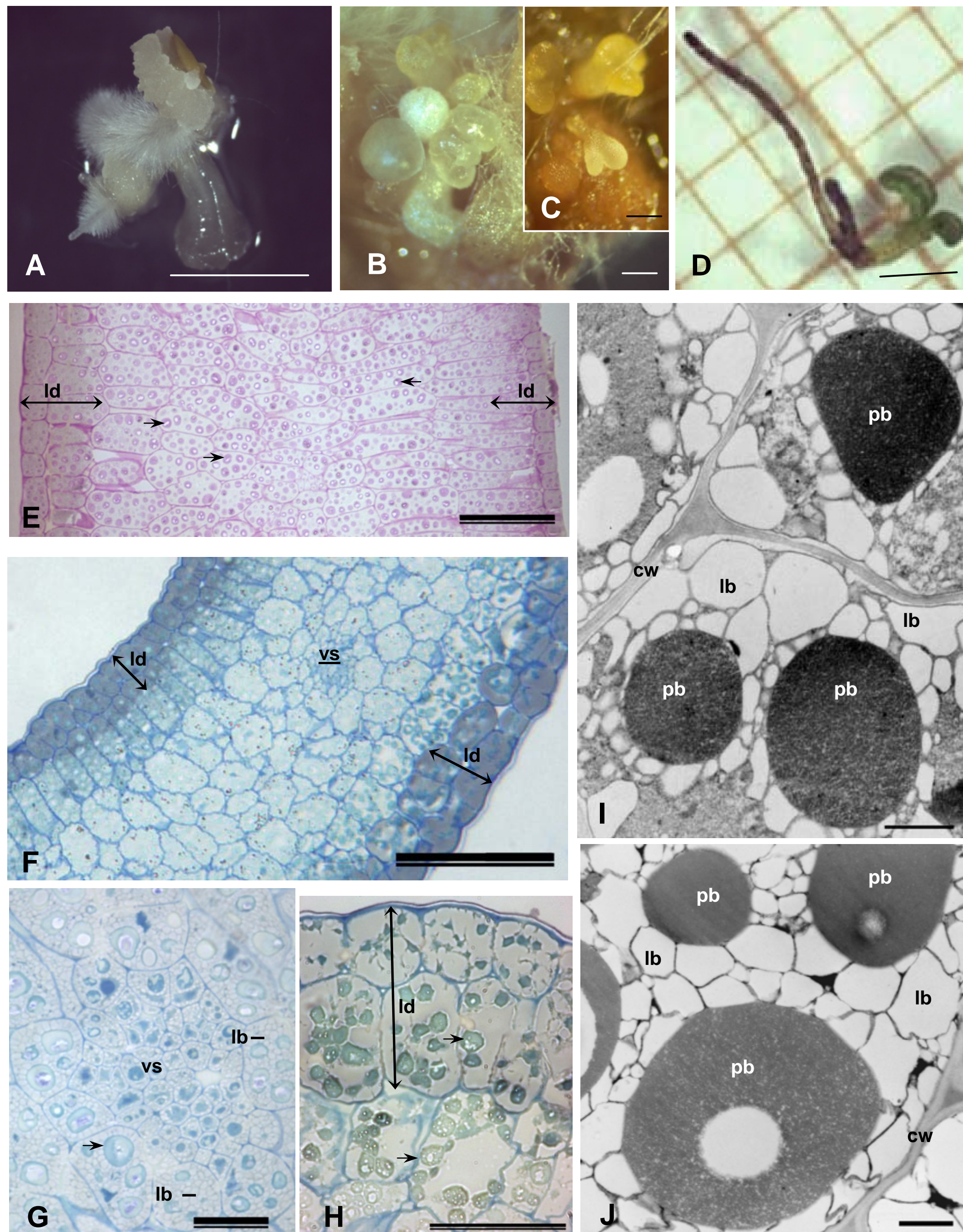


Plate 1. A-D Different aspects of somatic embryo formation and development. **A**) Explant after three week on induction medium. *Bar* 1cm. **B**) Globular somatic embryos. *Bar* 1mm. **C**) Cotyledonar somatic embryos. *Bar* 1mm. **D**) Germinated cotyledonar somatic embryo. *Bar* 1mm. E-J Histocytological aspects of the cotyledonar region of zygotic embryos used as explants. **E**) Cross section of cotyledon stained with PAS, showing also protein bodies (arrows). *Bar* 50µm. **F**) Cross section of cotyledon stained with toluidine blue. *Bar* 50µm. **G**) Detail of the lipid deposition zone and partially digested protein bodies (arrow). *Bar* 1µm. H) Detail of a newly formed vascular strand. *Bar* 1µm. **I)** Ultrastructural view of cells packed with protein and lipid bodies. *Bar* 50µm. J) Aspect of partially digested protein bodies. *Bar* 1µm. Legends: **cw:** cell wall; **lb**: lipid bodies; **ld**: lipid deposition; **pb**: protein bodies; **vs**: vascular strand.

On average, 33% of cells showed fully stain for lipids, which was mostly evident for the epidermal cells and subjacent mesophyll layer cells (Fig 1, Plate 1.F, G). These explants were also rich in undigested and partially digested protein bodies (Plate 1. E-J). Epidermal cells were covered with wax, and were isodiametric, in general smaller (one third to half) than those of the mesophyll and had high deposition of lipids (Plate 1.E-G). Ducts were seen only sporadically and always located at the abaxial epidermis.

Table 1. Relative percentages of vascular, mesophyll and epidermis tissues occupation during induction period. (average ± standard error).

	Vascular strands	Palisade parenchyma	Undifferentiate parenchyma	Spongy parenchyma	Abaxial epidermis	Adaxial epidermis
Week 0	3.95± 1.2	23.13± 4.3	53.87± 2.1	0.00	6.90± 0.8	6.70± 1.2
Week 1	6.34± 2.5	15.04± 1.6	54.31± 6.0	0.00	5.96± 1.8	6.55± 0.5
Week 3	16.57±8.8	13.67± 3.0	26.78± 16.3	26.45± 18.4	11.79± 9.0	7.34± 0.7

Week 1

One week after induction, explants still resembled the initial cotyledon explants, but having one layer of palisade parenchyma and a more differentiated spongy parenchyma. This was particularly evident for non mitotically responsive regions where spongy mesophyll cells frequently accumulated larger amounts of lipids than cells of the adaxial margin (Plate 1.A). This margin had an epidermis showing phenolisation. Palisade mesophyll had large vacuolated cells (Plate 2.A, B) with etioplasts (Plate 2.C, D). Starch granules distribution was difuse in mesophyll (Plate 2.B, Fig 1a). However, the amount of starch accumulated changed during the embryogenic process (Fig 1a).

During this period, lipid and protein digestion occurred, as shown by the decreases of the lipid occupation rate and the average number of protein bodies per cell (Fig 1a, b; Plate 2.E) and the reduction of the lipid bodies size compared to day 0 (Plate 1.F), suggesting a digestion from the inside from the outside of the explant. Lipid digestion was more intense at adaxial than at the abaxial margins (Plate 2.A). Often parenchyma cells with small vacuoles and rich cytoplasm (*e.g.* abundant endoplasmatic reticulum, dyctiossomes) were observed (Plate 2.F). By this time, meristematic activity was present in mesophyll regions, or near vascular bundles. In these meristematic regions some groups

of three or more vacuolated cells, with very thin walls, were surrounded by a thicker cell wall (Plate 2.B).

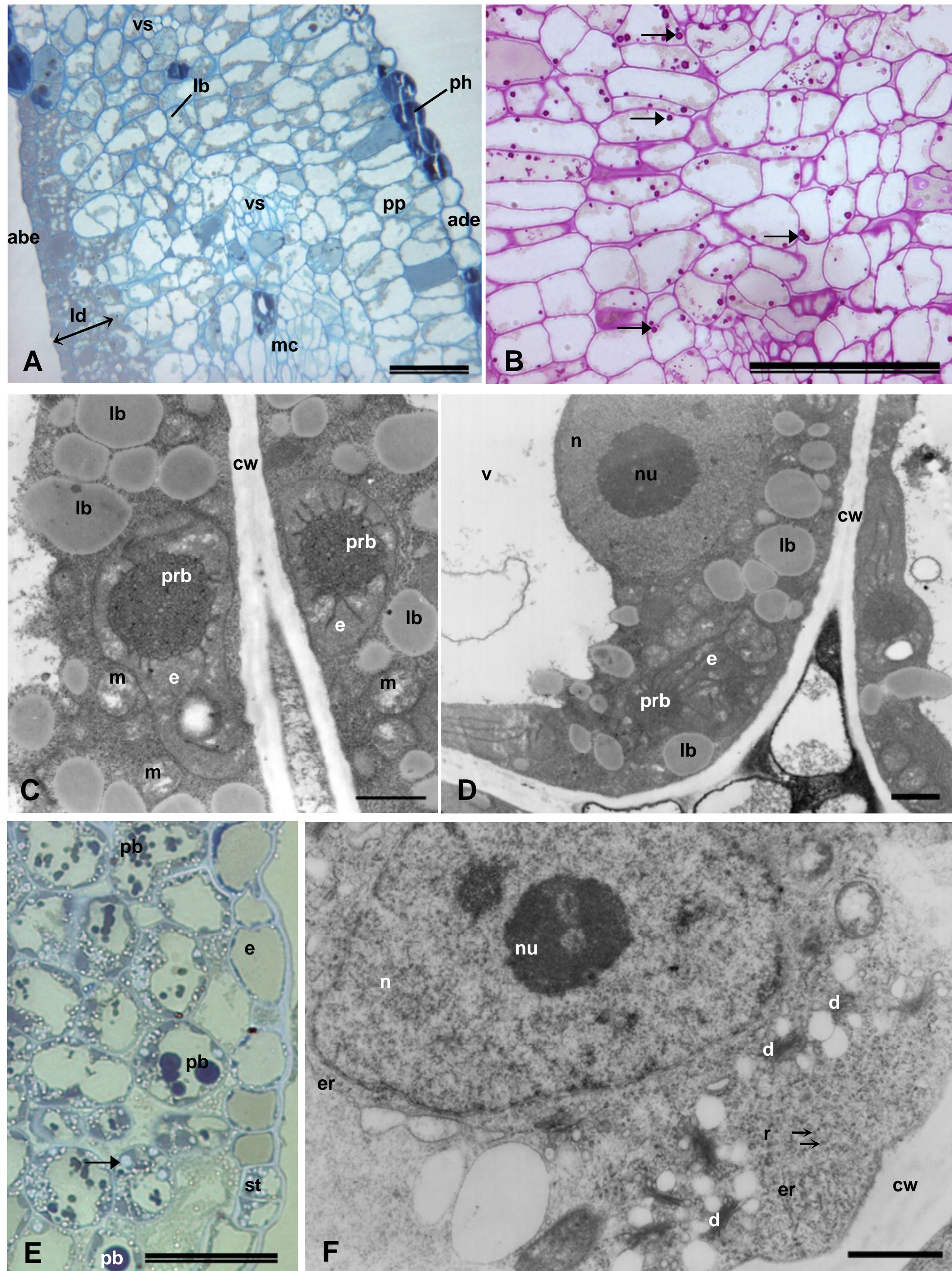
Week 3

After three weeks meristematic regions continued to increase causing explant enlargement. Meristematic cells were compressed and small, had thin walls (Plate 3.A), dense cytoplasm, small vacuoles, nucleus with prominent nucleolus, abundant endoplasmatic reticulum, and vesicules funded with the plasmalema suggesting active extrusion of material to cell wall synthesis (Plate 3.B, C). Non-meristematic neighboring callus cells were larger and highly vacuolated (Plate 3.A).

In regions with no meristematic activity starch accumulated near the vascular strands (Plate 3.D) and mesophyll differentiation proceeded (with evident etioplasts, Plate 3.E). These non mitotically responsive regions showed partially digested protein bodies (Plate 3.F) while in the responsive regions no protein bodies were seen (Plate 3.G).

By the end of this period, almost no lipids were seen. Phenolic compounds accumulated randomly in epidermal or mesophyl cells (Plate 3.A, D).

Plate 2. Different aspects of zygotic cotyledons after one week on induction medium. **A**) Transversal section stained with toluidine blue showing a weakly differentiated palisade parenchyma, meristematic regions, vascular strands and some lipid deposition. *Bar* 50µm. **B**) Transversal section stained with PAS showing starch with diffuse distribution (arrows). *Bar* 50µm. **C** and **D**) Mesophyll cells evidencing etioplasts and lipid bodies. *Bar* 1µm. **E**) Section stained with bromophenol blue showing partially digested protein bodies and starch granules (arrows). *Bar* 50µm. **F**) Parenchyma cell near vascular strands with small vacuole, prominent nuclei, many dyctiossomes and endoplasmatic reticulum. *Bar* 1µm. Legends: **ade**: adaxial epidermis; **abe**: abaxial epidermis; **cw:** cell wall; **d**: dyctiossomes; **e**: etioplast/etio-amyloplast; **er**: endoplasmatic reticulum; **ph**: phenolic compounds; **lb**: lipid bodies; **ld**: lipid deposition; **m**: mitochondria; **mc**: meristematic cells; **n**: nucleus; **nu**: nucleolus; **pb**: protein bodies; **pp**: palisade parenchyma; **prb**: prolametar bodies; **r**: ribosome; **v**: vacuole; **vs**: vascular strand.



(Plate 2, see page 96)

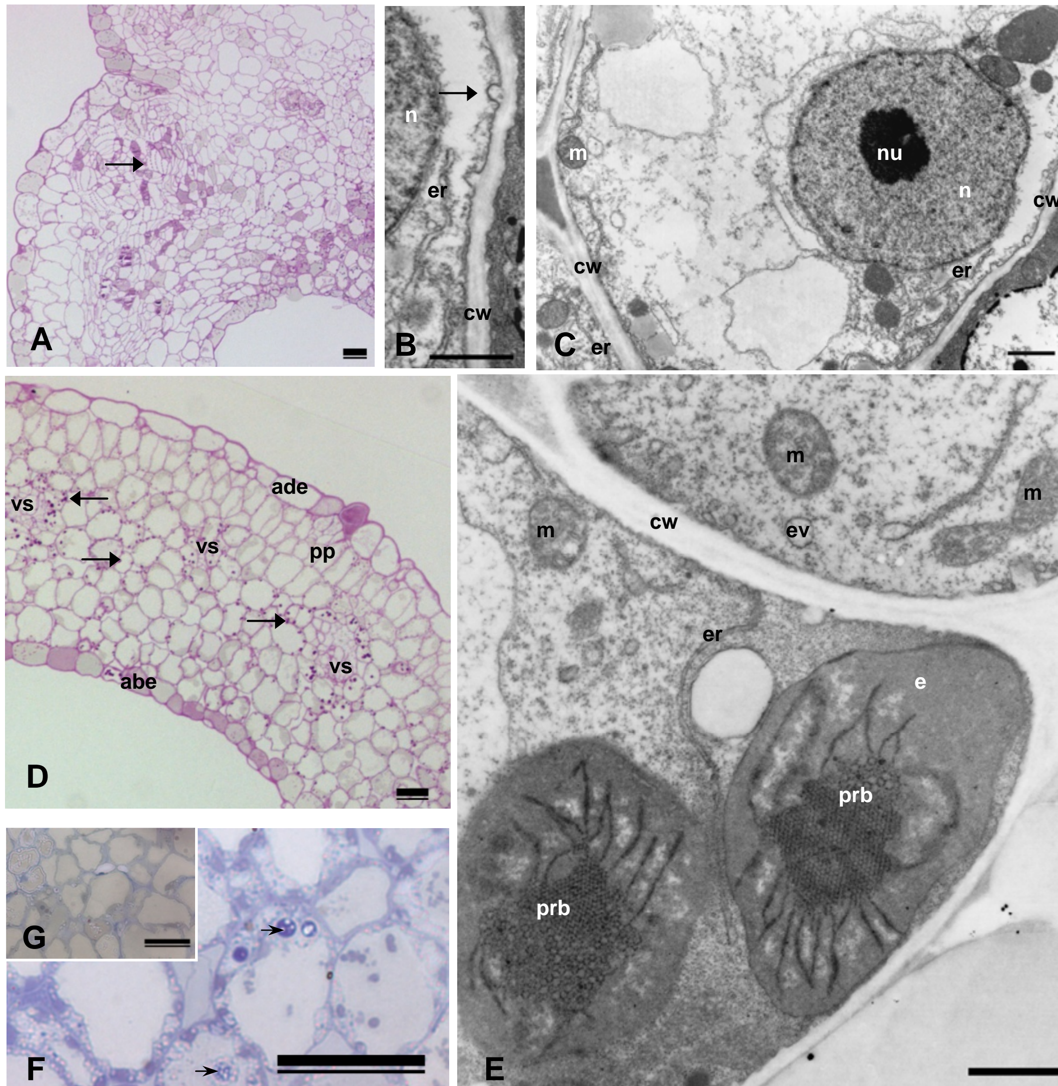


Plate 3.A-E) Zygotic cotyledons (explants) after three weeks on induction medium. **A**) PAS staining of a mitotically responsive region, also showing xylogenesis (arrow). *Bar* 50µm. **B**) Ultrastructural detail showing cell material extrusion (arrow) to cell wall. *Bar* 1µm. **C**) Ultrastructural detail showing a cell rich in endoplasmatic reticulum, extrusion vesicules, small vacuoles and a prominent nucleolus. *Bar* 1µm. **D**) PAS staining of a non responsive region with starch deposition (arrows) around vascular strands. *Bar* 50µm. **E**) Ultrastructural details of mesophyll cells showing etioplasts in a non-responsive region. *Bar* 1µm. **F**) Cross section of a non-responsive region stained with bromophenol blue evidencing parcial digestion of protein bodies (arrows). *Bar* 50µm. **G**) Cross section of a mitotically responsive region stained with bromophenol blue with no protein bodies. *Bar* 50µm. Legends: **ade**: adaxial epidermis; **abe**: abaxial epidermis; **cw:** cell wall; **e**: etioplast/etio-amyloplast; **er**: endoplasmatic reticulum; **m**: mitochondria; **n**: nucleus; **nu**: nucleolus; **pb**: protein bodies; **pp**: palisade parenchyma; **prb**: prolametar bodies; **vs**: vascular strand.

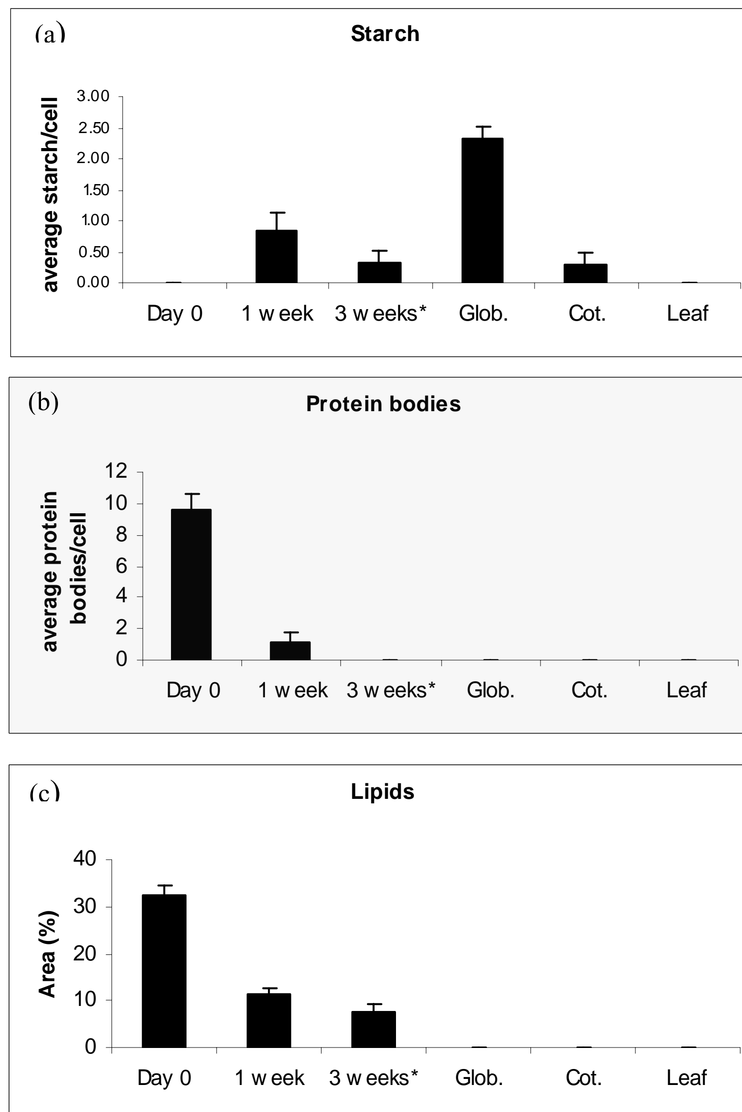


Figure 1: Changes in reserves accumulation during primary somatic embryogenesis in *E. globulus*. **a)** protein; **b)** starch; **c)** lipids. The symbol * reports to mitotically responsive regions.

Expression period

Primary somatic embryos

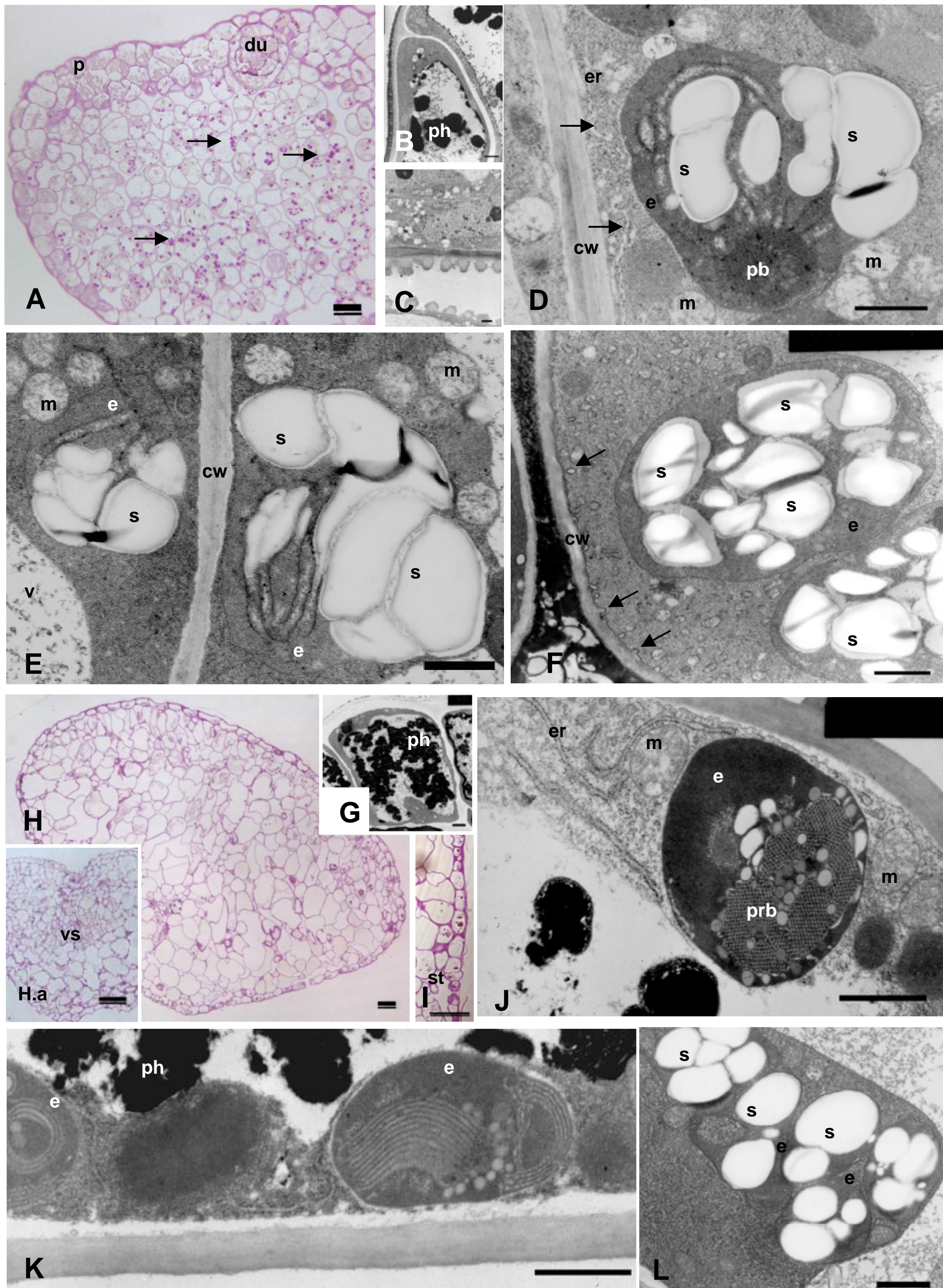
Although somatic embryos occasionally formed before three weeks oj culture, it was after this period that the majority of globular somatic embryos were observed as whitish and round protuberances.

Globular embryos emerged from the callus/explant, and their complete independence from the underlying cells was histologically confirmed. Globular embryos had a protoderm consisting of vacuolated cells accumulating phenolic compounds (Plate

4.A, B). This protoderm surrounded a mass of parechymatous like vacuolated cells, loosely packed and few meristematic regions were seen (Plate 4.A). Tracheary elements were rare and diffusely distributed (Plate 4.A, C). Curiously, even in young globular embryos, ductus delimited by secreting cells wew seen (Plate 4.A). At this stage, starch granules, but no proteins, were present (Figure 1).

Parenchymatous like cells contained a high number of etioplast-amyloplasts (containing prolamelar bodies, with non functional grana, and also several large starch granules) (Plate 4.D, F). Newly formed cells had dense cytoplasm rich in endoplastic reticulum, mitochondria and dictyossoms with vesicles funded with the plasmalema (Plate 4.F). These cells were also abundant in endoplasmatic reticulum and mitochondria, together with evident high rates of extrusion of material (Plate 4.D, F).

Plate 4. A-F) Primary globular somatic embryo. **A**) Globular embryo section showing a protoderm surrounding parenchymatous like cells. PAS staining shows several starch granules (arrows). *Bar* 50µm. **B**) Ultrastructural aspects of phenolic compound rich protoderm cells. *Bar* 1µm. **C**) Ultrastructural detail of a tracheary element. *Bar* 1µm. **D**) ultrastructural detail of a parenchymatous cell with ribosomal (arrow) rich cytoplasm and etio-amyloplasts with pro-lamelar bodies and starch granules. *Bar* 1µm. **E-F**) Ultrastructural detail of etio-amyloplasts and dense cytoplasm with abundant extrusion vesicles (F, arrows). *Bar* 1µm. G-L) Primary cotyledonar somatic embryo. **G**) Ultrastructural detail of a phenolic compound rich protoderm. **H-I**) Cotyledon cross section stained with PAS showing a protoderm, vascular strand, parenchymatous cells (H, Ha) and stomata (I). *Bar* 50µm. **J** and **K**) Ultrastructural detail of parenchymatous cells with rich cytoplasms, and having etioplasts with different pro-lamelar organisations. *Bar* 1µm. **L**) Detail of an etio-amyloplasts. *Bar* 1µm. Legends: **cw:** cell wall; **d**u: ductus; **e**: etioplast/etio-amyloplast; **er**: endoplasmatic reticulum; **ph**: phenolic compounds; **m**: mitochondria; **p**: protoderm **pb**: protein bodies; **pp**: palisade parenchyma; **prb**: prolametar bodies; **s**: starch granules; **st**: stomata; **v**: vacuole; **vs**: vascular strand.



(Plate 4, see page 100)

During somatic embryos evolution, assynchronism became evident but the most abundant embryo forms were globular and cotyledonar. Cotyledonar embryos had well developed cotyledons and root structures and well defined vascular systems. Transversal sections of the cotyledonar region showed a protodermis surrounding parenchyma cells and a procambial zone in the center (Plate 4.H, Ha).

In these embryos, phenolic compounds accumulated in the vacuoles of protoderm cells (Plate 4.G). Besides, some stomata were already formed (Plate 4.I). The most abundant plastids were, at this stage, etioplasts (Plate 4.J, K) as most the etioplasts-amyloplasts almost disappeared (Plate 4.L). Confirming this observation, histochemical staining showed a lower amount of starch grains at this stage compared to globular embryos (Fig. 1.b).

Leaf from SE- derived plant

Leaves from converted plants showed a typical dicotyledons leaf histology (Plate 5.A). Although stomata were present in both margins (amphistomatous leaves) they wre predominantly in abaxial margins (Plate 5.B). Also, abaxial epidermal cells were larger than those of the adaxial margin. Adjacent to the adaxial epidermis, one layer of palissade parenchyma was evident, having rare and small intercellular spaces (Plate 5.A). Besides, 4-5 layers of poorly differentiated mesophyll (suggesting later differentiation into spongy tissue) was present. These cells were isodiametric, highly vacuolated and presented fewer chloroplasts than palisade cells (Plate 5.A). Occasionally vascular strands were seen, with well differentiated tracheids (Plate 5.C). Plastids with different thylakoid organization levels and with irregular shapes were seen, although plano-convex or biconvex form predominated (Plate 5.D, E). Also, occasionally small starch granules were observed.

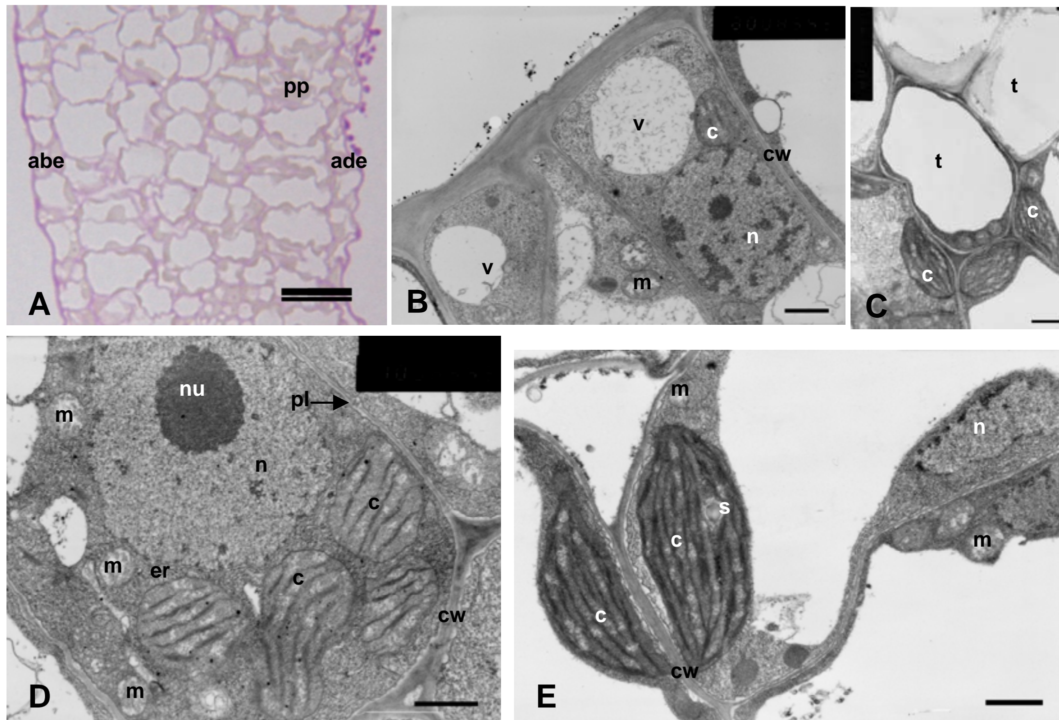


Plate 5. A-E) Leaf cross sections SE-derived plantlet. **A**) PAS stained cross section with very few starch reserves. *Bar* 50µm. **B**) Ultrastructural detail of a stomata guard cells. *Bar* 1µm. **C**) Ultrastructural detail of tracheid elements. *Bar* 1µm. **D**) Ultrastructural detail of a cell with a rich cytoplasm and plastids with poorly granar organization and several plasmadesmata evidencing communication between cells. *Bar* 1µm. **E**) Ultrastructural detail of vacuolated mesophyll cells with regular shaped chloroplasts with still poorly organised grana. *Bar* 1µm. Legends: **ade**: adaxial epidermis; **abe**: abaxial epidermis; **c**: chloroplast; **cw:** cell wall; **m**: mitochondria; **n**: nucleus; **pl**: plasmadesmata; **pp**: palisade parenchyma; **s**: starch granules; **t**: tracheid; **v**: vacuole.

Discussion

Though much attention has been paid to conditions leading to SE in several species of Myrtaceae (e.g. Muralidhan and Mascarenhas 1995, Canhoto *et al.* 1999, Pinto *et al.* 2002) little attention was given to histological and cytological changes during the whole process (from induction to plant conversion).

Our observations confirmed that the mature seeds of *E. globulus* are non-endospermic, containing the storage reserves in the cotyledons as refereed for other species (Canhoto *et al.* 1996) Histologically, the explant (cotyledonar region) showed a poorly differentiated mesophyll with cells rich in lipid and protein bodies as reserve substances. In *E. nitens* (Bandyopadhyay and Hamill 2000) somatic and zygotic embryos had, in

general, similar lipid rich globular bodies in their cells, although cotyledonar cells of somatic embryos had levels of lipid reserves than zygotic ones.

Reserves are crucial to in vitro morphogenesis (e.g. Branca *et al*. 1994). These authors reported high levels of polysaccharides at the beginning of in vitro development in tomato. On the other hand, the consumption of these compounds was correlated with the development of organogenesis and somatic embryogenesis (Mangat *et al*. 1990, Martin *et al*. 2000)

Starch has been considered to be a primary source of energy for cellular proliferation and growth (Cangahuala-Inocente *et al.* 2004), but *E. globulus* initial explants were richer in lipids and proteins lacking starch. Starch appeared during first days suggesting reserve accumulation, but while it continued to increase in non mitotically responsive tissues (data not shown), it decreased in responsive ones, that eventually lead to embryogenic responses. This starch consumption in mitotically/pre-embryogenic tissues was already reported for other species (e.g. Barciela and Vieitez 1993, Canhoto and Cruz 1996). Globular embryos showed higher starch content that was rapidly consumed during embryo evolution, probably to support energetic demands of mitosis and morphogenesis events. In *Feijoa sellowiana* the presence of few starch granules and abundant protein bodies were observed in the globular and early torpedo stages, while in torpedo and cotyledonary-stages an enhanced synthesis of starch granules was associated with the accumulation of reserves to be used during conversion (Cangahuala-Inocente *et al.* 2004. Although slight differences were found in the timing of starch accumulation and consumption among other species, it seems that a general pattern of starch pre-accumulation followed by consumption (e.g. Canhoto *et al.* 1996, Rodriguez and Wetzstein 1998, Cangahuala-Inocente *et al*. 2004) seems to occur in many dicotyledonous SE process. These data support Ho and Vasil (1983) who hypothesised that starch can be used as an early embryogenic marker since embryogenic cells usually do contain this compound providing energy for metabolic and mitotic activity (Stamp 1987).

With respect to lipid and protein accumulation, our findings suggest that during the normal ontogenic process, lipases and proteases activities may ply an important role, as was reported for *Feijoa sellowiana* mature zygotic explants (Canhoto *et al.* 1996). Merkle e*t al.* (1995) highlighted that the differences in lipid and fatty acid composition between zygotic and somatic embryos probably reflect the type of maturation protocol used.

Few studies concern lipid and protein reserve roles in SE process (Stamp 1987), and up to moment we do not know at what extent both species and protocol factors influence this reserve accumulation pattern. Bandyopadhyay and Hamill (2000) reported, for *E. nitens*, that cotyledons of somatic embryos accumulated lipids. We hypothesise that this discrepancy in reserves accumulation between zygotic cotyledons and somatic ones may condition conversion rates and that by increasing lipid and protein reserves in these embryos we may eventually increase conversion rates turning this SE protocol adequate to *Eucalyptus* breeding programs, but the roles of these reserves in *E. globulus* deserve urgent clarification.

First phenolisation symptoms were microscopicaly detected after one week increasing with time. Phenolisation in SE process has been largely described for other woody species mostly associated with neighbouring non embryogenic cells (*e.g*. Canhoto *et al.* 1996, Puigderrajols *et al.* 2000). Bandyopadhyay and Hamill (2000) suggested that high levels of brown exudates (probably phenolics) produced in embryogenic cultures of *E. nitens* could have protection functions. Cangahuala- Inocente *et al*. (2004) also referred the presence of polyphenolics in the meristematic centres that produced somatic embryos. Phenolics are suggested to be correlated with metabolic activity of auxins and other plant growth regulators, and are often associated with negative impacts in cultures, but their exact role in somatic embryogenesis process has to be re-evaluated. In *E. globulus*, addition of antioxidants to prevent phenolisation and increase explant response, had a detrimental effect (see chapter II 2)

Several authors usually report that somatic embryos may arise from an unicellular origin (frequently associated to an epidermal origin) or a multicellular origin (often associated with meristematic cell layers inside). Our studies have provided valuable information on *E. globulus* histocytodifferentiation during SE, but did not demonstrate, up to moment, embryo formation from either one or multiple cells. Besides, and according to Canhoto *et al*. (1996) the presence of a broad zone linking the embryo to the subtending tissues may result from a multicellular origin, suggesting that in *E. globulus*, at least, the embryos that also showed an apparent link to the surrounding tissue may have multicellular origin. The absence of a suspensor like structure also corroborated the idea of multicellular origin suggested by Arruda *et al.* (2000) in *E. urophylla*, where the globular embryo did not had a suspensor or a suspensor like structure.

Cotyledonar embryos showed typical histology of dark-adaptation, as etioplasts predominated. At this stage while starch grains decreased with respect to globular embryos. As already reported, these cotyledonar embryos had much lower reserves than zygotic cotyledons, a fact that probably hampers high rates plant conversion. In SE-converted plants, leaves showed typical histological organization with mesophyll differentiation and chloroplast dominance.

Conclusions

In conclusion, this is the first report in Myrtaceae covering histocytological and reserve accumulation analyses from induction to conversion stages by primary somatic embryogenesis (Figure 2). Despite the fact that the protocol is presently reliable, plant conversion continues to occur at low, non economically viable, rates. Therefore a deeper knowledge on the primary process may be useful not only to control the primary SE process *per se* (e.g. increase induction rates) but also to guarantee that those somatic embryos formed during the primary process may produce new cycles of secondary embryos. Besides, a correlation between maturation deficiencies (leading to low conversion rates) and simultaneous occurrence of secondary somatic embryogenesis was suggested by Kärkönen (2000) and although interesting it was not supported yet by our data needing further revision.

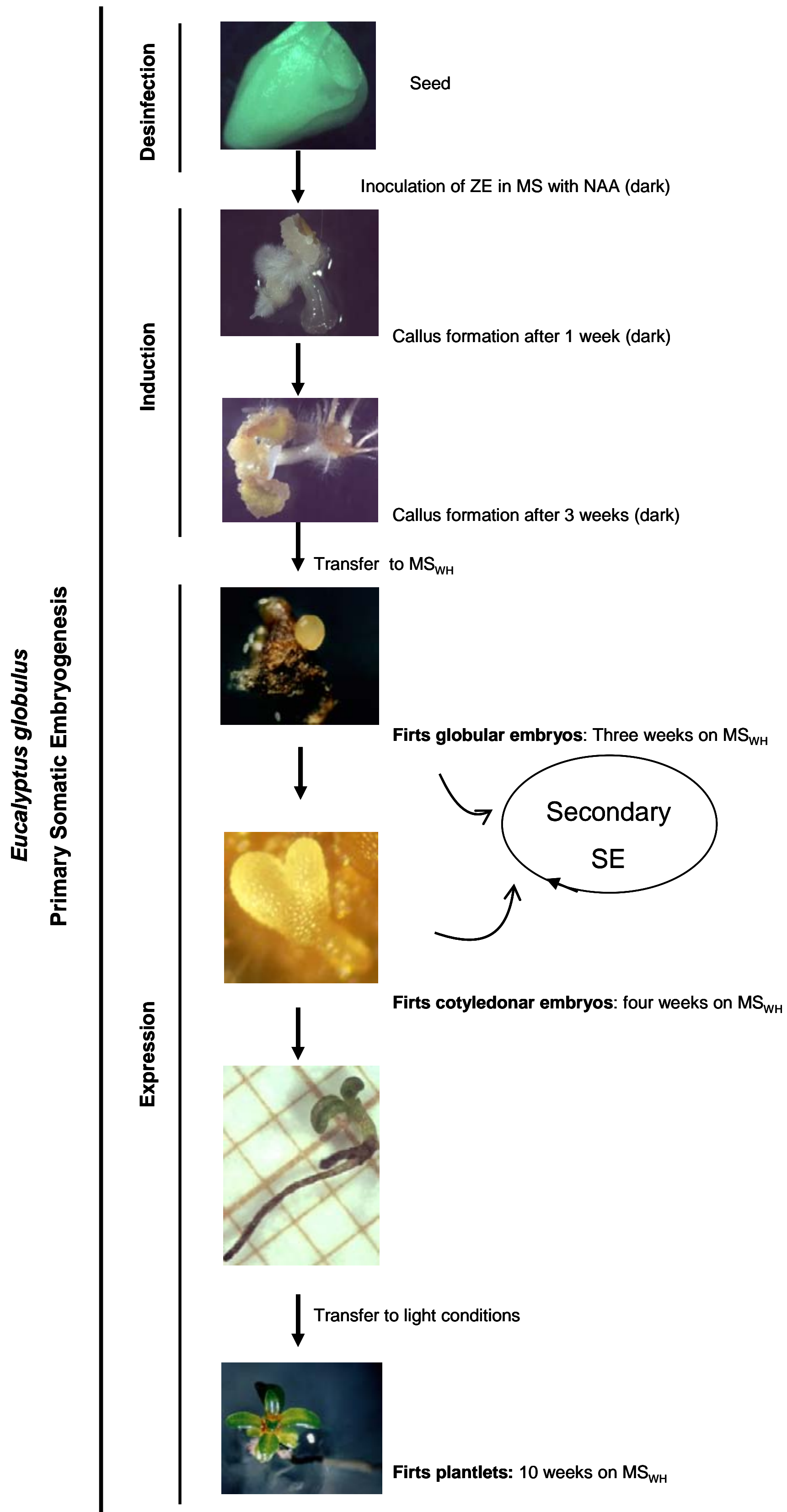


Figure 2: Global view of the main steps covering the whole process of *E. globulus* plant regeneration by primary SE from explant induction to plant conversion.

References

.Arruda SCC, Souza GM, Almeida M, Gonçalves AN (2000) Anatomical and biochemical characterization of calcium effect on *Eucalyptus urophylla* callus morphogenesis in vitro. Plant Cell Tissue Org Cult 63:143- 154

Bandyopadhyay S, Hamill JD (2000) Ultrastructural studies of somatic embryos of *Eucalyptus nitens* and comparisons with zygotic embryos found in mature seeds. Ann Bot 86:237–244

Barciela J, Vieitez AM (1993) Anatomical sequence and morphometric analysis during somatic embryogenesis on cultured cotyledon explants of camellia japonica. Ann Bot 71:395- 404

Branca C, Torelli A, Fermi P, Altamura MM, Bassi M (1994) Early phases in in vitro culture of tomato cotyledons: starch accumulation and protein pattern in relation to the hormonal treatment. Protoplasma 182:59–64

Cangahuala- Inocente GC, Steiner N, Santos M, Guerra MP (2004) Morphological analysis and histochemistry of *Feijoa sellowiana* somatic embryogenesis. Protoplasma 224:33-40

Canhoto JM, Cruz GS (1996) Histodifferentiation of somatic embryos in cotyledons of pineapple guava (*Feijoa sellowiana* Berg.). Protoplasma 19:34–45

Canhoto JM, Mesquita JF, Cruz GS (1996) Ultrastructural changes in cotyledons of Pineapple Guava (Myrtaceae) during somatic embryogenesis. Ann Bot 78:513- 521

Canhoto JM, Lopes ML, Cruz GS (1999) Somatic embryogenesis and plant regeneration in myrtle (Myrtaceae). Plant Cell Tiss Organ Cult 57:13- 21

Dodeman VL, Ducreux G, kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. J Exp Bot 48 (313):1493- 1509

Ho W-J, Vasil IK (1983) Somatic Embryogenesis in Sugarcane (*Saccharum officinarum* L.): Growth and Plant Regeneration from Embryogenic Cell Suspension Cultures. Ann Bot 51:719–726

Kärkönen A (2000) Anatomical study of zygotic and somatic embryos of *Tília cordata*. Plant Cell Tissue Org Cult 61:205- 214

Mangat BS, Pelekis MK, Cassels AC (1990) Changes in starch content during organogenesis in in vitro cultured *Begonia rex* stem explants. Physiol Plant 79:267-274

Martin AB, Cuadrado Y, Guerra H, Gallego P, Hita O, Martin L, Dorado A, Villalobos N (2000) Differences in the contents of total sugars, starch and sucrose in embryogenic and non-embryogenic calli from *Medicago arborea* L. Plant Sci 154:143–151

Merkle SA, Parrott WA, Flinn BS (1995) Morphogenic aspects of somatic embryogenesis. In Thorpe TA (ed.) In Vitro Embryogenesis in Plants. Kluwer Academic Publishers, Dordrecht. pp. 155-203

Muralidharan EM, Gupta PK, Mascarenhas AF (1989) Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus citriodora*. Plant Cell Rep 8:41–43

Muralidharan EM, Mascarenhas AF (1987) In vitro plantlet formation by organogenesis in *E. camaldulensis* and by somatic embryogenesis in *Eucalyptus citriodora*. Plant Cell Rep 6:256–259

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

Pinto G, Santos C, Neves L, C Araújo (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21:208-213

Puigderrajols P, Celestino C, Suils M, Toribio M, Molinas M (2000) Histology of organogenic and embryogenic responses in cotyledons of somatic embryos of *Quercus suber*. Int J Plant Sci 161(3):353–362

Prakash MG, Gurumurthi K (2005) Somatic embryogenesis and plant regeneration in *Eucalyptus tereticornis* Sm. Curr Sci 88 (8):1311- 1316

Quiroz-Figueroa F, Fuentes-Cerda CF, Rojas-Herrera R, Loyola- Vargas VM (2002) Histological studies on the developmental stages and differentiation of two different somatic embryogenesis systems of *Coffea arabica*. Plant Cell Rep 20:1141-1149

Rodriguez APM, Wetzstein HY (1998) A morphological and histological comparison of the initiation and development of pecan (*Carya illinoinensis*) somatic embryogenesis cultures induced with naphthaleneacetic acid or 2,4-dichlorophenoxyacetic acid. Protoplasma 204:71–83

Ruaud J, Churchill K, Pepper S (1997) Somatic embryogenesis initiation in *Eucalyptus nitens*. Acta Hort 447: 185-186

Stamp JA (1987) Somatic embryogenesis in cassava: the anatomy and morphology of the regeneration process. Ann Bot 57: 451–459

Termignoni R, Wang PJ, Hu CY (1996) Somatic embryo induction in *Eucalyptus dunnii*. Plant Cell Tissue Org Cult 45:129– 132

Watt MP, Blakeway FC, Termignoni R, Jain SM (1999) Somatic embryogenesis in *Eucalyptus grandis* and *E. dunnii*. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 5. Kluwer, UK, pp 63–78

Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behavior of cells as an embryogenic group. Ann Bot 57:443–462

II 5: Genetic control of somatic embryogenesis induction in *Eucalyptus globulus* Labill.

Chapter submitted as an original paper to a SCI journal:

Pinto G, Park Y-S, Neves L, Araujo C, Santos C (2007) Genetic control of somatic embryogenesis in *Eucalyptus globulus* Labill. Theoretical and Applied Genetics (Submitted)

Abstract

A reproducible protocol for somatic embryogenesis (SE) induction in *Eucalyptus globulus* from mature zygotic embryos has been available since 2002, opening new opportunities for large-scale application of SE methodology to this economically important species. However, for use in tree breeding programs, the frequency of SE initiation needs to be improved and controlled, and this was investigated in 13 open-pollinated (OP) families over 3 consecutive years. Genetic control of SE induction was studied using a diallel mating design with five parent trees. Results showed that SE induction varies across *E. globulus* families and over the years of seed production tested. Somatic embryogenesis was initiated on explants from 84% of the OP families tested in 2002 and 100% of the families tested in 2003 and 2004. The best results for percentage of induction, and for total number of somatic embryos produced, were obtained in 2003. Results concerning genetic control showed that SE induction is under the control of additive genetic effects, as 21.96% of variation in SE initiation was due to general combining ability (GCA) effect, whereas 6.46% was due to maternal effects. Neither specific combining ability (SCA) nor reciprocal effects were significant.

Keywords: control pollination, *Eucalyptus globulus*, genetic control, primary somatic embryos, somatic embryogenesis

Introduction

Compared with other in vitro propagation methods (e.g. organogenesis), somatic embryogenesis (SE) offers several advantages (Jain 1999). A separate rooting step is not required because somatic embryos have both a shoot and a root meristem, SE usually forms propagules faster and in much higher numbers per explant, and furthermore, the SE process can be automated, meaning it will eventually become cheaper than other clonal propagation techniques currently in use (Park *et al.* 1998a). A well-established SE protocol will allow embryogenic clonal lines to be cryopreserved in liquid nitrogen, while corresponding trees are tested in the field, as is currently being done for conifers (Park 2002). Thus, high-value clonal varieties can be developed by retrieving from liquid nitrogen those clones that performed the best in the field test, and subsequently propagating them (Park *et al.* 1998a). Elite clones can then be used for both advanced breeding programs and commercial forestry (Aitken-Christie 2001).

Use of SE in *Eucalyptus globulus* improvement programs depends on whether this process can be applied to a broad range of genotypes. In fact, low embryogenic initiation rates are generally observed in *Eucalyptus* species, which may limit the number of genotypes that can be propagated by SE (Pinto *et al.* 2002, for review see Muralidharan and Mascarenhas 1995, Watt *et al.* 1999). Other limitations, such as recalcitrant germination of somatic embryos, genotype influences, and somaclonal variation, have hindered SE commercialization in a wide range of forest trees (Merkle 1995, Jain 2006). Pullman and Johnson (2002) also refer to similar factors that currently limit commercialization of SE for lobolly pine (*Pinus taeda* L.), including low initiation rates, culture decline causing low or no embryo production, and the inability of somatic embryos to fully mature and germinate.

The SE system for most spruce (*Picea* spp.) and some pine (*Pinus* spp.) species is sufficiently refined to be considered for commercial use (Park *et al.* 2006). For *Eucalyptus*, although SE is not yet ready to be used commercially, there is sufficient information about induction and the subsequent histodifferentiation, maturation, and germination stages to provide a reasonable starting point for developing an optimizing regeneration system based on SE. Concerning *E. globulus,* we have already reported a standard protocol for plant regeneration using SE (Pinto *et al.* 2002, 2004). This protocol allows new studies on the

genetic control of *E. globulus* SE induction by assaying SE potential in OP and control-pollinated (CP) families.

The most important role in SE is enabling the implementation of multi-varietal forestry (MVF), defined by Park *et al.* (2006) as the deployment of genetically tested tree varieties in plantation forestry and preferably integrated in tree improvement programs. When combined with conventional tree breeding, MVF offers additional advantages. When a vegetative propagation system is optimized, it can mass-produce the same varieties consistently over time (Park 2002). Sufficiently high SE initiation and subsequent plant conversion rates are important for maintaining genetic diversity of clonal plantations while achieving a high level of genetic gain.

Improving the induction rate has been a major area of SE research, and is influenced by several factors, such as tissue culture media, stage of maturity of the zygotic embryo (ZE) explants, and genetic influence. In fact the genetic influence during the SE process is well known (Merkle *et al.* 1995), and understanding genetic control is an important element in improving the SE process (Park *et al.* 1998a). Depending on the type and magnitude of genetic variation, improved SE initiation may be introduced in recalcitrant genotypes (Park *et al.* 1998b; Park 2002). Concerning Dicotyledonous woody species, Hernández *et al.* (2003) tested the influence of genotype at several steps during the whole process of SE regeneration of *Quercus suber* plants, and found a significant genetic influence in all steps, indicating that genetic improvement is possible. A genotypic effect on the induction of embryogenesis in *Q. robur* leaves has also been suggested by Cuenca *et al.* (1999).

Concerning the importance of the year of production, some authors suggested that most vegetative propagation techniques based on morphogenic processes are conditioned by season, which influences regulation of the cell cycle, and thus affects morphogenic processes (Hartmann and Kester 1983, Anderson *et al.* 2001). Collection date often significantly affects SE induction. An example of this was shown in the frequency of leaves producing SE of *Q. suber* (Hernández *et al.* 2003). Most *E. globulus* breeding programs now involve CP assessment (Silva *et al.* 2004), which allows specific crosses to be made between individuals of known genetic value, thus assuring high levels of genetic gains. Celbi was the first in the world to use control-pollination on a commercial scale in *Eucalyptus* to produce large (kilograms) quantities of seed for commercial plantation

establishment (Leal and Cotterill 1997). Establishments of plantations by Celbi since 1996 has been based on outstanding full-sib *E. globuuls* families produced by mass-pollination using a refinement of the “one-stop” (Harbard *et al.* 1999) pollination systems (Cotterill *et al.* 2000). In this case somatic embryogenesis could allow mass production of selected clones from relatively small quantities of control pollinated seed from controlled crosses where outstanding parents are difficult to flower and/or only a small quantity of seed is produced as well as a strategy to speed up the deployment of outstanding families identified in progeny trials.

Based on the quantitative genetic analysis of full-sib families derived by diallel crossing, we were able to partition the total genetic variation into separate genetic components. Genetic variance is usually partitioned into additive and non-additive components. This information is required to properly evaluate the potential for genetic gain from various breeding and deployment options used in the genetic improvement of forest trees.

The purpose of these experiments is to examine embryogenic capacity among the *E. globulus* families and variability in yearly production using a standard SE protocol. The degree of genetic control during SE was examined using control-crossed families, with a potential application to improve SE process and integration into breeding programs.

Materials and methods

Plant material

Two experiments were conducted: one was to study the effects of family and year of seed production, and the other the genetic control of SE induction. Two types of *E. globulus* seeds were used in the experiments.

Experiment 1: Effect of genotype and yearly variation

In Experiment 1, seeds from 13 OP families, numbered consecutively CB01 to CB13, were used over 3 consecutive years beginning in 2002. However, due to a lack of seed, no observations for CB02 and CB08 were made in 2004. These parents are a part of Celbi’s breeding program.

Experiment 2: Genetic control of SE induction using controlled crosses.

Plant material for Experiment 2 was derived by a five-tree diallel mating scheme in 2004. The five parent trees were selected based on the results of Experiment 1, conducted in 2002. Two trees were used from each of high SE potential parents (CB04 and CB06), intermediate SE potential parents (CB09 and CB11), and poor (or no) SE potential parents (CB03), for a total of five trees. The controlled pollination was performed at Celbi´s clonal seed-orchards located at Óbidos district by the method of one-stop pollination (OSP) as described by Harbard *et al.* (1999). One hundred flowers were emasculated at anthesis, followed by slicing the stigma and top of the style to provide a site for pollen adherence. Pollen is applied immediately, followed by isolation of the style from contaminating pollen by covering with a section of tubing. The controlled pollinations were made to produce a total of 20 CP families, including the reciprocals but excluding selfs. However, uunfavourable weather conditions during the crossing period in 2004 due to frost resulted in loss of flowers and low seed yield. Consequently, seeds from five controlled crosses were lost at this stage. Therefore, only 15 full-sib families were available for this experiment.

Initiation of somatic embryogenesis

In both experiments, we used a standard protocol (Pinto *et al.* 2002) and the same procedure. Seeds of *E. globulus* were surface sterilized with a mixture of absolute ethanol: hydrogen peroxide 30% (v/v) for 15 min, and washed twice in sterile distilled water, adding fungicide when necessary, for 10 min. Seeds were then left to imbibe overnight in sterile distilled water. Cultures were initiated from entire mature ZE explants after aseptically removing the seed coat. A total of 80 embryo explants were used in eight 90-mm diameter Petri dishes (Sarsted, Nümbrecht, Germany) by placing ten explants per Petri dish. The cultures were kept in the dark at 24 ± 1ºC for 3 weeks on MS medium (Murashige and Skoog 1962) with 3 mg l^{-1} NAA (16.1 μM, α-naphthalene acetic acid). All media used during this study were supplemented with 30 g l^{-1} sucrose and 2.5 g l^{-1} gelrite®; pH was adjusted to 5.8, and media were autoclaved at 121ºC for 20 min. Culture media, sucrose, gelrite and NAA were purchased from Duchefa (Haarlem, Netherlands).

After this 3-week period, explants were transferred for 4 months to expression medium, MS_{WH}, which was the same medium used in initiation but without growth

regulators (Figure 1). Explants were transferred monthly to fresh medium. The embryogenic potential of the explants, calculated as the percentege of explants showing SE, was determined cumulatively every time the explants were transfered to fresh medium.

At the end of each experiment, i.e., 16 weeks after transfer to MS_{WH} , we obtained an embryogenic response for the following parameters: percentage of explants showing SE, total number of somatic embryos, and type of somatic embryos, such as embryogenic mass, globular, cotyledonary, and plant.

Figure 1: Standard protocol used during the experience

Data analysis

For Experiment 1, data on the percentage of SE initiation among the 13 OP families studied in 3 consecutive years were subjected to analysis of variance (ANOVA) using the model:

$Y_{ijk} = \mu + T_i + F_j + TF_{ij} + e_{ijk}$,

where Y_{ijk} is the initiation percentage the kth Petri dish of the jth family in the ith year; μ is overall mean; T_i is the effect of ith year; F_j is the effect of jth family; TF_{ij} is the interaction effects of ith year and j^{th} family; and e_{ijk} is the random error component.

For Experiment 2, the data on the percentage of initiation among 15 full-sib families produced by the diallel mating were analyzed using the model:

$Y_{ijk} = \mu + G_i + G_j + S_{ij} + M_i + M'_j + R_{ij} + e_{ijk}$,

where Y_{ijk} is the kth observation of SE initiation for the family resulting from the ith female and j^{th} male; μ is the overall mean; G_i (G_j) is the general combining ability (GCA) effect of of the ith (j^{th}) parent; S_{ij} is the specific combining ability (SCA) effect of the ith and j^{th} parents; M_i is the maternal effect of ith parents such that M'_j = - M'_j; and R_{ij} is the reciprocal effects involving the reciprocal cross of ith and j^{th} parents; e_{ijk} is the random error component. The calculation was performed using a computer program by Schaffer and

Usanis (1969). The percentage data were transformed by taking the arcsine value of the square root ($Sin^{-1}(o)$) before ANOVA, but the results are reported in untransformed scale.

Results

Experiment 1

Analysis of variance indicated that there were significant differences in SE initiation among the years of seed production and among the OP families; however, there was no year x family interactions (Table 1). The SE initiation was best in 2003, with an overall mean of 12.9%, followed by 5.5% and 3.3% in 2004 and 2002, respectively. The percentage of SE initiation in 2003 was significantly different from that of 2002 and 2004 ($P < 0.05$). In 2002, only 11 of the 13 (84%) families produced somatic embryos; however, in 2003, all the families produced somatic embryos. Families CB002 and CB008, which were previously responsive, did not produce enough seed to be included in the 2004 experiment. Mean initiation percentage when averaged over the 3 years ranged from 3.9% to 13.3 % (Fig. 2). Furthermore, a greater number of somatic embryos were in an advanced stage of histodifferentiation (cotyledon phase) and somatic plants (Table 2). With the exception of families CB01, CB03, CB05, CB06, and CB10, all others succeeded in regenerating plants.

Table 1. Analysis of variance of SE initiation for open pollinated families for the three consecutive years

Source	df	Mean Squares	F-ratio	P-value
Year (Y)	2	2577.9	32.38	0.0000
Families (F)	12	213.3	2.68	0.0021
Y x F	22	95.2	1.20	0.2520
Error	241	25.8		

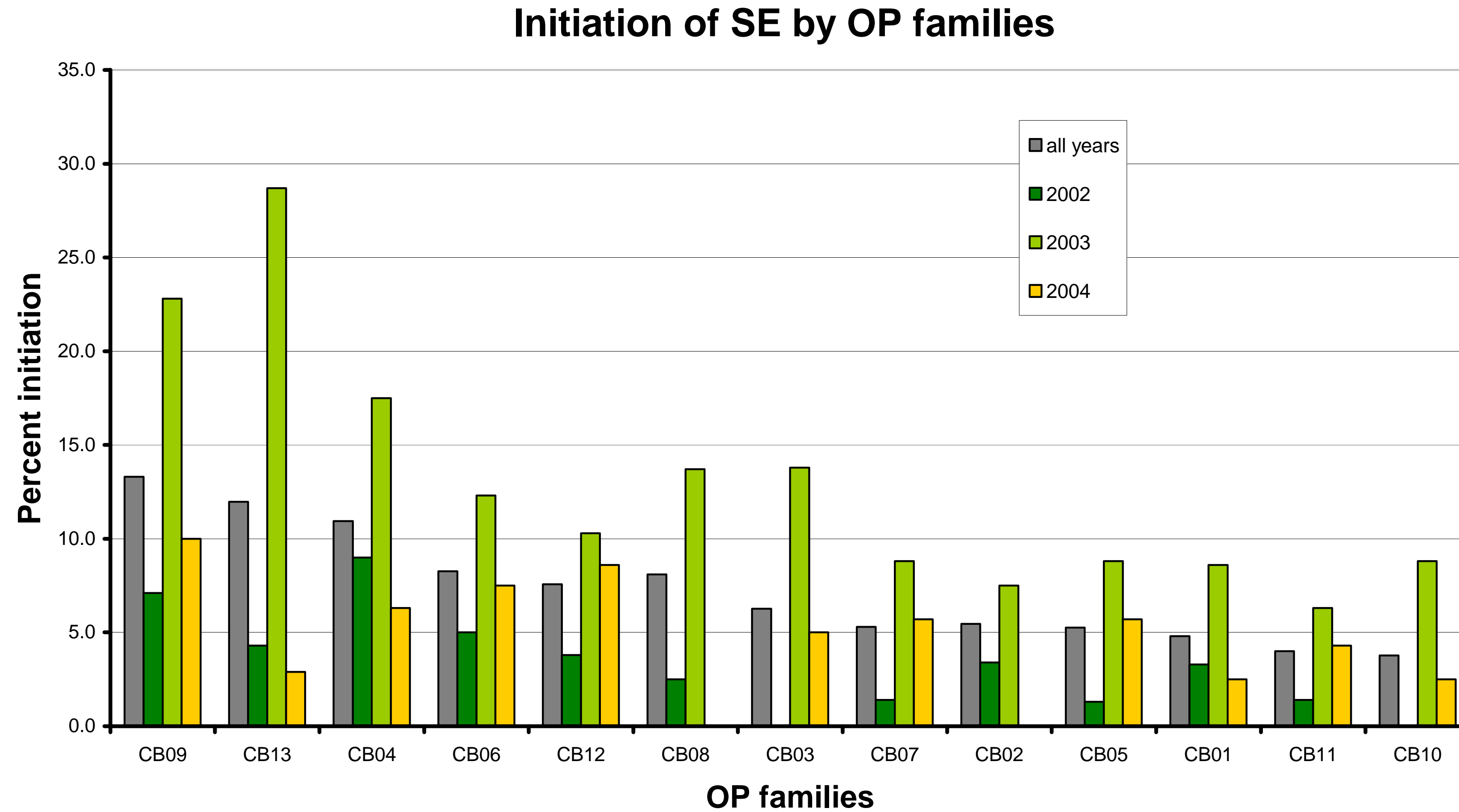


Figure 2: Mean initiation percentage of SE among open pollinated families for the total of the 3 years and for each year tested

Table 2: Characterization of embryogenic response of 13 OP seed families using mature zygotic embryos in *Eucalyptus globulus* after 16 weeks in expression medium for 2002, 2003 and 2004 (G: globular, C: cotyledonar, P: Plant)

OP family	% initiation			OP 2002 Type of Somatic Embryos			OP 2003 Type of Somatic Embryos			OP 2004 Type of Somatic Embryos			Ranking Mean of the 3 years
	2002	2003	2004	G	C	P	G	C	P	G	C	P	
CB01	3.3	8.6	2.5	4	6	0	5	3	0	2	0	1	7
CB02	3.4	7.5	*	1	1	0	20	5	1	-	-	-	3
CB03	0.0	13.8	5.0	-	-	-	9	30	0	3	2	0	13
CB04	9.0	17.5	6.3	23	0	0	13	12	2	15	0	0	5
CB05	1.3	8.8	5.7	4	1	0	9	6	0	2	3	0	9
CB06	5.0	12.3	7.5	3	5	0	12	1	0	16	2	0	1
CB07	1.4	8.8	5.7	6	0	0	4	4	3	6	0	0	10
CB08	2.5	13.7	*	5	1	0	19	8	1	-	-	-	4
CB09	7.1	22.8	10.0	14	0	0	54	23	1	31	4	0	2
CB10	0.0	8.8	2.5	-	-	-	5	2	0	2	0	0	12
CB11	1.4	6.3	4.3	1	0	0	5	3	0	6	0	0	11
CB12	3.8	10.3	8.6	2	1	0	3	4	4	8	3	0	8
CB13	4.3	28.7	2.9	6	0	0	33	30	4	2	0	0	6

The ranges of means were from 0.0 to 9.0% for 2002, from 6.3 to 28.7% for 2003, and from 2.5 to 10.0% in 2004. There was variation among families in different years, but the interaction was not significant. Although the SE potential varied among OP families, the response pattern during the experiment was consistent. For example, CB06 and CB09 are ranked as highly responsive, whereas the families that had low SE initiation (CB10 and CB11) tended to be low in each of the years.

Experiment 2: Genetic control of SE induction

The partitioning of total variation indicated that 21.96% of total variance was due to GCA effects, but there were no SCA or reciprocal effects. Furthermore, 6.46% of variation was accounted for by maternal effects. However, 71.58% of variation was due to error variance (Table 3). Therefore, SE initiation is under strong additive genetic control.

Table 3. Estimated variance components (Standard Deviation (SD) and their percentages to total variance from diallel cross experiment)

Variance	Estimate	SD	%
2 GCA	12.96	17.36	21.96
SCA	0.00	2.74	0.00
Maternal	3.81	2.16	6.46
Reciprocal	0.00	0.41	0.00
Error	42.25	5.89	71.58

Somatic embryogenesis was obtained from all 15 families derived from the diallel mating scheme, with means ranging from 1.3% to 13.8% (Table 4). The highest SE initiation percentage (14.1%) was obtained from a cross between CB06 (female) and CB09 (male). This particular cross also led to the highest number of somatic embryos in the cotyledon stage and of somatic plantlets (Table 4). Whenever conversion occurred, families CB 06 or CB 09 were involved. Mean initiation percentage of parents, when used as females, ranged from 1.9% (CB04) to 9.3% (CB6) whereas, when used as males, they ranged from 2.6% (CB11) to 8.2% (CB04) (Table 5).

Results also show that the induction stage is apparently influenced by the female parent. With the exception of CB04, parents had a slightly higher initiation percentage when used as females than males. Families CB06 and CB09 were consistently good parents in both OP and diallel tests.

Table 4: Characterisation of the embryogenic response after 16 weeks in expression medium (MS_{WH}) for the 15 controlled crosses tested. (**G**: globular, **C**: cotyledonar, **P**: Plant)

(Mother x Father)	% initiation	Total nº of somatic embryos	Average nº of somatic embryos per explant (n=80)	Type of SE (total) G	C	P	Petri Dish showing SE (n=8)
CB 0006 X CB 0003	6.9	13	0.16	10	3	-	3
CB 0006 X CB 0009	14.1	45	0.56	36	6	3	7
CB 0006 X CB 0011	3.8	16	0.20	15	1	-	3
CB 0006 X CB 0004	12.5	51	0.64	48	2	1	6
CB 0004 X CB 0011	1.3	1	0.01	-	1	-	1
CB 0004 X CB 0009	3.8	21	0.26	15	6	-	3
CB 0004X CB 0006	1.3	1	0.01	1	-	-	1
CB 0004 X CB 0003	1.3	6	0.07	6	-	-	1
CB 0009 X CB 0003	5.7	18	0.22	15	2	1	3
CB 0009 X CB 0006	11.3	26	0.32	23	2	1	7
CB 0011 X CB 0009	5.0	6	0.07	6	-	-	4
CB 0011 X CB 0004	3.8	7	0.09	6	1	-	3
CB 001 1X CB 0003	3.8	14	0.17	14	-	-	2
CB 0003 X CB 0006	6.3	15	0.19	14	1	-	6
CB 0003 X CB 0009	7.3	37	0.46	35	-	2	5

Table 5. Mean SE initiation percentage by the parents used in diallel cross.

Parent	Female	Male	Mid-parent	OP Families (2004)
CB03	6.7	4.4	5.6	5.0
CB04	1.9	8.2	5.1	6.3
CB06	9.3	6.3	7.8	7.5
CB09	8.5	7.5	8.0	10.0
CB11	4.2	2.6	3.4	4.3

Discussion

In this study we demonstrated that our standard protocol was effective for initiating SE in several OP families. From the 13 OP families tested, 84% showed SE induction in 2002 and 100% in 2003 and 2004. However, variation in the percentage of SE response was large, depending on the OP family. The effect of genotype in SE initiation is well known in different conifer species (Park *et al.* 1993, 2006) including, e.g., *P. taeda* (Becwar *et al.* 1990, MacKay *et al.* 2006), *P. sylvestris* (Keinonen-Mettälä *et al.* 1996), *P. strobus* (Garin

et al. 1998, Klimaszewska *et al.* 2001), *P. monticola* (Percy *et al.* 2000), and *P. pinaster* (Miguel *et al.* 2004), although there is a lack of information on woody dicotyledonous species.

Percy *et al.* (2000), in a first experiment involving 10 OP families of *Pinus monticola*, reported that SE initiation only occurred in one family, and at low rates (0.3%). Due to the strong family effect, the authors selected fewer, more responsive families for protocol optimization and plant production experiments in subsequent years (Percy *et al.* 2000). In a similar approach, Häggman *et al.* (1999) also emphasized the effect of seed family on the success of SE induction in Scots pine (*P. sylvestris*). Most of these studies reporting screening of SE induction in consecutive years were conducted in order to determine the best period of competence of the zygotic embryo for SE, as it is well known that this period may be extremely narrow in conifers (Park 2002). More recently, for *P. pinaster*, Miguel *et al.* (2004) also showed that the ability to initiate embryogenic masses was significantly influenced by collection date of the cones, and they correlated the optimal date with a stage prior to the development of cotyledon primordia of the zygotic embryo.

Our main aim was to understand the importance of the year of production in *E. globulus* SE initiation. For this reason, our experiments were initiated at the same time in 3 consecutive years (2002, 2003, and 2004), using a standard protocol and standard laboratory conditions. Högberg *et al.* (1998) noted that the use of a standard protocol is a practical necessity when dealing with a large number of genotypes. Using a single protocol (initially optimized for one cultivar), Chengalrayan *et al.* (1998) tested the genetic control of SE potential in 15 peanut genotypes and concluded that, although the protocol was effective for all genotypes, most of the variation in SE response depended on the genotypes. Similarly to this approach, we tested a standard protocol optimized for one family (CB04) and produced SE in the 13 OP families and 15 CP families used in the experiments. However, despite the success in using a standard protocol for the different *E. globulus* families tested here, we emphasize that further studies on induction and conversion media formulations should be encouraged in order to improve the initiation process (Pinto *et al.* 2006). We also note that, aside from the influence of genotype on SE induction, there is strong evidence that seed quality is an important factor. Seed quality is conditioned by intrinsic factors and by pre- and post-harvesting conditions.

Most of the genetic parameters reported to date for *E. globulus* are based on OP progenies (Lopez *et al.* 2002). The most widely used method for operational production of improved conifer seed has been OP seed orchards (Sutton 2002). The principal advantage of this approach is the comparatively low production cost (Silva *et al.* 2004). Deployment programs based on clonal forestry or on SCA of crosses exploit both additive and non-additive genetic effects (Högberg *et al.* 1998), whereas strategies based on OP seed orchards concentrate on the use of additive effects only. However, seed production by controlled crossing of selected individuals is an increasingly important method of capturing genetic gain from tree breeding and has been the adopted as the main deployment method by Celbi since 1990.

Genetic control of SE induction

Our results confirm the importance of genetic control in SE induction of *E. globulus,* and provide strong evidence as to the nature and magnitude of genetic control. Additive genetic effects explain SE induction, as 21.96% of total variation in SE initiation was due to the GCA effect, whereas 6.46% was due to maternal effects. Neither SCA nor reciprocal effects were significant. This means that SE initiation can be improved through the use of highly responsive parents. It also indicates that the direction of the cross can have an effect on SE initiation.

Our data show genetic control of SE in a dicotyledonous woody species, similar to what has been previously reported for conifers. For example, Niskanen *et al.* (2004) used controlled crosses of seven Scots pine trees, producing 49 families that included both reciprocals and selfings. In their study, the effect of parent genotypes was evaluated and the authors suggested that the effect of maternal parent was most pronounced at culture initiation. After 6 months in tissue culture, the maternal effect had decreased and the effects of both parents had become significant.

Park *et al.* (1993) demonstrated that the initiation of SE in spruce is under strong additive genetic control, with variance due to GCA accounting for 42% of the total phenotypic variance. The genetic effect in the subsequent maturation and germination phases was less strong (Park *et al.* 1994), showing that it is the initiation phase that can be most effectively manipulated by breeding. A similar model may be applicable in *E. globulus*, although further analyses must be undertaken.

In 2004, it was possible to compare the explant response from OP vs. CP seed families from the same orchard. The mean initiation percentage for the OP test was similar (5.5%) to the mean of the diallel families (6.0%), and CB06 and CB09 consistently responded well (Table 5). Percy *et al.* (2000) conducted a similar study in *P. monticola* and concluded that the overall initiation frequencies were similar for both CP and OP seed, 0.9% and 0.8%. They also found that, in pines, seeds of OP families may respond to SE initiation better than seeds of CP families (Percy *et al.* 2000, Mackay *et al.* 2006).

Our results showed that crosses between CB06 (female) and CB09 (male) gave rise to the highest SE induction response and also led to the highest number of somatic embryos in the cotyledonary stage and of SE-derived plantlets. These families also show the best response with OP seed and occupied the top two places in the ranking.

Based on the OP results in 2002, CB03 was selected as a poorly responding family as no SE was obtained. Subsequently, CB03 produced 13.8% and 5.0% in 2003 and 2004, respectively. Thus, CB003 could not be considered as a consistently poorly responding family. Therefore, the possibility of improving SE potential by controlling pollination with this parent could not be confirmed, although such a possibility exists because SE is under additive genetic control. In order to use this finding in practice, a large number of maternal parents must be screened to correctly identify SE potential and perform controlled pollinations. In addition, although our standard SE protocol produced SE from all the OP families included in the experiment, further optimization of media protocol should be explored by fine tuning the media components, such as macro and micronutrients and plant growth regulators.

Conclusion

The application of SE in breeding requires further research and development because SE in *Eucalyptus* is substantially different compared with operational conifer SE systems. In most conifer SE systems, the phases of SE, i.e., initiation, proliferation, maturation, and germination, are highly controllable. For example, conifer embryogenic tissue will continuously proliferate as long as it is subcultured on fresh maintenance medium and, when maturation of somatic embryos is desired, withdrawal of certain plant growth regulators (e.g. 2,4-D) and addition of ABA can trigger the maturation phase. However, such control is difficult for *Eucalyptus*. Although this study concerned primary SE, we also

looked at secondary SE, mostly because, without sustained embryonic culture, SE translates to only a few plantlets. Merkle (1995) reported that this conversion means the end of the culture regarding clonal propagation. This author therefore states that primary somatic embryos fail to mature and instead give rise to successive cycles of new embryo production (Merkle 1995). Therefore, a deeper knowledge of genetic control of secondary SE may improve our understanding of how those somatic embryos formed during the primary process produce new cycles of secondary embryos.

References

Aitken-Christie J (2001) Somatic embryogenesis for large-scale clonal testing and propagation of elite material. In: Proceedings of the International conference on wood, breeding, biotechnology and industrial expectations, Bordeaux, France, pp 9

Anderson JV, Chao WS, Horvath DP (2001) A current review on the regulation of dormancy in vegetative buds. Weed Sci 49:581–589

Becwar MR, Nagmani R, Wann SR (1990) Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can J For Res 20:810–817

Chengalrayan K, Mhaske VB, Hazra S (1998) Genotypic control of peanut somatic embryogenesis. Plant Cell Rep 17:522–525

Cotterill PP, Araújo C, Lencart P (2000) Project 3D: Celbi's advanced-generation *Eucalyptus globulus* improvement. Celbi Investigation Report No. 75, Volume 8, pp. 67-178

Cuenca B, San- José MC, Martínez MT, Ballester A, Vieitez AM (1999) Somatic embryogenesis from stem and leaf explant of *Quercus robur* L. Plant Cell Rep 18:538–543

Garin E, Isabel N, Plourde A (1998) Screening of large numbers of seed families of *Pinus strobus* L. for somatic embryogenesis from immature and mature zygotic embryos. Plant Cell Rep 18:37–43

Häggman H, Jokela A, Krajnakowa J, Kauppi A, Niemi K, Aronen T (1999) Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction. J Exp Bot 50:1769–1778

Harbard JL, Griffin AR, Espejo J (1999) Mass Control pollination of *Eucalyptus globulus*: A pratical reality. Can J For Res 29 (10): 1457-1463

Hartmann HT, Kester DE (1983) Plant propagation: principles and practices, 4thed, Prentice-Hall, London

Hernández I, Celestino C, Alegre J, Toribio M (2003) Vegetative propagation of Quercus *suber* L. by somatic embryogenesis II Plant regeneration from selected cork oak trees. Plant Cell Rep 21:765–770

Högberg K-A, Ekberg L, Norell L, Arnold SV (1998) Integration of somatic embryogenesis in a tree breeding programme: a case study with *Picea abies*. Can J For Res 28:1536–1545

Jain SM (1999) An overview on progress of somatic embryogenesis in forest trees. In: Altman A, Ziv M. Izhar S (eds) Plant biotechnology and in vitro biology in the 21st century. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 57–63

Jain SM (2006) An update on overall recent progress on somatic embryogenesis in forest trees. In: Plantation Technology in Tropical Forest Science. K Suzuki, K Ishii, S Sakurai and S Sasaki (Eds.). Springer- Verlag, Tokyo, Japan. Pp 113-122

Keinonen-Mettälä K, Jalonen P, Eurola P, von Arnold S, von Weissenberg K (1996) Somatic embryogenesis of *Pinus sylvestris*. Scand J For Res 11:242–250

Klimaszewska K, Park Y-S, Overton C, MacEacheron I, Bonga JM (2001) Optimized somatic embryogenesis in *Pinus strobus* L. In Vitro Cell Dev Biol Plant 37:392–399

Leal AMC, Cotterill PP (1997) Mass control-pollination of *Eucalyptus globulus*. Proc., IUFRO Symposium on Silviculture and Genetic improvement of *Eucalyptus*. Salvador, Brazil-August

Lopez GA, Potts BM, Dutkowski GW, Apiolaza LA, Gelid P (2002) Genetic variation and inter-trait correlations in *Eucalyptus globulus* base population trials in Argentina. For Genet 9:223–237

MacKay JJ, Becwar MR, Park YS, Corderro JP, Pullman GS (2006) Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genet Genom 2:1–9

Merkle SA (1995) Strategies for dealing with limitations of somatic embryogenesis in hardwood trees. Plant Tiss Cult Biotech 1:112–121

Merkle SA, Parrott WA, Flinn BS (1995) Morphogenic aspects of somatic embryogenesis. In: Thorpe TA (ed) In vitro embryogenesis in plants. Kluwer Academic Publ, Dordrecht, The Netherlands, pp 155–203

Miguel C, Gonçalves S, Tereso S, Marum L, Maroco J, Oliveira MM (2004) Somatic embryogenesis from 20 open-pollinated families of Portuguese plus trees of maritime pine. Plant Cell, Tissue and Organ Culture 76: 121–130

Muralidharan EM, Mascarenhas AF (1995) Somatic embryogenesis in Eucalyptus. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants, vol. 2. Kluwer, Dordrecht, pp 23–40

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473–497

Niskanen A-M, Lu J, Seitz S, Keinonen K, Weissenberg KV, Pappinen A (2004) Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus sylvestris*). Tree Physiol 24:1259–1265

Park YS (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Ann For Sci 59:651–656

Park YS, Barrett JD, Bonga JM (1998a) Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell Dev Biol Plant 34:231–239

Park YS, Bonga JM, Mullin TJ (1998b) Clonal forestry In: Mandal AK,Gibson GL (eds) Forest genetics and tree breeding, CBS Publ & Distrib,New Delhi, India, pp 143–167

Park YS, Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K, Bonga JM (2006) Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. Plant Cell Tiss Org Cult 86:87–101

Park YS, Pond SE, Bonga JM (1993) Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for tree breeding.Theor Appl Genet 86:427–436

Park YS, Pond SE, Bonga JM (1994) Genetic control in somatic embryos exposed to storage, maturation treatments, germination, and cryopreservation. Theor Appl Genet 89:742–750

Percy RE, Klimaszewska K, Cyr DR (2000) Evaluation of somatic embryogenesis for clonal propagation of western white pine. Can J For Res 30:1867–1876

Pinto G, Loureiro J, Lopes T, Santos CV (2004) Analysis of genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry. Theor Appl Genet 109:580–587

Pinto G, Santos C, Neves L, Araújo C (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21(3):208–213

Pinto G, Silva S, Coutinho J, Araujo C, Neves L, Santos C. (2006) Importance of media mineral composition on the induction of somatic embryogenesis in *Eucalyptus globulus* Labill. 2º Simposio Iberoamericano de *Eucalyptus globulus*. 17- 20 of October. Pontevedra, Spain

Pullman GS, Johnson S (2002) Somatic embryogenesis in loblolly pine (*Pinus taeda* L.): improving culture initiation rates. Ann For Sci 59:663–668

Schaffer HE, Usanis RA (1969) General least squares analysis of diallel experiments - A computer program - DIALL. Genetics Department Research Report No. 1. North Carolina State University, Raleigh, NC. USA

Silva JC, Borralho NMG, Potts BM (2004) Additive and non-additive genetic parameters from clonally replicated and seedling progenies of *Eucalyptus globulus*. Theor Appl Genet 108:1113–1119

Sutton B (2002) Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. Ann. For Sci. 59: 657–661

Watt MP, Blakeway FC, Termignoni R, Jain SM (1999) Somatic embryogenesis in *Eucalyptus grandis* and *E. dunnii*. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 5. Kluwer, UK, pp 63–78

Chapter III

<u>Secondary Somatic Embryogenesis</u>

III 1: Analysis of the genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry

Chapter published as an original paper in a SCI journal:

Pinto G, Loureiro J, Lopes T, Santos C (2004) Analysis of the genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry. Theoretical Applied Genetics 109: 580- 587

Abstract

The nuclear DNA content of *Eucalyptus globulus* Labill. somatic and zygotic embryos and leaves were determined by flow cytometry in order to assay if somatic embryogenesis induces DNA content and ploidy changes in this species. Mature zygotic embryos derived from open-pollination orchard families were collected in the Centre of Portugal. One group was kept for nuclear DNA content and ploidy analysis and another group was used for establishing embryogenic cultures. Mature zygotic embryos were grown on Murashige and Skoog medium supplemented with 3% (w/v) sucrose and with 3 mg l^{-1} α-naphtalene acetic acid. After three weeks explants were transferred to MS medium without growth regulators. Globular somatic embryos from approximately eight months old embryogenic cultures were used in this assay. Flow cytometry with propidium iodide staining was employed to estimate DNA ploidy levels and nuclear DNA content of mature zygotic embryos, of somatic embryos and of leaves from the mother field tree. Zygotic embryos had a nuclear DNA content of 1.32 pg/2C, somatic embryos had a nuclear DNA content of 1.39 pg/2C and leaves from the field tree had a nuclear DNA content of 1.40 pg/2C. The values found for somatic embryos and mother plant do not differ statistically ($P \leq 0.05$) but both differ from the content of zygotic embryos ($P \leq 0.05$). Results apparently indicate no ploidy changes induced during the embryogenic process. However, the differences found between field plants and zygotic embryos may suggest that some aspects must be evaluated carefully, as propidium iodide fluorescence may be potentially influenced by secondary compounds (e.g. anthocyanins, tannins) present in *E. globulus* somatic embryos and mature leaves. Therefore we believe that the somatic embryogenesis methodology used did not induce major genetic changes in the somatic embryos and the primary goal of “true-to-type” propagation was assured.

Keywords: *Eucalyptus globulus*, flow cytometry, ploidy stability, nuclear DNA content, secondary compounds, somatic embryogenesis

Introduction

The genus *Eucalyptus* comprises approximately 700 species and varieties although only 1% of them are used for industrial purposes (Watt *et al.* 1999). *Eucalyptus* kraft pulp industry is undoubtedly one of the most important applications and it is based largely on two species, namely *E. globulus* and *E. grandis* hybrids. The natural genetic diversity within *Eucalyptus* species is enormous and can be further enhanced by interspecific hybridisation making it an attractive genus for breeding (Eldridge *et al.* 1993). Due to its versatility, fast growth and fibre characteristics, *E. globulus* is nowadays grown worldwide. In Portugal, it was introduced 150 years ago, representing nowadays the third forestry species, covering approximately 672,140 ha of forest (Direcção Geral de Florestas 2001).

Both seed and clonal propagation have some disadvantages in *Eucalyptus globulus* breeding, namely the high level of heterozygosity found in seeds and the very irregular adventitious rooting behaviour (5-64%) (Marques *et al.* 1999) found in the vegetative propagation of some desired genotypes. Somatic embryogenesis has the capacity to provide large scale propagation systems for many species including forest trees and the advantage that both a root and a shoot meristem are present simultaneously in somatic embryos. This methodology also simplifies the conservation methods, as a large number of genotypes may be stored in a limited space, while they are being field-tested for genotype *vs.* environment interactions. The knowledge of these interactions, as in other forestry species, is of particular importance in *Eucalyptus* spp. (Zobel 1993).

A reproducible protocol for somatic embryogenesis in *E. globulus* from mature zygotic embryos was finally established by Pinto *et al.* (2002), opening new perspectives for a large-scale application of this methodology to this economically important species. Although somatic embryogenesis in woody plants has long been regarded as a safe methodology, in what concerns to somaclonal variation, some recent evidence showed that this might not be the case (Endemann *et al.* 2002). Therefore to minimize the risk for genetic variation a rapid screening for possible changes has to be applied to assure the primary goal of “true-to-type” propagation.

Numerical chromosomal changes which are the most frequently reported changes in broadleaf and conifer species (e.g. DeVerno 1995, Bueno *et al.* 1996) are not seen in RAPD analysis (Fourré *et al.* 1997). Conventional cytogenetic techniques, as karyotyping,

for this type of detection are laborious and time consuming. This drawback can be circumvented by applying flow cytometry, provided that it is sensitive enough to detect DNA content differences caused by the presence or absence of one single chromosome (Pfosser *et al.* 1995). However, very few reports have used this technique to assay somaclonal variation in woody plants (e.g. Awoleye *et al.* 1994; Bueno *et al.* 2000; Endemann *et al.* 2002) and only one report concerned to *Eucalyptus globulus* Labill. plants derived from organogenesis (Azmi *et al.* 1997).

Flow cytometry was originally developed as a method for rapid counting and analysis of blood cells (Ayele *et al.* 1996), and presently it has been used in plant sciences mostly focused on DNA ploidy and nuclear DNA content analysis. Flow cytometry analysis of the nuclear DNA content is based on the use of DNA-specific fluorochromes and on the analysis of the relative fluorescence of stained nuclei (Dolezel 1991). In most plants, analysis of relative DNA content of nuclei isolated from young tissues yields a histogram showing a dominant peak corresponding to nuclei at the G_0/G_1 phase of the cell cycle and a minor peak corresponding to G_2 nuclei. To estimate ploidy level, the position of the G_1 peak on a histogram of an unknown sample is compared to that of a reference plant with known ploidy (Dolezel 1997).

The flow cytometric assay has some important advantages over chromosome counting. It is convenient (sample preparation is easy), rapid (several hundreds of samples can be analysed in one working day), it does not require dividing cells, sample preparation requires only a few milligrams of tissue, and can detect mixoploidy (Dolezel 1997).

In the present study, mature zygotic embryos from several open-pollination orchard families and a field plant were used to quantify the DNA content of *E. globulus*. Also, in order to estimate if somatic embryogenesis induced DNA content and/or ploidy changes, somatic embryos obtained from one of the studied families were compared with zygotic embryos and with mature leaves from the mother plant. To complement these analyses the nuclear DNA content of leaves of in vitro micropropagated plantlets derived from mature zygotic embryos of the same family was estimated and compared with the values found for this family.

Materials and methods

Induction of somatic embryogenesis

Half-sib seeds of *Eucalyptus globulus* Labill. (Celbi, Leirosa, Portugal) collected in the Centre of Portugal from open-pollination orchard families (EG01, EG02, EG04, EG06, EG09, EG10, EG11, EG12, EG13) were sterilized as described in Chapter II.2. Somatic embryogenesis cultures were initiated from zygotic embryos in accordance with the protocol established by Pinto *et al.* (2002, see also Chapter II.2). Briefly, the seed coat was removed from the mature zygotic embryos and they were transferred to Murashige and Skoog (1962) medium (MS) supplemented with 3 % (w/v) sucrose and with 3 mg l^{-1} α-naphtalene acetic acid (NAA) to induce somatic embryogenesis. After three weeks on induction medium, explants were transferred to MS medium without growth regulators (MSWH). Thereby, they were maintained on this medium. Eight months old somatic embryos were used for ploidy comparison with the mature zygotic embryos.

Laser flow cytometry analysis

Nuclear suspensions from mature zygotic embryos dissected from the seeds, from somatic embryos, and from in vitro and field leaves were prepared according to Galbraith *et al.* (1983). In brief, to release nuclei from the cells, samples of this material were chopped with a razor blade together with a young leaf of the internal reference standard *Lycopersicon esculentum* cv. Stupicke (2C = 1.96 pg DNA; kindly provided by Dolezel J, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic) in Marie's isolation buffer (Marie and Brown 1993) containing: 50 mM glucose, 15 mM NaCl, 15 mM KCl, 5 mM EDTA Na_2, 50 mM sodium citrate, 0.5% Tween 20, 50 mM HEPES (pH 7.2). The suspension of nuclei was filtered through a 50 μm nylon filter to remove fragments and large tissue debris. Then 50 μg mL^{-1} of propidium iodide (PI) (Fluka, Buchs, Switzerland) and 50 μg ml^{-1} of RNAse (Sigma, St. Louis, MO, USA) were added to the samples to stain the DNA. Samples were analysed within a 15-min period in a flow cytometer.

The relative fluorescence intensity of PI-stained nuclei was measured by a Coulter EPICS XL (Coulter Electronics, Hialeah, Florida, USA) flow cytometer. The instrument was equipped with an air-cooled argon-ion laser tuned at 15 mW and operating at 488 nm. Integral fluorescence together with fluorescence pulse height and width emitted from

nuclei was collected through a 645 dichroic long-pass filter and a 620 band-pass filter and converted on 1024 ADC channels. Prior to analysis, the instrument was checked for linearity with fluorescent check beads (Coulter Electronics, Hialeah, FL) and the amplification was adjusted so that the peak corresponding to *Eucalyptus globulus* nuclei was positioned approximately at channel 200. This setting was kept constant. The results were obtained in the form of three graphics: linear-fluorescence light intensity (FL), forward angle (FS) *vs*. side angle (SS)-light scatter and FL pulse integral *vs.* FL pulse height. This last cytogram was used to eliminate partial nuclei and other debris, nuclei with associated cytoplasm and doublets (these events have a higher pulse area but the same pulse height as single nuclei) (Price and Johnston 1996). In this cytogram an “interest zone” was defined such that only single intact nuclei were included in the FL histogram (Brown *et al.* 1991).

To estimate ploidy level, the position of the G_0/G_1 peak of the sample on a histogram was compared with the internal reference plant with known ploidy. For each sample at least 5,000-10,000 nuclei were analysed.

Nuclear genome size of *Eucalyptus globulus* Labill. was calculated according to the following formula:

E. globulus 2C nuclear DNA content (pg) =

$$\frac{\textit{E. globulus}\ G_0/G_1 \text{ peak mean}}{\textit{L. esculentum}\ G_0/G_1 \text{ peak mean}} \times 1.96$$

Four to fifteen zygotic embryos of each family (EG01, EG02, EG04, EG06, EG09, EG10, EG11, EG12, EG13) were analysed to assay *E. globulus* nuclear DNA content. To detect possible changes induced by somatic embryogenesis the ploidy levels of 21 somatic embryos and 15 mature zygotic embryos from the same family (EG12) were analysed. The nuclear DNA content of leaves from two in vitro germinated plantlets (EG12) and nine leaves from a field tree (EG12) was also assayed and the values obtained were then compared with the ones obtained for zygotic and somatic embryos.

Test for inhibitors

In order to see if *E. globulus* extracts have some compounds that may affect PI fluorescence, the following experiments were performed according to Price *et al.* (2000).

Briefly, in the first experiment (**A**), nuclei suspensions were obtained from one-half of a tomato leaf simultaneously processed (co-chopped) with: a) mature zygotic embryos, b) somatic embryos, or c) field leaves. These samples were then stained with PI. The second experiment (**B**) consisted of nuclei suspensions that were obtained from the other half of the tomato leaf processed independently. These samples were also stained with PI. After staining for 15 min, samples from experiments A and B were analysed at the flow cytometer for mean PI fluorescence, after which they were mixed and re-analysed.

Statistical analysis

Statistical analyses were performed using a one-way ANOVA (SigmaStat for Windows Version 2.03, SPSS Inc., USA) to analyse possible differences between mature zygotic embryos of open pollinated trees of *E. globulus*, and to analyse possible differences between zygotic embryos, somatic embryos, leaves of in vitro germinated plantlets and field leaves. A multiple comparison Tukey-Kramer test was applied to determine exactly which groups were different.

Results and Discussion

Forward angle light scatter (FS) is proportional to cell-surface area or size, and side angle light scatter (SS) is proportional to cell granularity or internal complexity. The representation of these two parameters gives us useful information about the nuclei and most of flow cytometry reports on plant material do not contemplate these aspects. Figure 1a-d shows that there is a general homogeneity among the nuclei derived from zygotic embryos (Fig. 1a), somatic embryos (Fig. 1b), leaves of in vitro germinated plantlets (Fig. 1c) and field leaves (Fig. 1d) in what concerns the characteristics reported above, and that eucalypt and tomato nuclei have almost the same size as it is notorious by the overlapping between particles from both species.

Linear-fluorescence light intensity (FL) represents the linear fluorescence intensity stained with propidium iodide (PI) (Fig. 1e-h) and a cytogram with pulse integral *vs.* pulse height can be computed to define a gating region that excludes doublets and particles of plant debris (present in the bottom left corner of the cytogram) (Fig. 1i-l).

Throughout the experiment, and as expected, the internal standard had a tight 2C distribution (mean CV = 2.5 %; Fig. 1). The histograms of relative nuclear DNA content

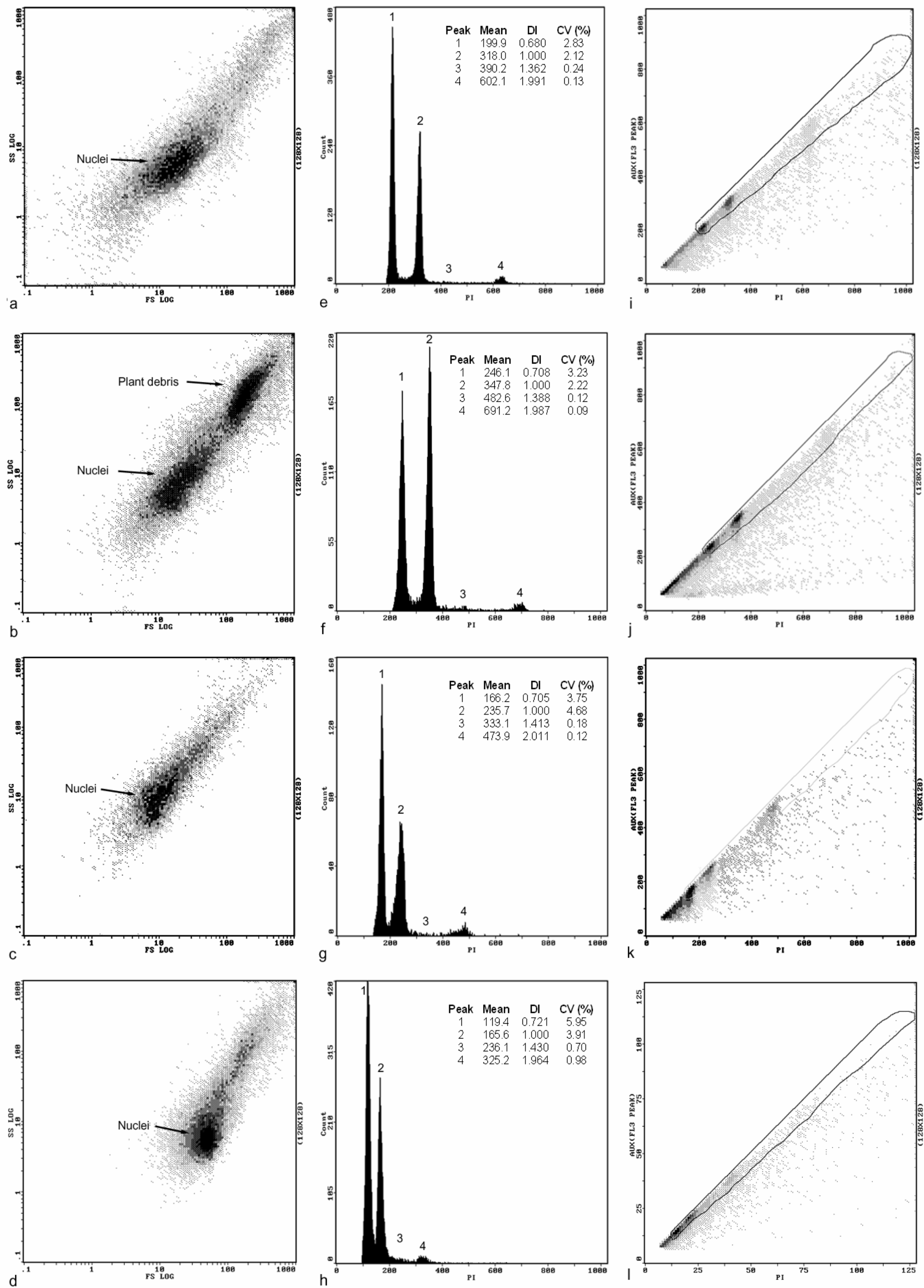
showed distinct G_0/G_1 peaks with coefficients of variation (CV) ranging from 2.2 % to 4.0 % for zygotic embryos (mean CV = 3.03 %; Fig. 1e), from 2.2 % to 4.5 % for somatic embryos (mean CV = 3.16 %; Fig. 1f), from 3.5 % to 5.5 % for leaves of in vitro germinated plantlets (mean CV = 4.51 %; Fig. 1g) and from 4.6 % to 6.8 % for field leaves (mean CV = 5.72 %, Fig. 1h). The importance of showing CV values in this type of studies is pointed out by Marie and Brown (1993) as they consider it an elementary criterion for assessing a cytological method. These authors also referred a range of 1 to 2 % for top quality analyses in plant cells, and around 3 % as a routine value (Marie and Brown, 1993). On the other hand, Galbraith *et al.* (2002) defined a CV of < 5 % as the acceptance criterion. However, for most recalcitrant species, as woody plants, to obtain these recommended values is up to the moment very difficult or in some cases impossible. Grattapaglia and Bradshaw (1994), in *Eucalyptus* spp. reported CV values ranging from 6.3 % to 12.8 %. Zoldos *et al.* (1998) working with oak species reported CV values ranging from 3.3 % to 6.9 %. Usually there is a correlation between higher CV values and higher background noise (Emshwiller 2002). High background noise is not unusual in the lower channel numbers (Kudo and Kimura 2001), and has been alternatively ascribed to broken cells damaged during the extraction procedure or to autofluorescence of chloroplast in the cytosol (Emshwiller 2002). In this latter point, Galbraith *et al.* (2002) referred that the contribution of chlorophyll autofluorescence is more critical in species where the nuclear DNA content is smaller, where this autofluorescence can overlap PI-induced nuclear fluorescence on one-dimensional histograms, affecting CV values. Unfortunately, many reports (some of them concerning *E. globulus*) do not show this information leading to some practical questions about the methodology used and constraining the interpretation of results.

In general, a small G_2 peak was observed for *E. globulus*. Similar results can be found in woody plants as *Q. robur* embryos (Endemann *et al.* 2002), but these authors did not present an explanation for the fact. In other species like rice and wheat no G_2 peak was found (Arumuganathan and Earle 1991) and this fact was attributed to the developmental stage of the leaves. Also, no G_2 peak was observed in *Vitis* spp. (Lodhi and Reisch 1995), and these authors attributed the obscurity of the 4C peaks to the high fluorescent background of the plant debris. Respectively to *E. globulus*, the small or even the absence of G_2 peak in zygotic embryos and in field leaves can be explained in the first case by the

seed dormancy, as low background noise was detected (Fig. 1e), and in the latter by the mature stage of the leaves and the presence of some background noise (Fig. 1h). In somatic embryos and in in vitro leaves, although the background found is not high (Figs. 1f, 1g) when compared with other woody plant species (Lodhi and Reisch 1995, Schwencke *et al.* 1998, Zoldos *et al.* 1998) it may be sufficient to under estimate the quantity of nuclei present in this peak.

The mean nuclear DNA fluorescence index (DI = $2C_{Eucalyptus}/2C_{Lycopersicon}$) for *E. globulus* ranged from 0.672 (mature zygotic embryos) to 0.715 (field leaves) (Table I). Despite this range of variation the assignment of peaks to 2C and 4C level values was not affected. If tetraploid levels were to be observed a DI of 1.344 was expected. Therefore, the results obtained sustain that all the samples analysed have the same ploidy level, i.e., diploid, and that no polyploidy was observed in somatic embryos and in leaves of in vitro germinated plantlets. Therefore we believe that there is a relative genetic stability at this level during the embryogenic process used for *E. globulus*. This result differs from some reports, in other species, where polyploidization was found during somatic embryogenesis (Kubaláková *et al.* 1996, Kudo and Kimura 2001, Endemann *et al.* 2002). However, Endemann *et al.* (2002) found that tetraploidy occurred only in 8% of the tested clones over a culture period of seven years. Despite no polyploidization was found in *Eucalyptus* in vitro cultures, the CV values obtained and the range of variation observed could mask the possible occurrence of small differences in nuclear DNA content and therefore the presence of a low level of aneuploidy should not be excluded.

Figure 1*:* Histograms of forward angle (FS) *vs.* side angle (SS)-light scatter (a-d), relative fluorescence intensity (FL) (e-h) and relative FL pulse integral *vs.* relative FL pulse height (i-l) obtained after simultaneous analysis of nuclei isolated from *Lycopersicon esculentum* cv. Stupicke (2C = 1.96 pg DNA, as an internal reference standard) and *Eucalyptus globulus*: a,e,i) mature zygotic embryo of the family EG12, b,f,j) somatic embryo of the family EG12, c,g,k) leaves of in vitro germinated plantlets, d,h,l) leaves of field plant of the family EG12. In all FL histograms (Figs. 1e-h) four peaks were observed: 1 – nuclei at G_0/G_1 phase of *E. globulus*; 2 – nuclei at G_0/G_1 phase of *L. esculentum* leaves; 3 - nuclei at G_2 phase of *E. globulus*; 4 – nuclei at G_2 phase of *L. esculentum* leaves. In FL pulse integral *vs.* relative FL pulse height cytograms (Figs. 1i-l) a gating region was defined to exclude doublets and particles of plant debris (present in the bottom left corner of the cytogram).



(Figure 1, see page 142)

Complementary studies, such as chromosome counting analysis, to evaluate this situation are under occurrence in our laboratory (data not shown). Recently Roux *et al.* (2003), combining flow cytometry and chromosome counting, showed that flow cytometry could rapidly detect aneuploidy situations in *Musa* sp.

The determination of the nuclear DNA content of *E. globulus* in absolute units ranged from 1.28 to 1.40 pg/2C, and these estimates were reproducible for a given sample source, with low standard deviations (Table I). The mean nuclear DNA content of *E. globulus* zygotic embryos was 1.32 ± 0.035 pg/2C (Family EG12: 1.32 ± 0.044 pg/2C). On the other hand this value raised to 1.39 ± 0.016 pg/2C in *E. globulus* somatic embryos and to 1.39 ± 0.001 pg/2C in leaves of in vitro germinated plantlets. In leaves of the field plant the value obtained raised to 1.40 ± 0.029 pg/2C, although fluorescence in absolute units was reduced. Statistical analysis (one-way ANOVA) showed significant differences for $P \leq 0.05$ among different groups. A multiple comparison Tukey-Kramer test showed significant differences ($P \leq 0.05$) between the values found for zygotic embryos (n=13) and those found for somatic embryos (n=21) and leaves of both in vitro (n=2) and field plants (n=9) (Table I). Despite these results a low variation of approximately 6 % was found between zygotic embryos and somatic embryos and between zygotic embryos and leaves of both in vitro and field plants.

Although significant, the interpretation of these results must be done with caution, as these differences in samples do not necessarily reflect real differences in nuclear DNA content but may be due to the presence of compounds that affect PI fluorescence or to chromatin structure differences between tissues. The presence of extra-nuclear compounds that reduce nuclear PI fluorescence was detected in some plant species, namely *Helianthus annuus* L. (Price *et al.* 2000) and *Coffea liberica* var. *dewevrei* (Noirot *et al.* 2000, 2002). Price *et al.* (2000) referred that it is likely that inhibitors that decrease fluorochrome fluorescence of plant nuclei are common in plants. Therefore, a test for naturally occurring inhibitors should be used in all flow cytometric studies. Noirot *et al.* (2000), working with coffee, highlighted cytosolic effects on dye accessibility to DNA, and that cytosolic compounds can bias nuclear DNA content estimates by up to 20 %. More recently Noirot *et al.* (2003) identified two of these cytosolic compounds that modify accessibility of the dye propidium iodide to *Petunia* spp. DNA (species used as internal standard for genome size evaluation): caffeine and chlorogenic acid (a precursor of polyphenols). These authors

also alerted that the presence of inhibitors compromises the reliability of estimations of nuclear DNA content, particularly if detection of small differences is desired.

Table 1: Nuclear DNA content of *Eucalyptus globulus* Labill. mature zygotic embryos, somatic embryos, leaves of in vitro germinated plantlets and leaves of field plants. The values are given as mean and standard deviation of the mean (SD) of DNA index relative to the internal standard *Lycopersicon esculentum* cv. Stupicke, as a mean and standard deviation of the mean (SD) of the nuclear DNA content (pg/2C) and as 1C genome size of *Eucalyptus globulus* Labill.

Families	Index	SD	Nuclear DNA content (pg/2C)	SD	1C Genome size (Mbp)*	*n*	Tukey-Kramer test grouping**
Zygotic embryos							
EG01	0.676	0.0189	1.33	0.038	650	4	A
EG02	0.682	0.0251	1.34	0.049	655	5	A
EG04	0.668	0.0162	1.31	0.032	641	6	A
EG06	0.667	0.0044	1.31	0.008	641	4	A
EG09	0.669	0.0083	1.31	0.016	641	6	A
EG10	0.654	0.0126	1.28	0.025	626	8	A
EG11	0.682	0.0296	1.34	0.058	655	8	A
EG12	0.673	0.0225	1.32	0.044	645	15	A
EG13	0.675	0.0299	1.31	0.047	641	8	A
	0.672	*0.0186*	*1.32*	*0.035*	644	*64*	*A*
Somatic embryos							
EG12	0.708	0.0083	1.39	0.016	680	21	B
Leaves of in vitro *germinated plantlets*							
EG12	0.710	0.0007	1.39	0.001	680	2	B
Leaves of field plants							
EG12	0.715	0.0146	1.40	0.029	689	9	B

*1 pg DNA = 978 Mbp (Dolezel *et al.*2003)

**Groups followed by the same letters a or b are not significantly different according to the multiple comparison Tukey-Kramer test at $P \leq 0.05$

In general, the amount and complexity of secondary products change with plant material ageing and among organs, interfering with the flow cytometric analyses. For this

reason, it is a usual request that plant organs comprise young and growing tissues for flow cytometry studies (Galbraith *et al.* 2002). This may explain the higher CV values observed in leaves respectively to embryos and also the decrease of peak resolution in histograms of leaves from field plants. On the other hand *Eucalyptus* somatic embryos, contrary to the zygotic ones, had a yellow-brownish colour and histograms with higher background noise were obtained, suggesting a higher content of secondary compounds (e.g. tannins, anthocyanins).

Data presented here show a notorious decrease of PI fluorescence from both Eucalyptus and tomato nuclei in samples obtained from leaves of field plants (Fig. 1h). This decrease of PI fluorescence was not observed in zygotic embryos, somatic embryos or in leaves of in vitro germinated plantlets. Also associated with this occurrence may be the fact that PI is a DNA intercalator sensitive to chromatin structure and that, as pointed out by Biradar and Rayburn (1994), chromatin structure may vary between organs/tissues.

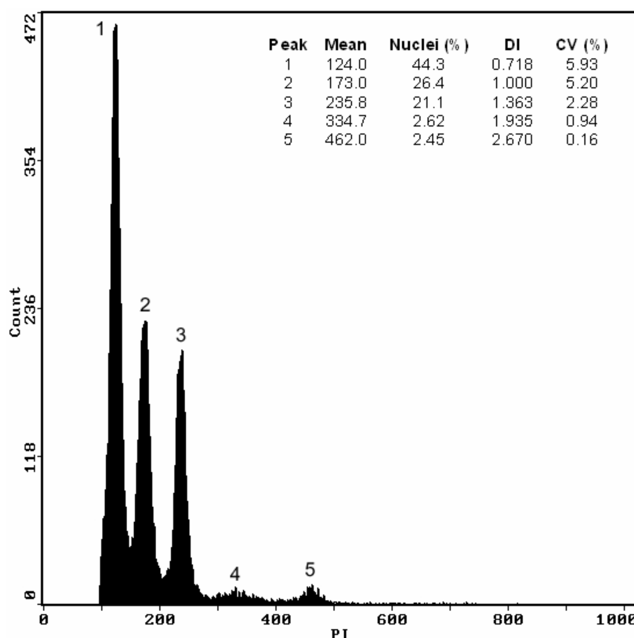


Figure 2: Flow cytometry histogram of PI-stained nuclei from simultaneously processed *E. globulus* field leaves (1) and *L. esculentum* leaves (2) to which PI-stained nuclei from independently processed *L. esculentum* leaves (3) were added. Peaks 4 and 5 refer, respectively, to nuclei at the G_2/M phase of simultaneously processed *L. esculentum* leaves and independently processed *L. esculentum* leaves. Peak 3 overlaps nuclei at the G_2/M phase of *E. globulus* field leaves.

In the inhibitors test a decrease of PI fluorescence was observed in tomato leaves simultaneously processed with *Eucalyptus* field leaves (Experiment A) when compared with the PI fluorescence of nuclei from independently processed tomato leaves. This result is even clearer when experiment A and experiment B were mixed and immediately re-

analysed (Fig. 2). Here, besides peaks 1 and 2 that represent, respectively, nuclei at the G_0/G_1 phase of Eucalyptus and tomato leaves that were simultaneously processed, a third peak (3) (that in the meantime probably already lost some fluorescence) was observed corresponding to nuclei at the G_0/G_1 phase of independently processed tomato leaves. In zygotic and somatic embryos this third peak was not observed. Instead when experiment A and experiment B were mixed an increase of the number of nuclei present in the tomato leaves peak was observed. This test reflects the presence of inhibitors in *Eucalyptus* field leaves that reduce PI fluorescence and that may be present in lower concentrations in other organs.

Table 2: Nuclear DNA content estimations for *Eucalyptus globulus*. obtained using flow cytometry.

Plant source	Nuclear DNA content (pg/2C)	Reference	Internal reference standard used
Lyophilized or frozen leaves	1.09	(Grattapaglia and Bradshaw 1994)	Chicken erythrocytes (2C = 2.34 pg)
Leaves	1.13	(Marie and Brown 1993)	Female chicken erythrocytes (2C = 2.33 pg)
Various tissues	1.13	(Azmi *et al.* 1997)	Chicken erythrocytes (2C = 2.33 pg)
Zygotic embryos	1.32	This report	*L. esculentum* nuclei (2C = 1.96 pg)
Somatic embryos	1.39		
In vitro leaves	1.39		
Field leaves	1.49		

The mean values of DNA content obtained are higher than the previously reported values for this species (Table II), although some of these data may be less reliable because no CV values and/or statistical analysis were presented. These differences may be however normal among laboratories due to the use of different methodologies as sample processing – e.g. Grattapaglia and Bradshaw (1994) used lyophilised or nitrogen frozen leaves – and buffer composition, and the use of different standards – e.g. all the reports found up to the moment for *E. globulus* used chicken erythrocytes (Marie and Brown 1993, Grattapaglia and Bradshaw 1994, Azmi *et al.* 1997). Inter-laboratory differences depend on the target/standard combination and ranged from 4.2%, for the *Arabidopsis thaliana/Rapanhus*

sativus combination to 15.6% for the *Glycine max/Zea mays* combination (Dolezel *et al.* 1998).

Conclusions

The main objective of this report was accomplished, as we could verify by flow cytometry that no major genetic instability was verified in the somatic embryos.

In conclusion this report shows that: a) DNA content of *E. globulus* is higher than the previously reported values for this species although this estimation (1.32 – 1.40 pg/2C) is the first based on the use of a plant DNA standard and is aware of some constraints that methodologies involving PI staining may pose in this species; b) homogeneity is found within the same sample source but some heterogeneity exists between zygotic embryos and the other sample sources tested; c) this heterogeneity and the decrease of PI fluorescence in leaves suggest that *Eucalyptus* contain extranuclear components that inhibit PI accessibility to DNA.

For all these reasons we believe that this species, as many woody plants, has several compounds that may interfere with PI fluorescence and that the observed differences are most probably artifactual. Therefore we believe that the somatic embryogenesis methodology used did not induce major genetic changes in the somatic embryos and the primary goal of "true-to-type" propagation was assured by flow cytometry.

References

Arumuganathan K, Earle E (1991) Estimation of nuclear DNA content of plants by flow cytometry. Plant Mol Biol Rep 9(3):229-233

Awoleye F, Van Duren M, Dolezel J, Novák F (1994) Nuclear DNA content and in vitro induced somatic polyploidization cassava (*Manihot esculenta* Crantz) breeding. Euphytica 76:195-202

Ayele M, Dolezel J, Van Duren M, Brunner H, Zapata-Arias F (1996) Flow cytometric analysis of nuclear genome of the Ethiopian cereal Tef [*Eragrostis tef* (Zucc.) Trotter]. Genetica 98(211):215

Azmi A, Noin M, Landre P, Prouteau M, Boudet AM, Chriqui D (1997) High frequency plant regeneration from *Eucalyptus globulus* Labill. hypocotyls: ontogenesis and ploidy level of the regenerants. Plant Cell Tiss Organ Cult 51(1):9-16

Biradar DP, Rayburn AL (1994) Flow cytometric probing of chromatin condensation in maize diploid nuclei. New Phytol 126(1):31-35

Brown S, Bergounioux C, Tallet S, Marie D (1991) Flow cytometry of nuclei for ploidy and cell cycle analysis. In: Negrutiu I, Gharti-Chherti G (eds) A laboratory guide for cellular and molecular plant biology. Bihuser, Basel, pp 326-345

Bueno M, Gómez A, Manzanera J (2000) Somatic and gametic embryogenesis in *Quercus suber* L. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants. Kluwer Academic Publishers, Dordrecht Boston London, pp 479-508

Bueno M, Gomez A, Vicente O, Manzanera J (1996) Stability in ploidy level during somatic embryogenesis in *Quercus canariensis*. In: Ajuha M, Bourjan W, Neale D (eds) Somatic cell genetics and molecular genetics of trees. Kluwer Academic Publishers, Dordrecht, pp 23-28

DeVerno L (1995) An evaluation of somaclonal variation during somatic embryogenesis. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants. Kluwer Academic Publishers, Dordrecht Boston London, pp 361-375

Direcção Geral de Florestas (2001) Inventário Florestal Nacional, 3rd. Lisbon, pp 3-162.

Dolezel J (1991) Flow cytometric analysis of nuclear DNA content in higher plants. Phytochem Anal 2:143-154

Dolezel J (1997) Applications of flow cytometry for the study of plant genomes. J Appl Genet 38(3):285-302

Dolezel J, Greilhuber J, Lucretti S, Meister A, Lysák M, Nardi L, Obermayer R (1998) Plant genome size estimation by flow cytometry: inter-laboratory comparison. Ann Bot (Lond) 82((Supplement A)):17-26

Dolezel J, Bartos J, Voglmayr H, Greilhuber J (2003) Nuclear DNA content and genome size of trout and human. Cytom Part A 51A(2):127-128

Eldridge K, Davidson J, Harwood C, van Wyk G (1993) Eucalypt domestication and breeding. Clarendon Press, Oxford

Emshwiller E (2002) Ploidy levels among species in the '*Oxalis tuberosa* Alliance' as inferred by flow cytometry. Ann Bot (Lond) 89(6):741-753

Endemann M, Hristoforoglu K, Stauber T, Wilhelm E (2002) Assessment of age-related polyploidy in *Quercus robur* L. somatic embryos and regenerated plants using DNA flow cytometry. Biol Plant (Prague) 44(3):339-345

Fourré JL, Berger P, Niquet L, Andre P (1997) Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. Theor Appl Genet 94(2):159-169

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell-cycle in intact plant-tissues. Science 220(4601):1049-1051

Galbraith DW, Lambert G, Macas J, Dolezel J (2002) Analysis of nuclear DNA content and ploidy in higher plants. In: Robinson J, Darzynkiewicz Z, Dean P, Hibbs A, Orfão A, Rabinovitch P, Wheeless L (eds) Current protocols in cytometry. John Wiley & Sons, Inc., New York

Grattapaglia D, Bradshaw HD (1994) Nuclear-DNA content of commercially important *Eucalyptus* species and hybrids. Can J For Res-Rev Can Rech For 24(5):1074-1078

Kubaláková M, Dolezel J, Lebeda A (1996) Ploidy instability of embryogenic cucumber (*Cucumis sativus* L.) callus culture. Biol Plant (Prague) 38(3):475-480

Kudo N, Kimura Y (2001) Patterns of endopolyploidy during seedling development in cabbage (*Brassica oleracea* L.). Ann Bot 87(2):275-281

Lodhi M, Reisch B (1995) Nuclear DNA content of *Vitis* species, cultivars, and other genera of the *Vitaceae*. Theor Appl Genet 90:11-16

Marie D, Brown S (1993) A cytometric exercise in plant DNA histograms, with 2C values for 70 species. Biol Cell (Paris) 78:41-51

Marques CM, Vasquez-Kool J, Carocha VJ, Ferreira JG, O'Malley DM, Liu BH, Sederoff R (1999) Genetic dissection of vegetative propagation traits in *Eucalyptus tereticornis* and *E. globulus*. Theor Appl Genet 99(6):936-946

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15(3):473-497

Noirot M, Barre P, Louarn J, Duperray C, Hamon S (2000) Nucleus-cytosol interactions - A source of stoichiometric error in flow cytometric estimation of nuclear DNA content in plants. Ann Bot (Lond) 86(2):309-316

Noirot M, Barre P, Louarn J, Duperray C, Hamon S (2002) Consequences of stoichiometric error on nuclear DNA content evaluation in *Coffea liberica* var. *dewevrei* using DAPI and propidium iodide. Ann Bot (Lond) 89(4):385-389

Noirot M, Barre P, Duperray C, Louarn J, Hamon S (2003) Effects of caffeine and chlorogenic acid on propidium iodide accessibility to DNA: consequences on genome size evaluation in coffee tree. Ann Bot 92(2):259-264

Pfosser M, Amon A, Lelley T, Heberle-Bors E (1995) Evaluation of sensitivity of flow cytometry in detecting aneuploidy in wheat using disomic and ditelosomic wheat-rye addition lines. Cytometry 21:387-393

Pinto G, Santos C, Neves L, Araújo C (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21(3):208-213

Price H, Johnston J (1996) Analysis of plant DNA content by Feulgen microspectrophotometry and flow cytometry. In: Jauhar P (eds) Methods of genome analysis in plants. CRC Press, Boca Raton, pp 115-131

Price HJ, Hodnett G, Johnston JS (2000) Sunflower (*Helianthus annuus*) leaves contain compounds that reduce nuclear propidium iodide fluorescence. Ann Bot (Lond) 86(5):929-934

Roux N, Toloza A, Radecki Z, Zapata-Arias F, Dolezel J (2003) Rapid detection of aneuploidy in *Musa* using flow cytometry. Plant Cell Rep 21:483-490

Schwencke J, Bureau JM, Crosnier MT, Brown S (1998) Cytometric determination of genome size and base composition of tree species of three genera of Casuarinaceae. Plant Cell Rep 18(3-4):346-349

Watt M, Blakeway F, Termignoni R, Jain S (1999) Somatic embryogenesis in *Eucalyptus grandis* and *E. dunnii*. In: Jain S, Gupta P, Newton R (eds) Somatic embryogenesis in woody plants. Kluwer Academic Publishers, Dordrecht Boston London, pp 63-78

Zobel B (1993) Clonal forestry in the *Eucalyptus*. In: Ahuja M, Libby W (eds) Clonal forestry. Springer, Berlin Heidelberg New York, pp 139-148

Zoldos V, Pape D, Brown S, Panaud O, Siljak-Yakovlev S (1998) Genome size and base composition of seven *Quercus* species: inter- and intra-population variation. Genome 41:162-168

III 2: Factors affecting maintenance, proliferation, and germination of secondary somatic embryos of *Eucalyptus globulus* Labill.

Chapter submitted as an original paper to a SCI journal:

Pinto G, Park Y-S, Silva S, Araújo C, Neves L, Santos C (2007) Factors affecting maintenance, proliferation, and germination of secondary somatic embryos of *Eucalyptus globulus* Labill. Plant Cell Tissue and Organ Culture (Submitted)

Abstract

Results reported here described an efficient protocol for repetitive (secondary) somatic embryogenesis (SE) in *Eucalyptus globulus,* producing many more somatic embryos than the previously reported primary SE protocol, and therefore, increasing the potential for plant regeneration. Primary somatic embryos formerly induced on MS medium supplemented with 3 mg l^{-1} NAA were isolated and transferred to the same medium, leading to new cycles of somatic embryos. This repetitive SE competence was maintained under these conditions for at least 2 years.

In order to understand the factors that may affect this SE maintenance and influence secondary somatic embryo competence, proliferation, germination, and conversion, several factors were investigated: basal media (MS and B5), plant growth regulators (auxins and cytokinins), and light.

The MS without growth regulators (MS_{WH}) was the most efficient medium for cotyledonary embryo formation and germination; $B5_{WH}$ also gave good results for globular secondary embryo proliferation, but not for more advanced stages of development. Reducing auxin (NAA) levels in the medium increased the proliferation of globular somatic embryos and allowed SE competence to be maintained on medium free of plant growth regulators.

Two cytokinins (BAP and KIN) were tested in secondary SE. Adding them to the MS medium did not improve proliferation of globular secondary embryos, but was found to be crucial during later stages of the SE process, in particular during germination and conversion. For example, the maximal number of cotyledonary somatic embryos was originated on MS supplemented with NAA and BAP, and kept in darkness, whereas the best germination results were achieved on MS supplemented with NAA, BAP, and KIN, in light conditions.

Data also show that, depending on the stage of the process, light can have an effect. Dark conditions stimulated maintenance and proliferation of globular secondary embryos, and should be maintained until the cotyledonary stage is reached, after which exposure to light is recommended.

Key words: *Eucalyptus***,** growth regulators, media composition, secondary somatic embryogenesis

Introduction

Secondary somatic embryogenesis (SE) is a phenomenon whereby new somatic embryos are initiated from other somatic embryos. Compared with primary SE, secondary SE offers several advantages, such as a much higher multiplication rate, independant explant source, and repeatability (Raemakers *et al.* 1995). Some cultures are able to retain their competency for secondary SE for many years, and thus provide useful material for various studies, as described for, e.g., *Vitis rupestris* (Martinelli *et al.* 2001), *Quercus suber* (Hernández *et al.* 2003), *Piper nigrum* (Nair and Gupta 2006), and *Myrtus communis* (Parra and Marco 1998). Additionally, for explants of some species primary SE is less efficient than secondary SE, as reported for cork oak (Pinto *et al.* 2002b, Hernández *et al.* 2003). Therefore, in plants with long life cycle, such as dicotyledonous woody plants, preserving embryogenic lines can be a cost-effective maintenance while those lines are being tested in field (Raemakers *et al.* 1995). Nevertheless, until the SE process is completely understood and optimized, the major limitations to most embyrogenic systems are maintaining embryogenic competence and the low conversion rate of somatic embryos, such as in *Castanea sativa* (Corredoira *et al.* 2003).

There is a wide range of protocols concerning the maintenance of embryogenic potential by secondary SE, and subsequent maturation, germination and conversion of secondary somatic embryos. Addition of plant growth regulators (PGRs) into the culture medium is the preferred way to induce morphogenetic responses in vitro in most plant tissue culture systems evaluated, SE being no exception, and this issue has been widely documented during recent decades (Jiménez 2005). Addition of PGRs seems to be crucial, not only in the induction phase of primary SE, but also in the induction and maintenance of secondary SE. Raemakers *et al.* (1995) highlighted that, in general, secondary SE requires no PGR in species with cytokinin-driven primary SE, whereas continuous exposure to PGRs is needed in species with cytokinin/auxin- or auxin-driven primary SE. Nevertheless, the period of culture in conjunction with continous exposure to high PGR concentrations might cause somaclonal variation (e.g., Berlyn *et al.* 1986).

Aside from PGRs, several other factors control secondary SE. In the research presented here, two more factors will be discussed: composition of the medium and presence of light. Most works concerning SE are based on empirical approaches and often use only one medium type during the whole process, even though this formulation may not

be optimal for the different stages of explant growth and development (Ramage and Williams 2002). As well, the same induction medium is also often used for primary and secondary SE processes (Von Arnold *et al.* 2002). Pinto *et al.* (2006) state that the basal medium composition determines somatic embryogenesis potential in *Eucalyptus globulus*; they tested several media (MS, B5, WPM, JADS, DKW), and demonstrated that, for this species, MS (Murashige and Skoog, 1962) and B5 (Gamborg *et al.* 1968) were the best. Also, B5 medium was used successfully in *E. citriodora* repetitive SE (Muralidharan and Mascarenhas 1995).

Concerning light conditions, Gaj (2004) reported that most authors use photoperiod or darkness during SE. Nevertheless, systematic studies on the effects of light on in vitro response of cultured explants (and in SE in particular) are limited.

In *Eucalyptus*, SE induction from zygotic embryos or seedling explants (Muralidharan and Mascarenhas 1995, Watt *et al.* 1999, Nugent *et al.* 2001, Pinto *et al.* 2002a, Prakash and Gurumurthi 2005, Oller *et al.* 2006) has been described. Although the protocol published by Pinto *et al.* (2002a) for *E. globulus* is presently reliable, plant conversion continues to occur at low, non-economically viable rates. Similar problems with primary SE were reported for other woody species, mostly because often only a single population of embryos is produced (e.g., Karkonen 2000), some of which mature and convert to plants (Merkle 1995). Although primary somatic embryos can be used as explants to initate secondary somatic embryos, in this genus, the occurrence of secondary SE was just reported for *E. gunnii* (Boulay 1987), *E. citriodora* (Muralidharan *et al.* 1989, Muralidharan and Mascarenhas 1995), and *E. globulus* (Pinto *et al.* 2004b, 2006). Muralidharan and Mascarenhas (1995) established an effective method for the induction of repetitive SE, allowing the large-scale propagation of *E. citriodora*. These authors reported that the embryogenic potential was maintained in darkness for over 9 years on B5 medium containing 5 mg l^{-1} NAA (α-naphthalene acetic acid), 500 mg l^{-1} CH (casein hydrolysate), 500 mg l^{-1} glutamine, and 3% (w/v) sucrose. This was the most recent report concerning secondary SE until 2004, when Pinto and colleagues induced and maintained secondary somatic embryos on MS with 3 mg l^{-1} NAA (Pinto *et al.* 2004a).

The main research objective reported here was to test factors that might improve maintenance of embryogenic competence, proliferation, and germination of secondary

somatic embryos of *E. globulus* derived from primary somatic embryos by investigating the effect of two different media (MS and B5), PGRs, and light, and thus open up opportunities to optimize the process and use repetitive SE on a large scale.

Materials and methods

Establishment of primary SE cultures and induction of secondary SE

Primary embryogenic cultures were established following the methods described by Pinto *et al.* (2002a, see also Chapter II.2). Briefly, half-sib seeds of *E. globulus* Labill. (Celbi, Leirosa, Portugal) were surface sterilized with a mixture of absolute ethanol:hydrogen peroxide 30 % (v/v) for 15 min, washed twice in sterile distilled water for 10 min each, and then rinsed with 0.1% (w/v) Benlate (Rhône-Poulenc) for 15 min. Seeds were then imbibed over night in sterile distilled water. Decoated seeds were inoculated in the dark at 24±1ºC for 3 weeks, on induction medium: MS medium with 3 mg l^{-1} NAA (MS_{3NAA}), 30g l^{-1} sucrose, 2.5 g l^{-1} gelrite®, and pH 5.8. After this period, explants were transferred to MS hormone-free medium (MS_{WH}) and incubated under the same conditions described above.

To obtain new cycles of secondary SE, primary somatic embryos were carefully detached from the explant, inoculated on the induction medium described above, and incubated in the dark at 24±1ºC. Embryogenic lines were routinely transferred to fresh medium every 30 days, which allowed embryo proliferation, by repetitive SE, for more than 2 years.

After this period, one embryogenic line was selected (line G4) as starting material for the research reported here. Small embryogenic clusters comprising 10–15 globular somatic embryos were cultured for 2 months, under different conditions. A total of 20 replicates per treatment were used. The following treatments were tested:

1) Influence of medium composition on secondary SE: The composition effect of two frequently used media on maintenance and proliferation of secondary somatic embryos was evaluated: MS basal medium with added vitamins, and B5 salt basal medium with added vitamins. Each medium was tested either free of PGRs (MS_{WH} and $B5_{WH}$) or supplemented with 3 mg l^{-1} NAA (MS_{3NAA} and $B5_{3NAA}$).

2) Effect of PGR on secondary SE: In this treatment, only MS medium was used, and the following PGR treatments were tested: a) PGR free (MS_{WH}) b) 0.2 mg l^{-1} NAA; c) 3 mg l^{-1} NAA; d) 0.2 mg l^{-1} NAA + 0.1 mg l^{-1} BAP (6- benzylaminopurine); and e) 0.46 mg l^{-1} NAA + 0.28 mg l^{-1} BAP + 0.53 mg l^{-1} Kinetin (KIN).

3) Effect of light on secondary SE: To evaluate the influence of light, the above treatments (1 and 2) were performed under two light conditions: one group was maintained in darkness and the other group was incubated under a 16-h photoperiod (23.50 ± 2 µmol $m^{-2}s^{-1}$ irradiation provided by cool white fluorescent lamps). Light exposure was increased gradually (first 5 days under 5.70 ± 2 µmol $m^{-2}s^{-1}$).

Repetitive SE response

The repetitive SE response was evaluated by counting the total somatic embryos present after 2 months in each cluster. The initial number of globular embryos present in the cluster at the beginning of the experiment was subtracted to obtain a final number. The influence of each treatment on the development of the somatic embryos (i.e., number of globular, cotyledonary, or germinated embryos) was also analyzed. Somatic embryos were counted under a stereozoom microscope (Olympus SZ60, Japan).

All cultures were maintained at 24 ºC ± 1ºC. All culture media were supplemented with 30 g l^{-1} sucrose, 2.5 g l^{-1} gelrite®, pH 5.8, and were autoclaved at 121ºC for 20 min. Media and reagents were supplied by Duchefa (Haarlem, Netherlands). For a schematic view of the strategy see Fig. 1.

Histological and cytological characterization of globular somatic embryos

Primary and secondary somatic embryos were fixed in 2.5% glutaraldehyde in 1.25% (w/v) piperazine-N,N'-bis-2-ethanesulfonic acid (PIPES) buffer (pH 7.4). Tissue was then transferred to 1% (w/v) osmium tetroxide in PIPES solution, dehydrated through a graded ethanol series, and embedded in an epoxy resin (Embed-812). Ultra-thin sections (80 nm) were cut using a LKB ultra-microtome (Leica Microsystems AG, Wetzlar, Germany). Sections were contrasted with uranyl acetate and lead citrate and observed with a Siemens Elmiskop-101 transmission electron microscope at 80 kV (Siemens AG, Germany). For light microscopy, semi-thin sections (app 1.0 µm) from the material embedded for electron

microscopy were stained by periodic acid-Schiff reaction (PAS) or toluidine blue. Samples were analyzed in a Nikon Eclipse 80i light microscope (Nikon Corporation, Kanagawa, Japan), and digital photographs were taken using a Leica DC 200 digital camera (Leica Microsystems AG, Wetzlar, Germany).

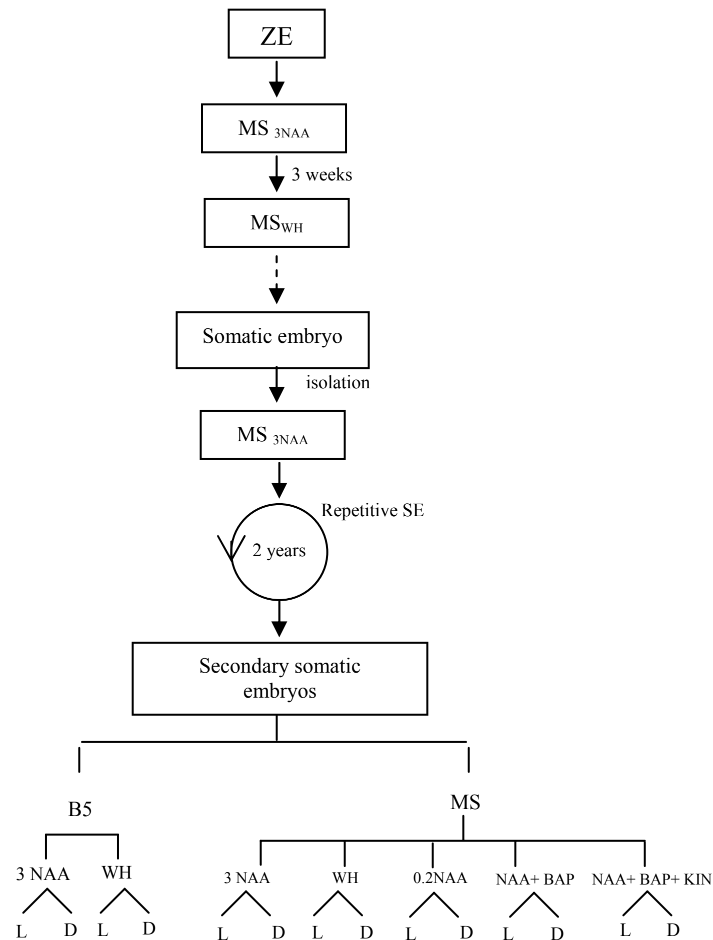


Figure 1: Schematic overview of the strategy followed in this study (L: Light; D: Dark)

Statistical analysis

Statistical analyses were performed using a two-way ANOVA (Sigmastat for Windows ver. 3.1, SPSS, USA) to analyze possible differences between treatments. A multiple comparison procedure (Duncan's Method) was applied to determine which groups were different ($P \leq 0.05$).

Results

SE reinduction in primary somatic embryos

The culture of primary embryo structures on the induction medium (MS with 3 mg l^{-1} NAA) was efficient for the production of masses of new globular secondary embryos. Any developmental stage of the primary somatic embryos could be used in this secondary SE re-induction strategy, but best results were achieved when embryo masses, or late/germinated cotyledonary embryos, were used rather than isolated globular or immature cotyledonary embryos. When germinated somatic embryos were used, the new somatic embryos were frequently observed in the hypocotyl–root transition region of somatic embryos (Plate 1.a). These repetitive masses produced several cycles of secondary embryos, leading to somatic embryo multiplication (Plate 1.b).

Somatic embryo development was asynchronous, and therefore, a broad spectrum of different developmental stages could be found simultaneously. Rhizogenesis was also a constant (Plate 1.c). Although intermediary stages were only rarely observed, these embryos evolved to the cotyledonary stage. Embryogenic competence was maintained in these media by subculturing several times in the dark over a 2-year period (Plate 1.c).

The capacity of *E. globulus* primary embryos for secondary SE is relatively low, depending on family tests and on the presence of NAA in the reinduction medium (data not shown).

Histological and cytological characterization of globular somatic embryos

Both primary and secondary somatic embryos had similar histological organization, consisting of a protoderm surrounding vacuolated parenchymatous-like cells (Plate 1D, G). However, secondary somatic embryos had in general smaller intercellular spaces than primary somatic embryos (Plate 1D,G), which was also confirmed by their ultrastructure (Plate 1.E,F,H,I), and starch accumulation had a more peripheral localization (near the protoderm) than the diffuse starch distribution found in primary somatic embryos (Plate 1D,G). Regions with meristematic activity were present in both somatic embryo types (Plate 1D,G). Plasmodesmata were highly abundant in both embryo types, mostly in newly formed cells with dense cytoplasm (rich in, e.g., mitochondria, endoplasmatic reticulum) and showing active cell material extrusion to the cell wall (Plate 1E, H). Plastids usually

had etio-amyloplast characteristics, with irregular shapes and large starch granules, but in secondary somatic embryos, thylakoidal organization was frequently seen (Plate 1E,F,H,I).

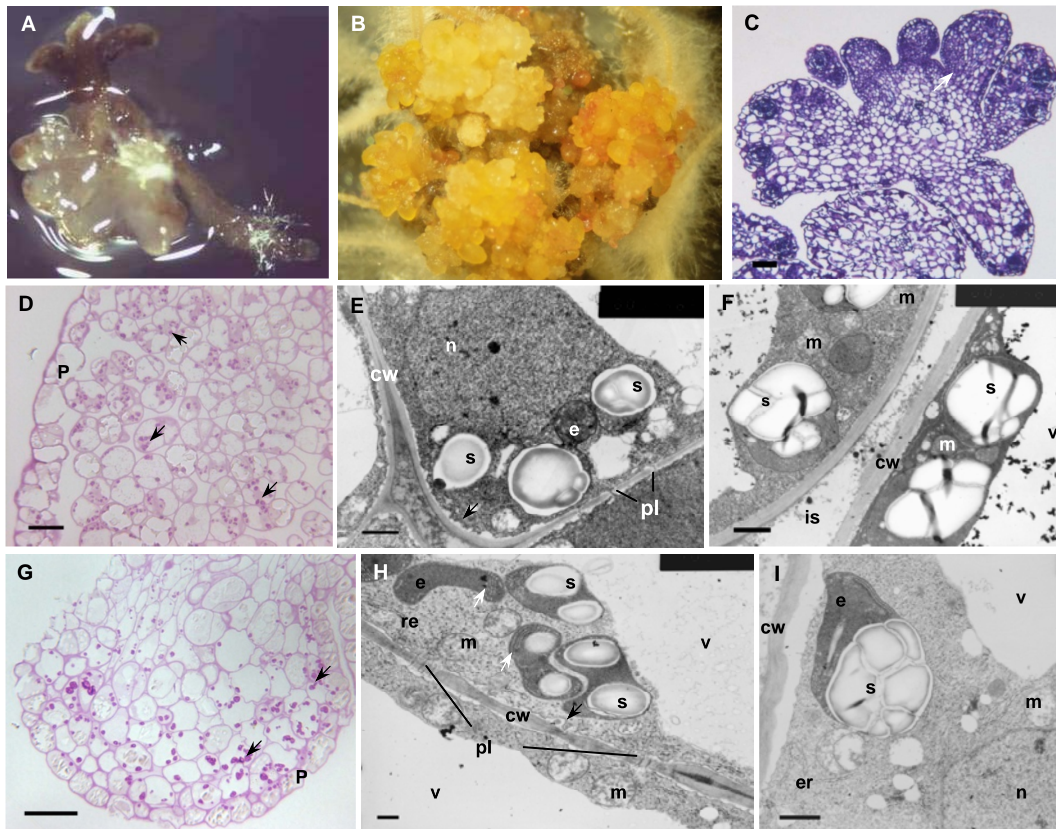


Plate 1. Comparative analysis of primary and secondary embryos in *E. globulus*. **A**) formation of new somatic embryos in the hypocotyl-root transition of a primary somatic embryo on MS_{3NAA}. **B**) Cluster of secondary somatic embryos maintained on MS_{3NAA} for two years. **C**) Secondary somatic embryos cluster cross section stained with toluidine blue, showing independence from the origin tissue (arrow). *Bar* 50µm. D-F) Primary globular somatic embryo. **D**) Embryo section showing a protoderm surrounding parenchymatous like cells rich in starch (arrows). *Bar* 50µm. **E** and **F**) Ultrastructural detail of a cell with a rich cytoplasm, etio-amyloplasts, plasmodesmata, extrusion vesicles (arrow) and intercellular spaces. *Bar* 1µm. G-I) secondary globular somatic embryo. **G**) Embryo section showing a protoderm surrounding parenchymatous like cells rich in starch (arrows). *Bar* 50µm. **H**-**I**) Ultrastructural detail of a cell with rich cytoplasm, abundant plasmodesmata, etio-amyloplasts with thylakoidal organization (white arrow) and extrusion vesicles (black arrow). *Bar* 1µm. Legends: **cw:** cell wall; **e**: etio-amyloplast; **er**: endoplasmatic reticulum; **m**: mitochondria; **n:** nucleous; **p**: protoderm; **s**: starch granules; **v**: vacuole; **is:** intercellular space; **pl:** plasmodesmata

Influence of different conditions on secondary SE

Concerning the effect of salt content in the medium on the production and maintenance of new globular somatic embryos, results show that, in the presence of 3 mg l^{-1} NAA , MS is more effective than B5 (Fig. 2a). As well, the average number of new globular somatic embryos formed increased significantly ($P \leq 0.05$) on PGR-free MS (MS_{WH}). Although $B5_{SH}$ medium gave excellent results for globular secondary embryo proliferation, MS_{SH} was equally efficient.

Concerning the influence of the medium composition on embryo evolution, Fig. 2b shows that no significant differences were found between MS and B5 when supplemented with NAA (3 mg l^{-1}). In the absence of NAA, an heterogeneous response was found, and MS_{WH} increased higher rates of histodifferentiation, relatively to MS with NAA, comparing with $B5_{WH}$ and B5 supplemented with NAA. Therefore, with regard to germination, MS_{WH} supports this stage significantly better than $B5_{WH}$ ($P \leq 0.05$). There was no statistically significant interaction between light conditions and these treatments (Fig. 2c).

The effects of PGRs on proliferation and germination of secondary somatic embryos are shown in Fig. 3. The decrease of auxin concentration (NAA) improved the formation of new globular somatic embryos. Absence of PGRs significantly increased the proliferation of globular somatic embryos per cluster compared with all other PGR conditions. Furthermore, absence of PGR gave better results (proliferation) than MS_{3NAA}, the medium on which they were maintained for approximately 2 years ($P \leq 0.05$, Plate 2a, c, e). However, addition of cytokinins (BAP and/or KIN) had a negative effect at this stage of proliferation (Fig. 3a), although the presence of cytokinins was advantageous for somatic embryo development (Plate 2g and h). For example, the addition of BAP alone significantly increased ($P \leq 0.05$) the number of cotyledonary somatic embryos recovered (Fig. 3b). Also, kinetin had a positive effect on embryo germination (Plate 2h), although not significantly different from results obtained with BAP alone or MS_{WH} (Fig. 3c). At this stage, a decrease in auxin (NAA) was also beneficial.

Data from Figs. 1a and 2a clearly show that the presence of light had a negative effect on the proliferation of new globular secondary somatic embryos. Light and NAA alone repressed secondary SE proliferation in both MS and B5 media, and completely

inhibited histodifferentiation and subsequent germination. In the presence of light, the clusters became green and compact and accumulated antochyanins (Plate 2b, 2f).

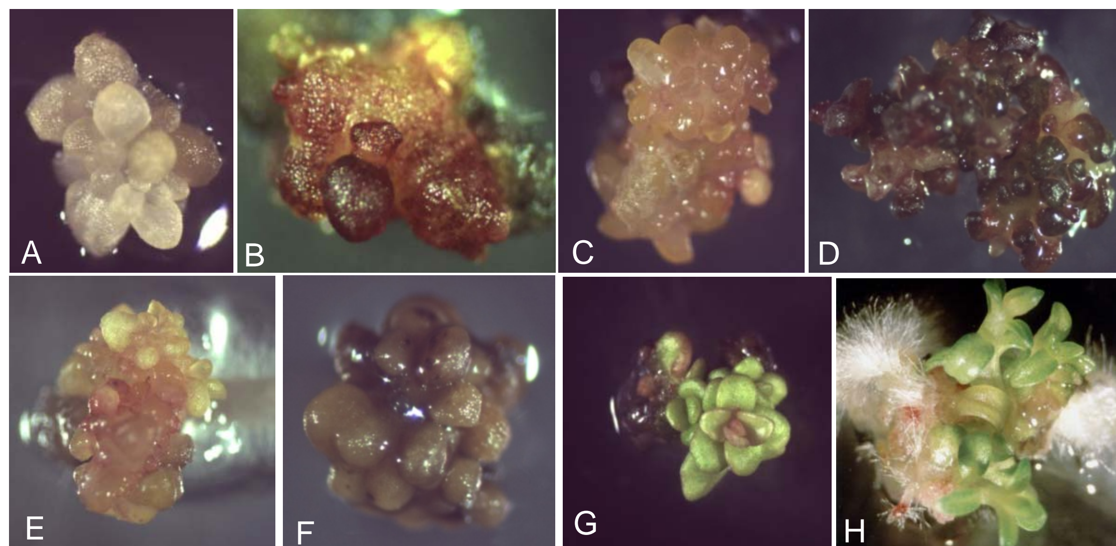
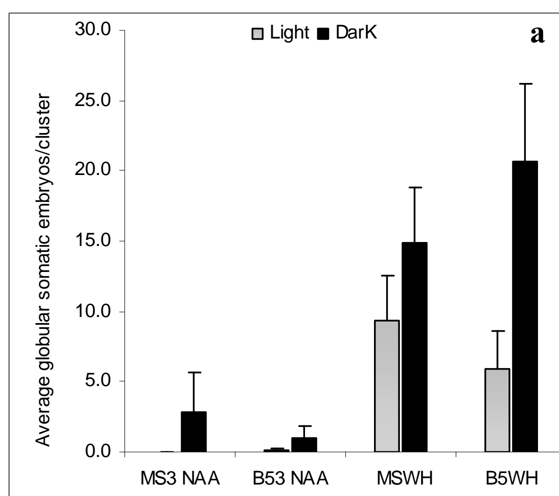


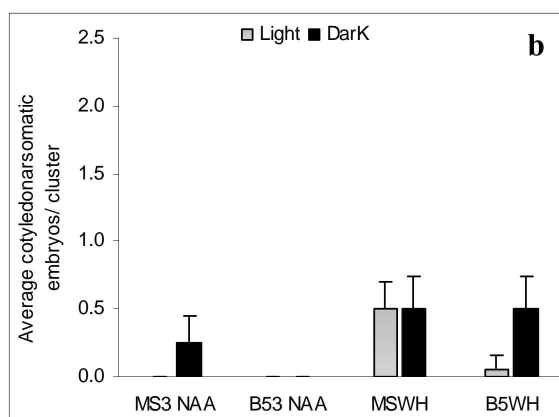
Plate 2: Different aspects of repetitive SE in *E. globulus* after eight weeks on MS medium with different PGRs and light conditions: **A** and **B**) Cluster of secondary somatic embryos maintained on MS_{3NAA} in dark (A) and light (B) (60x); **C** and **D**) Cluster of secondary somatic embryos maintained in MS_{WH} in dark (C) and light (D) (60x); **E** and **F**) Cluster in the presence of 0.2 mg l^{-1} NAA in dark (E) and light (F) (60x); **G**) cluster in the medium containing NAA and BAP with light (60x); **H**) Shoot elongation (40x).

Dark conditions seemed in general to have a more positive effect on SE response, significantly increasing embryo proliferation on $B5_{SH}$ compared with those obtained under light conditions ($P \leq 0.05$, Fig 2a). This positive effect of darkness on somatic embryo proliferation was enhanced in the absence of NAA (Plate 2c and d) for both MS and B5.

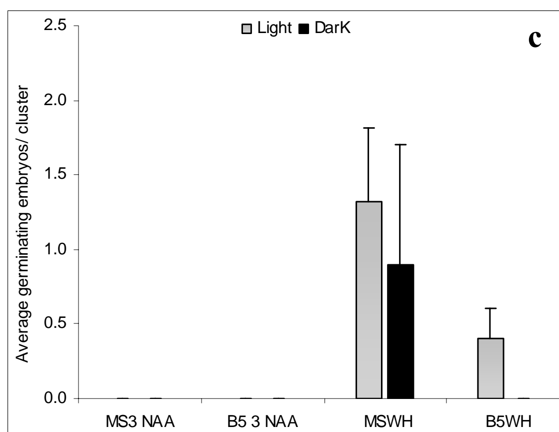
The effect of light shows a positive trend as the evolution of the somatic embryos progresses on MS_{WH} and $B5_{WH}$, and this is especially notable during the transition between the cotyledonary (Fig. 2b) to the germinating stages (Fig. 2c). However, when NAA and BAP are present in the media, darkness offered the best results ($P \leq 0.05$) for achieving the cotyledonary stage.



Source of Variation	DF	MS	F-Ratio	P-value
Light	1	1446.0	8.0	0.05
Treatment	3	1850.9	10.3	<0.001
L X T	3	370.6	2.1	0.101
Error	152	179.9		

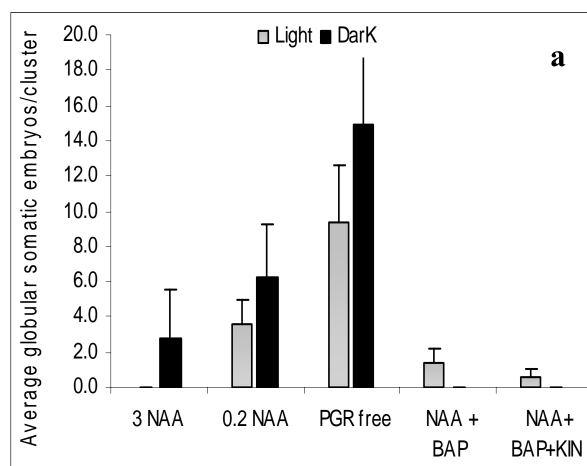


Source of Variation	DF	MS	F-Ratio	P-value
Light	1	1.22	2.52	0.114
Treatment	3	1.85	3.81	0.011
L X T	3	0.47	0.98	0.404
Error	152	0.48		

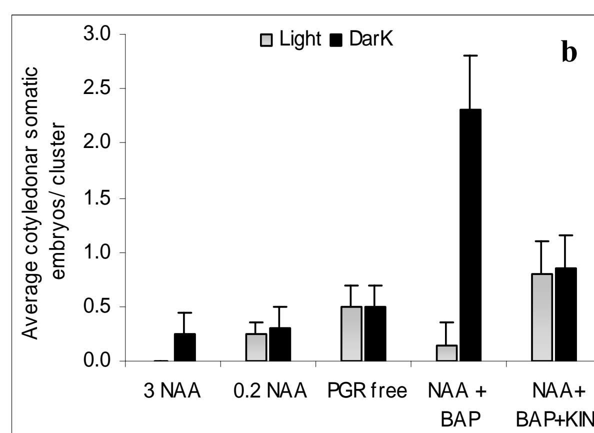


Source of Variation	DF	MS	F-Ratio	P-value
Light	1	1.4	0.680	0.411
Treatment	3	10.5	5.088	0.002
L X T	3	0.5	0.229	0.876
Error	152	2.1		

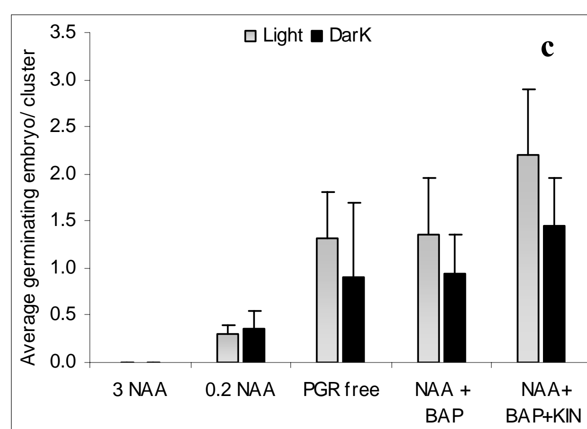
Figure 2: Effect of medium composition (with or without NAA) and light on secondary SE response after two months. **a**) New globular somatic embryos. **b**) Cotyledonar somatic embryos. **c**) Germinating somatic embryos. Values are means ± Standard error (SE). At right, the respective tables of analysis of variance (Two Way ANOVA) using Duncan's Method ($P < 0.05$).



Source of Variation	DF	MS	F-Ratio	P-value
Light	1	163.8	1.8	0.183
PGRs	4	980.9	10.7	<0.001
L X PGRs	4	79.6	0.9	0.483
Error	190	91.5		



Source of Variation	DF	MS	F-Ratio	P-value
Light	1	12.5	9.3	0.003
PGRs	4	7.8	5.8	0.003
L X PGRs	4	8.6	6.4	<0.001
Error	190	1.3		



Source of Variation	DF	MS	F-Ratio	P-value
Light	1	4.2	1.0	0.313
PGRs	4	20.9	5.1	<0.001
L X PGRs	4	1.1	0.3	0.904
Error	190	4.2		

Figure 3: Effect of PGRs on secondary SE response after two months. **a**) New globular somatic embryos. **b**) Cotyledonar somatic embryos. **c**) Germinating somatic embryos. Values are means ± Standard error (SE). At right, the respective tables of analysis of variance (Two Way ANOVA) using Duncan's Method ($P < 0.05$).

Discussion

Repetitive SE

The SE re-induction strategy described here led to the maintenance of embryogenic potential for more than 2 years. As reported for other species, maintenance of a repetitive

SE process makes a permanent source of morphogenetic material available for prolonged periods of time (Neves *et al.* 1999). Data obtained here confirm that the use of clustered somatic embryos is crucial for maintaining competence during first developmental (globular) stages, whereas in later stages (cotyledonary, germinated somatic embryos), isolation is not inhibitory. One possible explanation for the apparent correlation between embryo isolation and development for repetitive competence is the small size of these embryos at the earlier stages. In fact, previous attempts to isolate globular embryos, in this study, revealed that they were unable to undergo repetitive SE (data not shown). Muralidharan and Mascarenhas (1995) reported the isolation of somatic embryos in early stages for *E. citriodora,* but used liquid culture in the presence of auxin (NAA).

Furthermore, in the cotyledonary or germinated stages, new embryos usually arise from the transition zone between root and hypocotyl. Similarly, in *E. citriodora,* secondary SE developed at the radicle of the primary embryos (Muralidharan and Mascarenhas 1995). Also in cork oak, the later developmental stages are preferable for repetitive SE (Pinto *et al.* 2002b, Hernández *et al.* 2003). However, in other embryogenic systems, the early developmental embryo stages (e.g. globular and heart stages) showed higher potential for secondary embryogenesis compared with later stages (Neves *et al.* 1999, Nair and Gupta 2006).

Although global histocytological aspects were similar in both primary and secondary embryos, the latter showed an apparently more organized parenchymatous tissue, with less intercellular spaces and more localized starch granules, together with a thylakoidal organization in plastids. The potential correlation between these characteristics and the greater ability of secondary somatic embryos to evolve and regenerate plants is still unknown and any explanation would be speculative. The high abundance of plasmodesmata in these embryos indicates high connections between cells, and Canhoto *et al.* (1996) suggested that, in early phases of somatic embryo differentiation, connections are essential for the normal course of somatic embryo development, which is supported by our findings in *E. globulus*.

Medium composition, PGRs and light

Data reported here show that the tested factors (medium composition, PGRs, and light) influenced repetitive SE in different ways.

Although $B5_{SH}$ medium gave excellent results for globular secondary embryo proliferation, it is not suitable for supporting more advanced somatic embryo stages. However, MS_{SH} was equally efficient for globular embryo proliferation and further evolution and germination. Therefore, the data support a recommendation to use MS medium in similar repetitive SE systems within *Eucalyptus*. Comparative mineral studies among different culture media (e.g., B5 and MS) and *E. globulus* explant composition also support this suggestion (Pinto *et al*. 2006). Results reported here highlight the importance that salt content of media could have on the different steps of the SE process, although most of the protocols published use the same medium during the entire process.

With regard to PGRs, our data show that a decrease in auxin concentration increased secondary somatic embryo proliferation, and also that, in the later stages, the presence of BAP alone (for the cotyledonary stage) or in combination with KIN (for the germinating stage) had a stimulatory effect. NAA was already used for the initiation of *E. globulus* embryogenic cultures (Pinto *et al.* 2002a) and was also effective for maintaining embryogenic competence. However, results reported here prove that a reduction of NAA is desirable and will significantly improve the rate of proliferation of new globular somatic embryos. The possibility of maintaining the embryogenic competence in a PGR-free medium is a significant result of this work. Also, the suitability of PGR-free medium for secondary embryogenesis has been reported in different embryogenic systems (Fernández-Guijarro *et al.* 1995, Neves et al. 1999, Pinto *et al.* 2002b). Zimmerman (1993) suggested that once embryogenesis is induced, the auxin roles changes, and embryos begin to synthesize their own auxins and thus require lower auxin.

The risk of somaclonal variation may be reduced when, after initiation, somatic embryos are transferred to and maintained on PGR-free media, based on the assumption that high levels of exogenous PGRs can induce somaclonal variation (Berlyn *et al.* 1986). Pinto *et al.* (2004a) demonstrated for *E. globulus* that embryogenic clusters maintained for 8 months in the presence of NAA showed ploidy stability by flow cytometry. Despite these results with NAA, we believe that removing PGR from the medium will reduce the probability of somaclonal variation in *E. globulus*, increasing the benefits that long-term cultures may bring to *Eucalyptus* SE breeding programs.

In the present study, the highest number of cotyledonary somatic embryos was obtained in MS supplemented with NAA and BAP under dark conditions, whereas best

germination (with apical shoot promotion and elongation) was achieved in the presence of NAA, BAP and KIN under light. Also, in germinating somatic embryos of myrtle, BAP combined with gibberellic acid induced shoot bud elongation and proliferation, although root growth was inhibited (Canhoto *et al.* 1999). Also, maximal conversion of somatic embryos in pepper was observed on MS with BAP and kinetin (Yusuf *et al.* 2001).

In general, SE protocols report light conditions (often darkness for SE induction), but systematic studies on the light effect in SE potential are limited. The induction of primary SE in *E. globulus* occurs in the dark (Pinto *et al.* 2002a).The presence of light at the proliferation stage had a negative effect on the culture, and our results suggest that darkness is preferable for maintenance and proliferation of globular secondary embryos and should be the preferred condition until the cotyledonary stage is reached. However, in this species, as the somatic embryos evolved, and particularly during the germination phase, the effect of light often gained in importance. In *E. citriodora*, the proliferation of embryogenic masses also occurred in the dark. A similar strategy is used in black pepper (*Piper nigrum* L.), where light is used when the main objective is germination, conversion, and ex vitro establishment of SE-derived plantlets (Nair and Gupta 2006).

Conclusions

In conclusion, this is the first report that defines the optimal culture conditions necessary to induce and sustain repetitive cycles of secondary SE in *E. globulus*. The reported protocol is highly efficient for repetitive SE in *E. globulus,* producing a much larger number of somatic embryos than in primary SE, and therefore, increasing the plant's potential for regeneration. The most significant result from this investigation is the possibility of maintaining embryogenic competence in a PGR-free medium as an alternative to continuous exposure to an auxin (NAA). Considerable improvements were also achieved by adding cytokinis in the later stages of somatic embryos to potentially improve the plant propagation system in this species by secondary SE. By maintaining secondary SE, researchers can now access a permanent source of material over long period.

References

Berlyn GP, Bock RC, Renfroc MH (1986) Tissue culture and the propagation and genetic improvement of conifers. Tree Physiol 1:227–240

Boulay M (1987) Recherches preliminaires sur l'embryogénèse somatique d'*Eucalyptus gunnii*. Annales de Recherches Silvicoles, Association For &Cellulose, pp. 23–37

Canhoto JM, Mesquita JF, Cruz GS (1996) Ultrastructural changes in cotyledons of pineapple Guava (Myrtaceae) during somatic embryogenesis. Ann Bot 78:513–521.

Canhoto JM, Lopes ML, Cruz GS (1999) Somatic embryogenesis and plant regeneration in myrtle (Myrtaceae). Plant Cell Tiss Organ Cult 57:13–21

Corredoira E, Ballester A, Vieitez AM (2003) Proliferation, maturation and germination of *Castanea sativa* Mill. Somatic embryos originated from leaf explants. Ann Bot 92:129–136

Neves LO, Duque SRL, de Almeida JS, Fevereiro PS (1999) Repetitive somatic embryogenesis in *Medicago truncatula* ssp. Narbonensis and *M. truncatula* gaertn cv. Jemalong. Plant Cell Rep 18:398–405

Fernandez-Guijarro B, Celestino C, Toribio M (1995) Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber.* Plant Cell Tiss Organ Cult 41:99–106

Gaj MD (2004) Factors influencing somatic embryogenesis induction and plant regeneration with particular reference to *Arabidopsis thaliana* (L.) Heynh. Plant Growth Regul 43 (1):27–47

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Hernández I, Celestino C, Alegre J, Toribio M (2003) Vegetative propagation of *Quercus suber* L. by somatic embryogenesis. II. Plant regeneration from selected cork oak trees. Plant Cell Rep 21:765–770

Jiménez VM (2005) Involvement of plant hormones and plant growth regulators on in vitro somatic embryogenesis. Plant Growth Regul 47:91–110

Kärkönen A (2000) Anatomical study of zygotic and somatic embryos of *Tília cordata*. Plant Cell Tiss Organ Cult 61:205–214

Martinelli L, Candioli E, Costa D, Poletti V (2001) Morphogenic competence of *Vitis rupestris* S. secondary somatic embryos with a long culture history. Plant Cell Rep 20:279–284

Merkle SA (1995) Strategies for dealing with limitations of somatic embryogenesis in hardwood trees. Plant Tissue Culture and Biotechnology 1:112–121

Muralidharan EM, Gupta PK, Mascarenhas AF (1989) Plantlet production trough high frequency somatic embryogenesis im long term cultures of *Eucalyptus citriodora*. Plant Cell Rep 8:41–43

Muralidharan EM, Mascarenhas AF (1995) Somatic embryogenesis in *Eucalyptus*. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 2-Angiosperms. Kluwer Academic Publishers, Dordrecht, pp 23–40

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nair RR and Gupta SD (2006) High- frequency plant regeneration trough cyclic secondary somatic embryogenesis in black pepper (*Piper nigrum* L.). Plant Cell Rep 24:600–707

Nugent G, Chandler S, Whiteman P, Stevenson T (2001) Somatic embryogenesis in *Eucalyptus globulus*. Plant Cell Tiss Organ Cult 67:85–88

Oller J, Celestino C, López-Vela D, Alegre J, Toval G, Toribio M (2006) Induction of somatic embryogenesis in mature zygotic embryos of *Eucalyptus globulus* Labill. 2nd Simposio Iberoamericano de *Eucalyptus globulus*. 17–20 October, Pontevedra, Spain

Parra R, Amo-Marco JB (1998) Secondary somatic embryogenesis and plant regeneration in myrtle (*Myrtus communis* L.) Plant Cell Rep 18:325–330

Pinto G, Loureiro J, Lopes T, Santos CV (2004a) Analysis of genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry. Theor Appl Gen 109:580–587

Pinto G, Santos C, Neves L, C Araújo (2002a) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21:208–213

Pinto G, Silva S, Coutinho J, Araujo C, Neves L, Santos C. (2006) Importance of media mineral composition on the induction of somatic embryogenesis *in Eucalyptus globulus* Labill. 2nd Simpósio Iberoamericano de *Eucalyptus globulus*. 17–20 October, Pontevedra, Spain

Pinto G, Silva S, Santos C, Neves L, Araújo C (2004b) Somatic embryogenesis of *Eucalyptus globulus* labill. and assessement of genetic stability. In: Borralho NMG, Pereira JS, Marques C, Coutinho J, Madeira M, Tomé M (eds.) Eucalyptus in a changing world. Proc IUFRO Conf, 11–15 October, Aveiro, Portugal, pp 543–544

Pinto G, Valentim H, Costa A, Castro S, Santos C (2002b) Somatic embryogenesis and plant regeneration from leaf calli of mature *Quercus suber* L. plants. In Vitro Cell Dev Biol Plant 6 (4):569–572

Prakash MG, Gurumurthi K (2005) Somatic embryogenesis and plant regeneration in *Eucalyptus tereticornis* Sm. Curr Sci 88 (8):1311–1316

Raemakers CJJM, Jacobsen E, Visser RGF (1995) Secondary somatic embryogenesis and aplications in plant breeding. Euphytica 81:93–107

Ramage CM, Williams RR (2002) Mineral nutrition and plant morphogenesis. In Vitro Cell Dev Biol Plant 38:116–124

Von Arnold S, Sabala I, Bozhkov P, Dyachaok J, Filanova L (2002) Developmental pathways of somatic embryogenesis. Plant Cell Tiss Organ Cult 69:233–249

Watt MP, Blakeway FC, Termignoni R, Jain SM (1999) Somatic embryogenesis in *Eucalyptus grandis* and *E. dunnii*. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 5. Kluwer Academic Publishers, Dordrecht, pp 63–78

Williams EG, Maheswaran G (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. Ann Bot 57:443–462

Yusuf A, Tyagi RK, Malik SK (2001) Somatic embryogenesis and plantlet regeneration from leaf segments of *Piper colubrinum.* Plant Cell Tiss Organ Cul 65:255–258.

Zimmerman JL (1993) Somatic embryogenesis: a model for early development in higher plants. Plant Cell 5:1411–1423

III 3: Acclimatization of Secondary Somatic Embryos Derived Plants of *Eucalyptus globulus* Labill.: An Ultrastructural Approach

Chapter submitted as an original paper to a SCI journal:

Pinto G, Silva S, Loureiro J, Costa A, Araújo C, Neves L, Santos C (2007) Acclimatization of Secondary Somatic Embryos Derived Plants of *Eucalyptus globulus* Labill.: An Ultrastructural Approach. Annals of Botany (Submitted)

Abstract

An efficient protocol for plant regeneration by repetitive somatic embryogenesis (SE) in *Eucalyptus globulus* was recently developed. For industrial application, the acclimatization of these SE-derived plants (emblings) to ex vitro conditions must be achieved. Therefore, by knowing the changes occurring in embligs during acclimatization, the process may be better controlled and optimized.

Secondary somatic embryo clusters were maintained for one year on Murashige and Skoog (MS) medium without growth regulators. Clusters with cotyledonar embryos were subcultured on elongation medium to promote shoot elongation. Prior to acclimatization, emblings were analysed for morphological, and for ploidy abnormalities by flow cytometry. Emblings were transferred to peat:perlite and acclimatized in a phytotron, with progressive reduction of relative humidity and increase of light intensity. Histocytological analyses were performed in order to follow histocytological and reserves accumulation changes in emblings' leaves during acclimatization.

The acclimatization protocol described here allowed the recovery of ex vitro emblings. Emblings used looked morphologically normal and had no ploidy or DNA content changes. Histocytological comparative analyses of in vitro and ex vitro acclimatized plants showed significant changes along time, mostly in stomata shape and aperture, starch reserves and chloroplast morphology. Mesophyll differentiation also showed major changes after two weeks.

Key words: Emblings, *Eucalyptus*, histological differentiation, Myrtaceae, plant acclimatization, ploidy stability, secondary somatic embryos, ultrastructural studies

Introduction

The success of any in vitro propagation process on a commercial scale depends on the ability to regenerate a high number of plantlets and to efficiently acclimatize them to ex vitro conditions (Barry- Etienne *et al*. 2002, Hazarica 2006). Although, conditions for SE induction and plant regeneration are well established in many species, acclimatization continues to be a major bottleneck in the commercial application of these SE protocols, as a high percentage of emblings are lost or damaged when transferred to greenhouse or field (e.g. Pospisilova *et al*.1999).

Overall, plantlets obtained by SE (emblings) are regenerated in vitro in a semi-solid medium and later acclimatized (once they have a few pair of leaves and a root system). In vitro, these plantlets grow in a unique aseptic microenvironment with special conditions in air-tight cultivation vessels, under low light intensity, on a medium containing sugar and nutrients to allow the heterotrophic growth and in an atmosphere with high level of humidity (Pospisilova *et al*. 1999, Hazarika 2003, 2006). These conditions frequently lead to abnormal morphology, anatomy and physiology, often characterised by, e.g. poor photosynthetic efficiency, retardation in development of the cuticle, non functional stomata apparatus, or by changes in wax deposition on leaf surface (Pospisilova *et al.* 1999, Hazarica 2006). Absence of palisade cells, large intercellular spaces and large chlorophyll-rich cells are also common features of in vitro grown plantlets, as reported for *Eucalyptus saligna* (Jones *et al.* 1993).

During this period, transpiration rates may be controlled by the plant by developing an effective stomatal regulation of gas exchange, correlated with changes in leaf anatomy (Hazarica 2006). It has been demonstrated that epicuticular wax, cuticule and periclinal cell walls are very important barriers against water loss. Wetzstein and Sommer (1992) and Gilly *et al.* (1997) reported an increase in thickness of these structures during acclimatization of *Liquidambar styraciflua* and *Hedera helix*, respectively. In *Eucalyptus saligna*, the number and volume of chloroplasts in palisade parenchyma, number of thylakoids per granum and volume of starch granules were the main changes verified during acclimatization (Jones *et al.* 1993). Changes in morphology, dimension and frequency/distribution of stomata were also shown to be strongly conditioned during acclimatization (Harazika 2006).

The loss of in vitro formed plantlets (obtained by shoot multiplication) during the acclimatization process was reported as common in *Eucalyptus* genus, often due to the formation of hyperhydric plants (Louro 1999). With respect to *Eucalyptus* emblings, most works are restricted to germination frequency, conversion into plantlets and survival rates during acclimatization (Muralidharan *et al*. 1989, Watt *et al.* 1991, Pinto *et al*. 2002a, Prakash and Gurumurthi 2005), with no focus on the undergoing morphological and physiological processes**.** Understanding the physiological and morphological behaviour of in vitro *Eucalyptus* emblings and the changes that occur during the acclimatization process should facilitate the development of an efficient acclimatization protocol.

Also, the production of “true-to-type” plants is a prerequisite for mass clonal propagation, including SE. Somaclonal variation is often found in many plant species (Endemann *et al.* 2002). Somaclonal variations can be analysed at the level of the phenotype or of the genotype (Kaeppler *et al.* 2000) and therefore, several phenotypic and genetic markers may be used, each giving valuable but restricted information. Among genotypic variations, genomic mutations affect the number of chromosomes and can be detected by flow cytometry (FCM) or chromosome counting (Tremblay *et al.* 1999). FCM, has recently been preferred to rapidly screen for ploidy/DNA content changes in SE-derived plantlets (*e.g.* Pinto *et al.* 2004a; Loureiro *et al.* 2005, Santos *et al*. 2007), often combined with other phenotypic or genotypic evaluations (e.g. Santos *et al.* 2007)

The aim of this work was to acclimatize *E. globulus* emblings (showing ploidy stability) to ex vitro conditions, and to comparatively follow morphological, histocytological and reserves accumulation during this process in order to better understand and control, in the future, the process.

Material and Methods

Plant material and plant regeneration from secondary somatic embryos

Eucalyptus globulus Labill. secondary somatic embryos were obtained according to Pinto *et al.* (2004 a-c), and were maintained on MS medium for one year without growth regulators (MS_{WH}). Clusters of cotyledonary embryos were transferred to MS elongation medium (Celbi internal report) supplemented with 30 g l^{-1} sucrose and 2.5 g l^{-1} gelrite. The pH was adjusted to 5.8 prior to autoclaving and incubated under a photoperiod of 16 h

with a light intensity of 23.5 µmol m^{-2} s^{-1}. To improve root elongation, emblings were transferred to MS medium with 1 mg l^{-1} indole-3-butyric acid (IBA) for 10 days and incubated at 24 ± 1 ºC in the dark. Emblings were then transferred to MSWH and incubated under a photoperiod of 16h with a light intensity that was gradually increased up to 23.5 µmol m^{-2} s^{-1}.

Assessment of emblings ploidy stability by flow cytometry

Nuclear suspensions from leaves of 24 emblings were prepared according to Galbraith *et al.* (1983) as described by Pinto *et al.* (2004a). In brief, samples were chopped together with a young leaf of the internal reference standard *Solanum lycopersicum* cv. Stupicke (2C = 1.96 pg DNA; kindly provided by J Doležel, Laboratory of Molecular Cytogenetics and Cytometry, Institute of Experimental Botany, Olomouc, Czech Republic) in Marie's nuclear isolation buffer (Marie and Brown 1993). The nuclear suspension was filtered through an 80 µm nylon filter. Then 50 µg ml^{-1} of propidium iodide (PI; Fluka, Buchs, Switzerland) and 50 µg ml^{-1} of RNAse (Fluka) were added to the samples. The relative fluorescence intensity of PI-stained nuclei was measured by a Beckman Coulter® EPICS XL (Beckman Coulter®, Hialeah, USA) flow cytometer. Instrument calibration and sample analysis were performed according to Pinto *et al.* (2004a). For each sample at least 5,000-10,000 nuclei were analysed. Nuclear genome size was calculated according to:

$$\textit{E. globulus}\text{ 2C nuclear DNA content (pg)} = \frac{\textit{E. globulus}\ G_0/G_1\text{ peak mean FL}}{\textit{L. esculentum}\ G_0/G_1\text{ peak mean FL}} \times 1.96$$

Emblings acclimatization to greenhouse

Forty plantlets (minimum 2 cm long) were transferred to pots containing sterilized peat:perlite (3:2) and were weekly watered with a commercial solution of 5 ml l^{-1} Complesal-Calcium® (Agrevo). Occasionally, Previcur® (1.5 ml l^{-1}) and Derosal® (0.75 g l^{-1}) were added to both plantlets and soil. During acclimatization, plantlets were grown in a phytotron (Snijders) at 24 ºC with a photoperiod of 16 h and a light intensity of 480 µmol m^{-2} s^{-1}. Initial relative humidity (RH) was higher than 95 %, and was weekly decreased until 50- 60 % RH. For a general overview of the process, see Figure 1.

Histological and cytological analyses

For histological and cytological characterisation, samples were collected from: a) leaves from emblings growing in vitro; b) leaves from emblings at six different stages of acclimatization (three hours, three days, one, two and four weeks and three months).

For transmission electron microscopy (TEM), samples were fixed in 2.5 % glutaraldehyde in 1.25 % (w/v) piperazine-N,N'-bis-2-ethanesulfonic acid (PIPES) buffer (pH 7.4) for 3 h and washed in PIPES. Tissues were transferred to 1.0 % (w/v) osmium tetroxide in PIPES buffer for 1h, rinsed in the same buffer, dehydrated through a graded ethanol series and embedded in a graded low-viscosity epoxi resin (Embed-812). The blocks were polymerised at 60 ºC for 48 h. Ultra-thin sections were cut with a LKB ultra-microtome (Leica Microsystems AG, Germany), stained with uranyl acetate for 15 min and lead citrate for 10 min and observed with a Elmiskop-101 transmission electron microscope at 80 kV (Siemens AG, Germany). For light microscopy, semi-thin sections (0.5-1.5 µm) were stained with 0.1 % (w/v) toluidine blue or by the periodic acid-Schiff (PAS) reaction. Samples were analysed in a Nikon Eclipse 80i light microscope (Nikon Co, Kanagawa, Japan) and photographs were taken using a Leica DC 200 digital camera (Leica Microsystems AG, Germany).

For scanning electron microscopy (SEM) analysis, material preparation and fixation were performed as previously described by Pinto *et al*. (2002b). Briefly, leaf samples were fixed with 2.0 % (v/v) glutaraldehyde in PIPES buffer, at 4 ºC for 16 h. Dehydration was achieved by successive immersions in aqueous ethanol solutions of increasing concentration (30 % - 100 %, v/v), acetone solutions of increasing concentration (30 % - 100 %, v/v) and finally in a critical point device (Baltec CPD 030, USA) using CO_2 as transition agent. Samples were fixed on steel supports and coated with gold using a JEOL metalizer (FFC-1100,Japan) at 1100-1200 V, 5 mA for 10 min. Samples were observed in a scanning electron microscope (Hitachi, S4100, Japan) at 20 kV. PIPES buffer was acquired in Duchefa (Haarlem, The Netherlands), while the remaining chemicals were purchased to Agar Scientific (Essex, UK).

Morphometric studies

Predefined selected regions in microphotographs from the different conditions (n ≥10 for each condition) were measured using ImageTool for Windows (vs 3.00, Univ.Texas Health Science Center, San Antonio,USA). The following parameters were assessed: leaf thickness (µm), palisade and spongy parenchyma thicknesses (µm), areas occupied by intercellular spaces (μm^2), chloroplasts area (μm^2), number of starch granules per cell and number of plastoglobules per chloroplasts.

Statistical analyses

Statistical analyses were performed using a one-way ANOVA on Ranks (SigmaStat for Windows Version 3.1, SPSS Inc., USA) to assay for possible differences in the morphometric measures among samples from different acclimatization stages. A multiple comparison Dunn's test was applied to determine which groups were different.

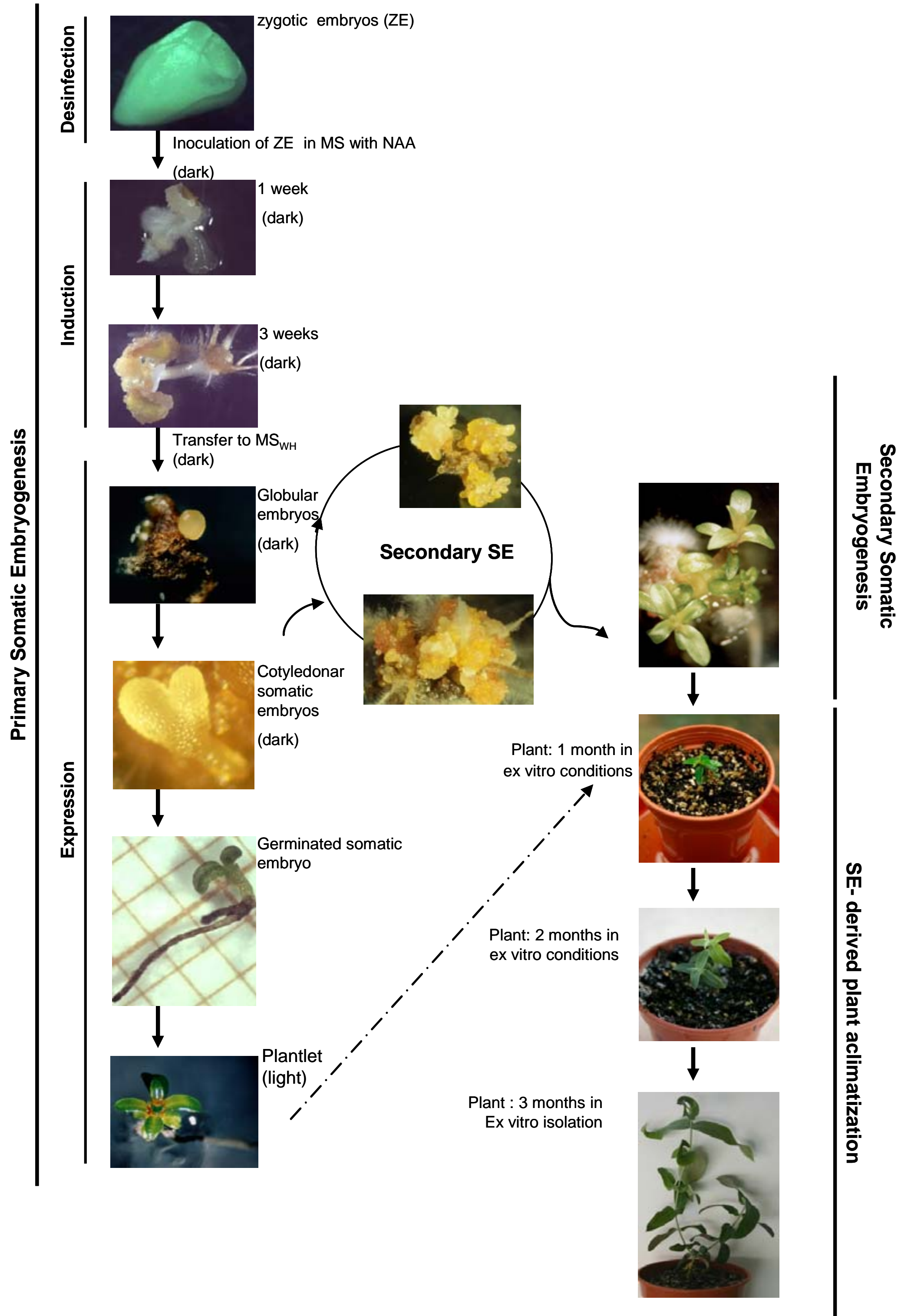


Figure 1: Overview of the whole process of *E. globulus* plant regeneration by SE; covering both primary SE process (left side) and secondary SE together with plant acclimatization (right side).

Results

Plant regeneration from secondary somatic embryos

In the somatic embryogenic clusters, maintained for one year, it was possible to follow the differentiation of somatic embryos to the cotyledonar stage (with well defined cotyledons, a vascular system and a root pole) (Plate 1A). Cotyledonar embryos in isolated clusters were able to elongate on elongation medium (Plate 1B) and, then, it was possible to separate emblings from the cluster without damaging the roots (Plate 1C). However, this separation was often difficult to achieve due to both cluster compacticity and the fragility of the emblings' root system (often consisting of only one thin, poorly elongated and phenolized root). In order to overcome this problem and improve root quality, SE-derived plantlets were transferred to a rooting medium before hardening. This strategy resulted in a higher number of plants that were ready for acclimatization (Plate 1D).

Emblings under in vitro conditions presented round opened stomata in both leaf sides (Plate 1E). Emblings with 2 cm long (on average) were selected for acclimatization and after two weeks (in a phytotron), leaves acquired longer oval stomata with prominent epicuticular wax (Plate 1 F). By this time, plants survival rate was approximately 50 % (Plate 1G). With time, emblings showed a normal morphological development, with well developed leaves and internodes, no signs of stunting, hyperhidricity or chlorosis (Plate 1 I-K). In fact, no morphological differences (e.g. similar shoot diameter/form and similar root system) were seen between these emblings and seedlings (Plate 1H).

Plate 1: A) Longitudinal section of a cotyledonary phase somatic embryo, showing well developed cotyledons, vascular bundles and apical and root poles (bar = 1mm). B) Plantlet elongation (bar = 1 cm). C) SE-derived plantlet. D) SE-derived plantlets selected for acclimatization studies (bar = 2 cm). E) Round and opened stomata from an embling under in vitro conditions (SEM, bar= 10 μm). F) Longer and oval stomata with prominent epicuticular wax from one plant two weeks after transfer to ex vitro conditions (SEM, bar = 10 μm). G) SE-derived plants two weeks after transfer to ex vitro conditions. H) Plants derived from zygotic embryo (ZE) and from somatic embryo (SE) conversion, being evident no significant morphological changes among them independently of their origin. I) SE-derived plant 3 months after transfer to ex vitro conditions. J and K) SE-derived plant before and after, respectively, transfer to open greenhouse.

(Plate 1, see page 182)

Assessment of emblings ploidy stability by flow cytometry

Emblings were also screened with respect to ploidy stability and all (24) presented a highly homogenous diploid genome size of 1.38 ± 0.02 pg with a typical G_0/G_1 dominant peak and a smaller G_2 peak (Figure 1). Mean CV values of 3.05 % and low background debris were obtained. This data is in perfect agreement with previous analysis (Table 1) and suggests that no major somaclonal variations are observed among the analysed emblings. Therefore, a "true-to-type" of these emblings propagation was assured, concerning the measured morphological and ploidy parameters.

Table 1: Nuclear DNA content of *Eucalyptus globulus* Labill. emblings. Previous estimations for somatic embryos and leaves of field trees are also given. The values are given as the mean and standard deviation of the mean (SD) of the nuclear DNA content (pg/2C) and as 1C genome size of *Eucalyptus globulus* Labill.

Plant material	Nuclear DNA content (pg/2C)		1C Genome size (Mbp)*	*n*
	Mean	SD		
Leaves of emblings	1.38	0.02	675	24
Somatic embryos**	1.39	0.02	680	21
Leaves of field plant**	1.40	0.03	689	9

*1 pg DNA = 978 Mbp (Doležel *et al.* 2003)
**Data taken from Pinto *et al.* (2004a, see Chapter III.1)

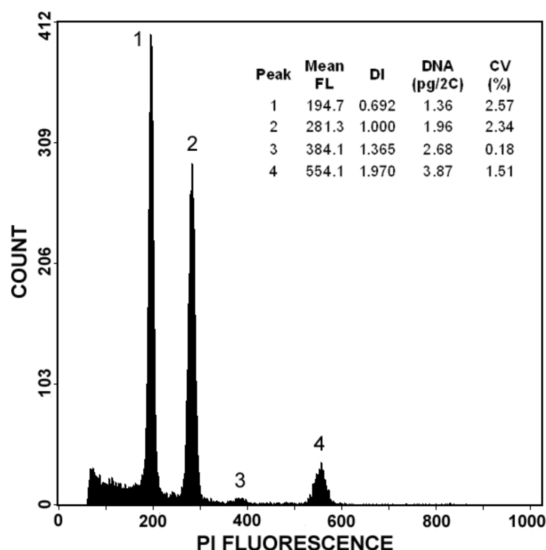


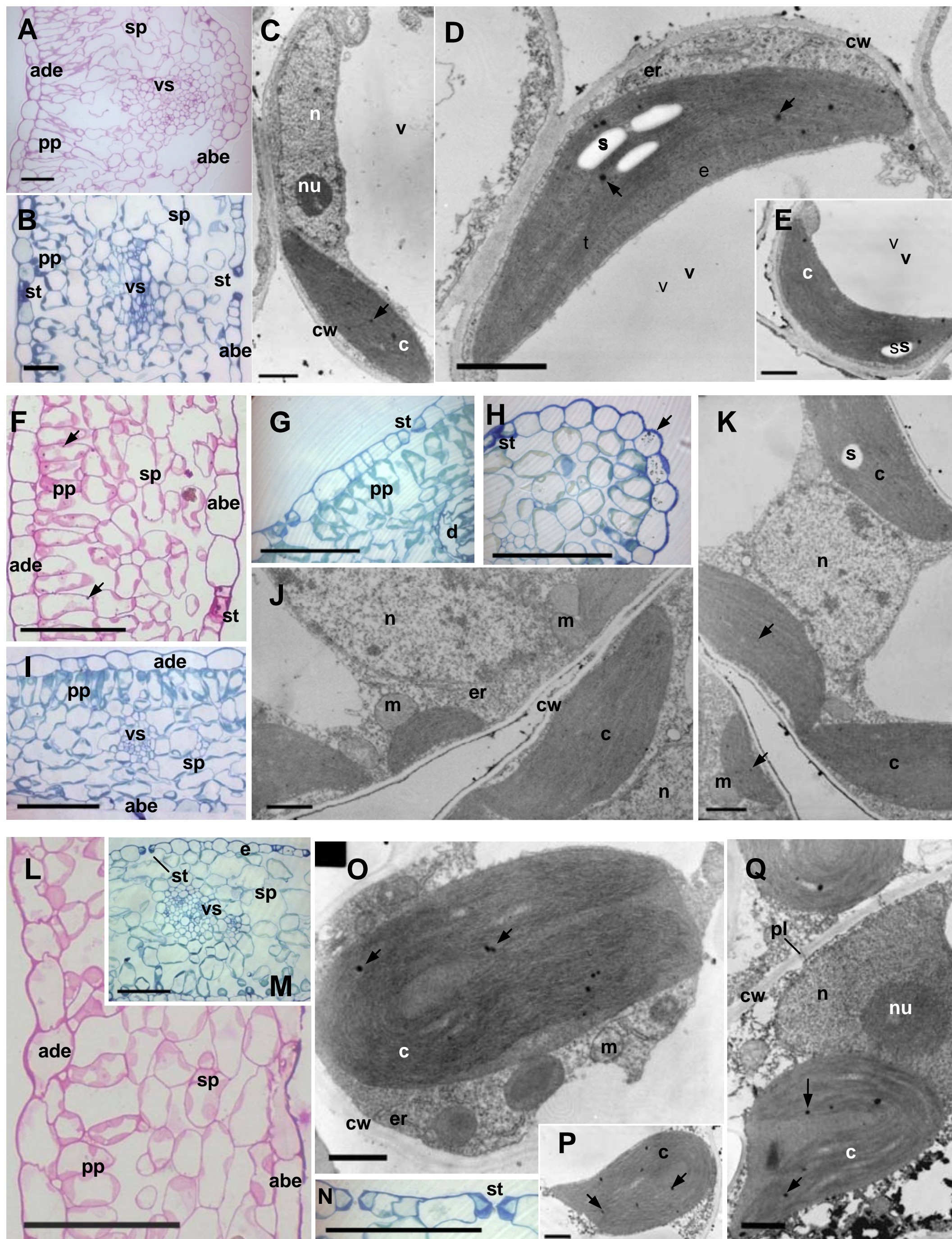
Figure 2: Histogram of relative fluorescence intensity (PI fluorescence) obtained after simultaneous analysis of nuclei isolated from leaves emblings and *Solanum lycopersicum* cv. Stupicke (2C = 1.96 pg DNA, as an internal reference standard). The following four peaks were observed: 1 – nuclei at G_0/G_1 phase of *E. globulus*; 2 – nuclei at G_0/G_1 phase of *S. lycopersicum*; 3 - nuclei at G_2 phase of *E. globulus*; 4 – nuclei at G_2 phase of *S. lycopersicum* leaves. The mean fluorescence (Mean FL, channel numbers), DNA index (DI, ratio between the mean channel number of sample and reference standard), nuclear DNA content (DNA, pg/2C) and coefficient of variation (CV, %) of DNA peaks are given.

Emblings acclimatization to greenhouse: histological and cytological characterization

In general, in vitro leaves had a thin cuticle (e.g. Plate 2A) and their mesophyll consisted of one single layer of poorly differentiated palisade cells, averaging 2.5±0.2 μm in length (Table 2). The spongy tissue had an average height of 6.4±0.3 μm (Table 2) and consisted of three to five small cell layers (Plate 2A and B). Chloroplasts were present in both palisade and spongy parenchyma with an average area of 6.5±0.7 μm^2 (Table 2). These chloroplasts had regular shapes, had poorly developed grana and small starch granules and some osmiophillic bodies (plastoglobulus) were detected (Plate 2C-E, Table 2). The low content of starch granules (0.3±0.1 granules/cell, Table 2) in these leaves was confirmed by PAS staining (Plate 2A). Also, no lipid or protein reserves (Plate 2B) were detected at this stage. Three hours after transferring to ex vitro conditions, stomata were in general closed, even when exposed to light (Plate 2F-H). No histological significant changes were observed in mesophyll differentiation (Plate 2F-I) with respect to in vitro leaves (Plate 2 A and B), but the density of starch granules increased (Plate 2F, 1.3±0.1 granules/cell, Table 2). Changes in chloroplasts shapes were detected, and plastoglobulus density increased (Plate 2J, K, Table 2), with respect to in vitro condition.

Three days after acclimatization, intercellular spaces in mesophyll increased (Plate 2L, M, Table 2), but stomata were already partially opened (Plate 2M and N) and starch granule density decreased (0.2±0.1 granules/cell) (Plate 2L, Table 2). Chloroplasts had increased dimensions (18.2±1.7 μm^2, Table 2) ($P<0.05$). Plastoglogulus deposition was abundant at this stage (Plate 2 O-Q, Table 2).

One week after acclimatization, overall no significant ($P<0.05$) differentiation of the mesophyll was seen (Plate 3A and B, Table 2), but palisade parenchyma was much richer in chloroplasts than spongy parenchyma (Plate 3A and B). Compared to 3 days of acclimatization, plastids decreased in size and reassumed flat-convex or biconvex shapes (Plate 3 C and D, Table 2). For plastoglobulus density, similar values were obtained (Plate 3C-E). PAS staining showed very low carbohydrate reserves (Plate 3A, Table 2) which was confirmed by the lack, or rare presence, of starch granules in chloroplasts (Plate 3C-E, Table 2). Also, no lipid or protein reserves were detected (Plate 3B).



(Plate 2, see page 187)

Plate 2 : A-E: In vitro leaf sections (immediately before transfer to ex vitro conditions, time 0): A and B) light microphotographs of transversal leaf sections, stained with PAS (A) and toluidine blue 0.1% (B), showing poorly differentiated mesophyll (bar= 50 µm). C-E) TEM microphotographs showing vacuolated mesophyll cells with chloroplasts with few thylakoidal membranes, starch granules and plastoglobules (arrow) (bar=1 µm). **F-K**: Leaf sections, three hours after transfer to ex vitro conditions: F-H) Light microphotograph of transversal leaf sections, stained with: F) PAS (arrows: carbohydrate stain). G-I) Poorly differentiated mesophyll, closed stomata (F, G and H) and epicuticular wax at the epidermal cells (H) (bar=50 µm; J, K) TEM microphotographs showing vacuolated mesophyll cells with chloroplasts with slight shape changes, few thylakoidal membranes, starch granules and plastoglobules (arrow), (bar=1 µm). **L-Q**: Leaf sections, three days after transfer to ex vitro conditions: L-N) Light microphotograph of transversal leaf sections, stained with: L) PAS (no carbohydrates stained); M,N) With toluidine blue showing poorly differentiated mesophyll, partially opened stomata (M, N) (bar=50 µm); O-Q) TEM microphotographs showing vacuolated mesophyll cells with chloroplasts with high irregular shapes and internal thylakoidal arrangements, and with plastoglobules (arrows) (bar=1 µm). Legends: **ade**: adaxial epidermis; **abe**: abaxial epidermis, **pp**: palisade parenchyma, **sp**: spongy parenchyma, **st**: stomata, **d**: ductus, **vs**: vascular strand, **cw**: cell wall, **c**: chloroplast, **er**: endoplasmatic reticulum, **m**: mitochondria, **s**: starch granule, **n**: nucleus, **nu**: nucleolus, **pl**: plasmodesmium, **v**: vacuole.

By the second week of acclimatization, leaf thickness increased ($P<0.05$, 22.2 ± 0.6 µm, Table 2), as well as cuticle thickness (Plate 3F). Palisade parenchyma was already well-differentiated (Plate 3F) and carbohydrate reserves were present mostly in the chloroplast rich palisade parenchyma (1.0 ± 0.2 starch granules per cell, Table 2, Plate 3G).

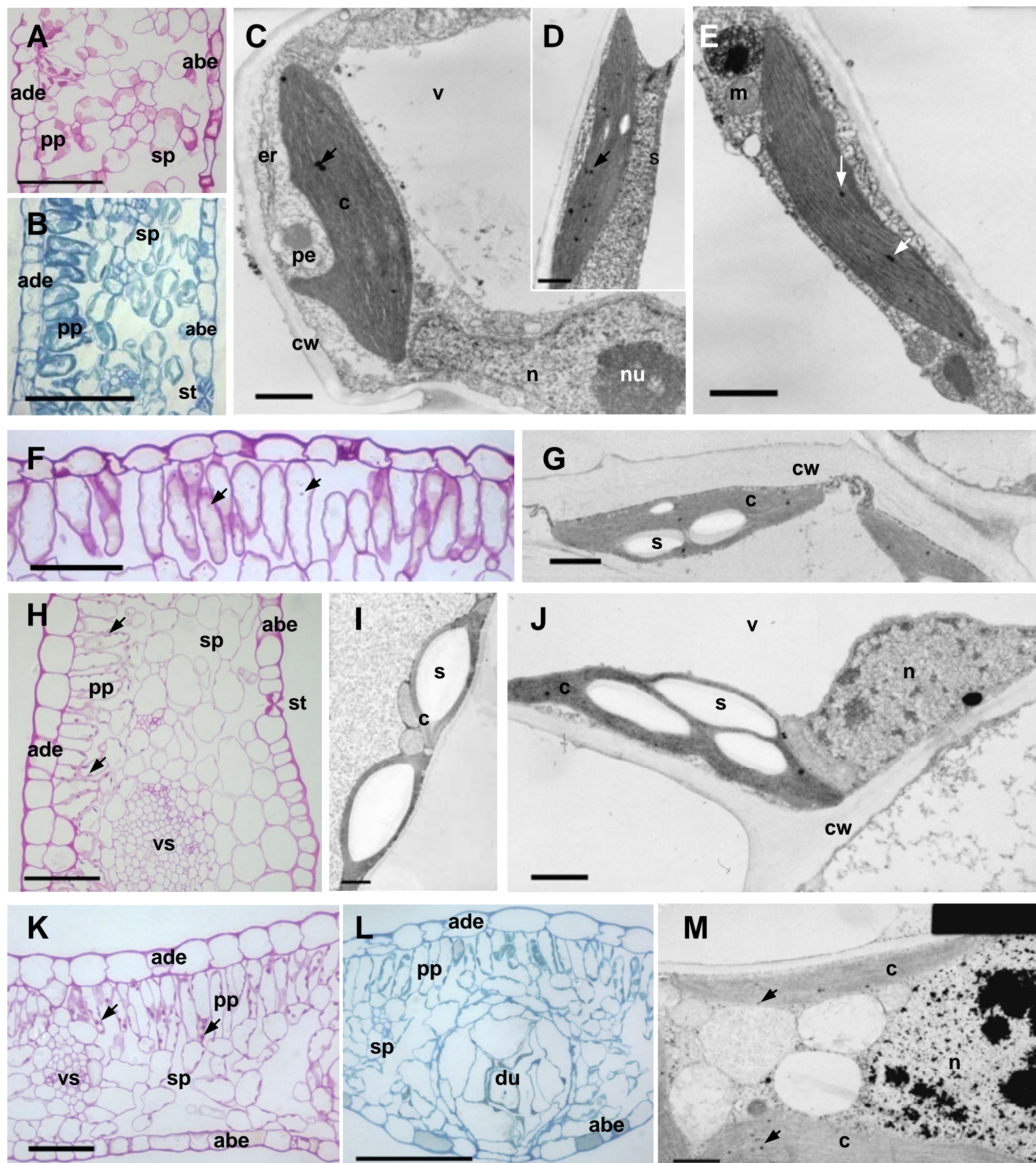
One month after acclimatization, leaf thickness was similar to that found after two weeks, but the main vascular strand was in general more developed (Plate 3H). Carbohydrate continued to accumulate in palisade parenchyma (2.4 ± 0.2 granules/cell, $P<0.05$, Table 2). Chloroplasts had one or more large starch granules (Plate 3I and J) while plastoglobulus density decreased (Plate 3I and J, Table 2).

Finally, after three months of acclimatization, leaves showed well differentiated palisade and spongy parenchyma with typical characteristics (e.g. predominance of chloroplasts in palisade cells) (Plate 3K and L). By this period, the density of starch granules was the highest so far (3.0 ± 0.2 granules/cell, Table 2, Plate 3K). Plastoglobulus density in chloroplasts increased but had low dimensions (Plate 3M).

Table 2: Dimensions (µm) of tissues and chloroplats as well as number of starch granules and plastoglobulus per cell in leaves of *E. globulus* emblings during acclimatization (average ± standard error). For the same parameter the same letters are not significantly different according to the multiple comparison Dunn´s test at P ≤ 0.05 (Within columns)

Time	Leaf thick. (µm)	Palisade Parenchyma thick. (µm)	Spongy Parenchyma thick. (µm)	Intercellular spaces ($µm^2$)	Chloroplast ($µm^2$)	Plastoglobulus / Chloroplast	Starch/ cell
0	10.7± 0.6a	2.5± 0.2a	6.4± 0.3a	2.7± 0.4a	6.5± 0.7b	2.3±0.9b	0.3±0.1a
3 h	11.6± 0.5b	2.7± 0.1a	6.4± 0.3a	2.7± 0.5a	6.6± 1.7b	2.7±1.3a	1.3±0.2b
3 d	11.6± 0.5b	2.1± 0.1a	7.8± 0.5a	9.8± 1.5b	18.2± 1.7c	8.3±1.4a	0.2±0.1a
1 w	11.6±0.1ab	2.5± 0.1a	6.6± 0.1a	6.7± 1.3ab	7.3± 1.1b	7.0±1.1a	0.1±0.05a
2 w	22.2± 0.6c	5.1± 0.2b	15.8±0.4c	13.7± 2.8bc	5.0± 0.1a	3.0±1.0a	1.0±0.2ab
4 w	20.3± 1.0c	4.8± 0.3b	11.2±0.5bc	14.4± 1.8c	6.8± 1.1b	1.3±0.4b	2.4± 0.2c
12 w	16.5± 0.3bc	4.0± 0.1b	8.2± 0.3ab	22.9± 3.c	5.6± 0.6b	4.5±0.6a	3.0± 0.2c

Plate 3 A-E) Leaf sections, one week after plant transfer to ex vitro conditions: A,B) Light microphotographs of transversal leaf sections, stained with: A) PAS and B) toluidine blue, showing poorly differentiated mesophyll (bar=50 µm); C-E) TEM microphotographs showing vacuolated mesophyll cells with regular shaped chloroplasts, with thylakoidal arrangements and with plastoglobulus (arrows) (bar=1 µm). **F-G)** Leaf sections, two weeks after plant transfer to ex vitro conditions: F) Light microphotograph of a transversal leaf section stained with PAS (arrows: carbohydrate staining) showing differentiated mesophyll (bar=50 µm). G) TEM microphotograph showing vacuolated mesophyll cells with regular shaped chloroplasts with thylakoidal arrangements starch granules (bar=1 µm). **H-J**) Leaf sections, one month after plant transfer to ex vitro conditions: H) Light microphotograph of a transversal leaf section stained with PAS (arrows: carbohydrate staining) showing well differentiated mesophyll and the main vascular strain (bar=5 µm). I and J) TEM microphotographs showing vacuolated mesophyll cells with regularly shaped chloroplasts rich in starch granules and plastoglobulus (bar=1 µm). **K-M**) Leaf sections, three months after plant transfer to ex vitro conditions: K) Light microphotograph of a transversal leaf section stained with PAS (arrows: carbohydrate staining) and L) Toluidine blue, showing well differentiated mesophyll and vascular strain (bar=50 µm); M) TEM microphotographs showing mesophyll cells with regularly shaped chloroplasts (bar=1 µm). Legends: **ade**: adaxial epidermis; **abe**: abaxial epidermis, **pp**: palisade parenchyma, **sp**: spongy parenchyma, **st**: stomata, **d**: ductus, **vs**: vascular strand, **cw**: cell wall, **c**: chloroplast, **er**: endoplasmatic reticulum, **m**: mitochondria, **s**: starch granule, **n**: nucleus, **nu**: nucleolus, **pl**: plasmodesmium, **v**: vacuole.



(Plate 3, see page 188)

Discussion

This paper shows that an efficient protocol of repetitive somatic embryogenesis was developed for *Eucalyptus globulus*, and follows the changes occurring in emblings' leaves during acclimatization.

To optimize the acclimatization process and increase the emblings rate survival (lower than 50% after one week), a better knowledge of the structural, histological and functional changes occurring in these emblings during acclimatization, is needed. Plant transfer from in vitro to ex vitro conditions leads to substantial changes in leaf morphology

and anatomy, such as in epidermal characteristics, leaf thickness, differentiation of leaf mesophyll, chloroplast number and structure (Pospilova *et al.* 1999, Hazarika 2003, 2006).

A noticeable feature of *E. globulus* in vitro emblings' leaves was the presence of a very thin cuticle, that increased during acclimatization process. Acclimatization studies with other species also showed that, in general, cuticle deposition increases as the acclimatization process advances (Pospilova 1999). The primary function of the cuticle is to limit water loss due to transpiration. Poor deposition of cuticle on in vitro leaves, allowing high rates of non-stomata transpiration, has been regarded as one of the most critical factors responsible for excessive water loss during acclimatization, which may decrease plant survival (Hazarika 2006). The production of epicuticular wax, observed in *E. globulus* embling' leaves during acclimatization, is heterogeneous in *Eucalyptus* species. *Eucalyptus saligna* produced epicuticular wax under field conditions, but this deposit seemed to decrease in in vitro cultures (Jones *et al.* 1993). Louro *et al.* (1999) also reported a weak epicuticular wax covering the leaf blade in in vitro *E. grandis* × *E. urophylla* hybrid shoots.

The stomata of *E. globulus* emblings showed an amphistomatous distribution in vitro and during acclimatization, supporting previous data for this species under field conditions (Pereira *et al.* 1987). Louro *et al.* (1999) described a similar distribution in in vitro plants of *E. grandis* × *E. urophylla* hybrid, which was then replaced by a hypostomatous organization after acclimatization. Curiously, the amphistomatous distribution in *E. globulus* was reported to be a characteristic of adult type leaves, while in juvenile leaves of up 1-3 years-old field plants, a hypostomatous distribution was found (James *et al.* 1999). One week after transfer to ex vitro conditions, stomata shapes changed from circular (characteristic of the in vitro cultured plants) to elliptical. Stomata behaviour also changed along the process of plantlet hardening – while in vitro leaves showed full opened stomata, during the first hours of acclimatization, a full closure was observed. This fact suggests that these emblings possess a short-term mechanism of stomata regulation that may rapidly evolve from one status to another, controlling aperture and water losses in recently acclimatized plants. The epicuticular wax deposition over the stomata, forming an outer chamber, may also have an important role in stomata ability to rapidly control water loss during acclimatization, as it was already suggested by Louro *et al*. (2003) for *Eucalyptus grandis* × *E urophylla* micropropagated plants.

The mesophyll of *E. globulus* in vitro leaves was poorly differentiated, as it has been already reported for other micropropagated plants like *Liquidambar stryraciflua* (Wetzstein and Sommer 1982) and *Eucalyptus* hybrids (Louro *et al*. 1999). In general, it was already shown that leaves of in vitro grown plants lacked a differentiated palisade parenchyma and presented a spongy parenchyma interspersed with large air spaces when compared to greenhouse-grown/acclimatized plants (Pospilova *et al.* 1999, Hazarika 2003, 2006). *E. globulus* in vitro leaves showed dorsiventral characteristics identical to those of ex vitro young leaves (James *et al.* 1999). Similarly, in *Eucalyptus* hybrids transferred from in vitro to ex vitro conditions, it was observed that together with an increase in the size and number of mesophyll cells, a higher distinction between palisade and spongy parenchyma could be made, although the same leaves in field conditions became isobilateral (Louro *et al.* 2003).

Carbohydrate reserves (given by the number of starch granules per cell) decreased slightly after three days to one week of acclimatization. This response is supposedly due to the adaptation period of leaves to new environmental conditions. In fact, during the first days of acclimatization stomata were kept continuously closed, compromising gas exchange efficiency, and also limiting transpiration. This response suggests that during the first days, plants are still most probably mixotrophic, with the transition for a fully autotrophic regime starting thereafter. This hypothesis is supported by the increase of starch contents after longer periods of acclimatization (15 days and, mainly, one and three months), which is in agreement with previous results for other species (e.g.Van Huylenbroeck and Debergh 1996). Only after this period, plants will probably become fully autotrophic. The development of photoautotrophy in micropropagated plants represents one of the most important turnovers during the transition from in vitro to greenhouse conditions (Piqueras *et al*. 1998). It has also been demonstrated that an increase of exogenous carbohydrate reserves (sucrose) leads to higher starch and sucrose contents in micropropagated plants, which improves the success of acclimatization and speeds up physiological adaptations (e.g. Piqueras *et al*. 1998). Such an increase may also advantageously decrease the medium osmotic potential to which the in vitro plants must adapt before acclimatization.

During *E. globulus* emblings acclimatization, most marked changes occurred in the chloroplasts. Immediately before being transferred to ex vitro conditions, in vitro SE-

derived plants showed chloroplasts with few starch granules and poorly organised internal membranes. Few hours/days after transfer to ex vitro conditions, these plastids acquired unexpected forms with the loosely organised internal thylakoidal membranes bending in several shapes.

Also starch content and plastoglobulus density suffered visible changes during the acclimatization period. As lipid and protein reservoirs, plastoglobulus are very dynamic particles being accumulated in young leaves (Kessler *et al.* 1999) or during thylakoid disassembly in senescing chloroplasts (Ytterberg *et al.* 2006). It was also showed that these structures may play a role in the removal of protein catabolites as part of thylakoid turnover (Smith *et al.* 2000). In the case of *Eucalyptus globulus* emblings acclimatization, the apparent transient plastoglobulus accumulation in plastid transition (decreasing in the later stage of acclimatization), though still not fully understood, may represent a lipid/protein reservoir that allows the rapid formation of thylakoids and supports the structural plastid polymorphism during the critical first stages of acclimatization.

Our results represent the first step towards a better histocytological understanding of SE-derived plants, prior and during ex vitro acclimatization in the *Eucalyptus* genus.

There are also some plant quality problems in somatic embryo derived plants namely the fragility of the root system that hampers plant isolation from clusters. Studies carried out on other species demonstrated that plantlets propagated by organogenesis frequently had deficient vascular connections between the root system and the stem and that roots formed in vitro were often non-functional (Grout and Aston 1977, Debergh and Maene 1981, Zimmerman 1981). Some difficulties in root elongation under in vitro conditions were already observed in primary SE process of *E. globulus*, where few plantlets could be obtained (Pinto *et al.* 2002a), constraining the application of primary SE of this species at least at an industrial level. This constraint in rooting formation and/or elongation is in accordance with the bad reputation in this particular aspect of this species within the *Eucalyptus* genus (Eldridge *et al.* 1993). The adopted strategy of transferring emblings to root elongation medium before acclimatization showed to be efficient and appropriate. A similar strategy has already been used with success in the SE process of *Castanea sativa* (Corredoira *et al.* 2003).

Before starting the acclimatization protocol, and considering that the SE-derived plantlets were obtained from clusters maintained in vitro for three years, all plantlets were screened

for morphological and ploidy abnormalities. This screening revealed that not only they had no morphological variations, but also that they were highly homogeneous with respect to ploidy/nuclear DNA content, supporting that the repetitive SE methodology is, considering the parameters measured, a “true to type” propagation method. These results are in accordance to what was previously reported by Pinto *et al*. (2004a), where ploidy stability during the first steps of the secondary SE process was observed. In addition, the 2C nuclear DNA content for these SE-derived plants was similar to the values reported for somatic embryos and leaves of field plants by Pinto *et al.* (2004a). The ploidy stability demonstrated here, together with previous data, supports that this SE-protocol allows long term in vitro cultures (at least up to three years) without inducing gross genetic changes, as polyploidy and aneuploidy, a problem frequently referred to occur in vitro in many woody plant species (*e.g*. DeVerno 1995).

Conclusions

In conclusion, this is the first report concerning emblings acclimatization to ex vitro conditions in *Eucalyptus*. Besides, these emblings proved to have no significant changes with respect to morphology and ploidy level analyses. Therefore, data presented is highly valuable for the improvement of a general strategy, already developed and summarised in Figure 1, for the application of SE in *Eucalyptus globulus* breeding programs. Finally, it was clearly demonstrated that, during acclimatization, emblings suffered profound changes in leaf morphology in order to successfully adapt to ex vitro conditions.

References

Afreen F, Zobayed SMA, Kozai T (2002) Photoautotrophic culture of *Coffea arabusta* somatic embryos: photosynthetic ability and growth of different stages embryos. Ann Bot 90:11- 19

Barry-Etienne D, Bertrand B, Vasquez N, Etienne H (2002) Comparison of Somatic Embryogenesis-derived Coffee (*Coffea arabica* L.) Plantlets Regenerated in vitro or ex vitro: Morphological, Mineral and Water Characteristics. Ann Bot 90:77-85.

Debergh PC, Maene LJ (1981). A scheme for commercial propagation of ornamental plants by tissue culture. Sci Hortic 14:335-345

DeVerno, LL (1995) An evaluation of somaclonal variation during somatic embryogenesis. In S. Jain, P. Gupta, and R. Newton (eds), Somatic embryogenesis in woody plants, vol. 1,. Kluwer Academic Publishers, Dordrecht pp 361–377

Endemann M, Hristoforoglu K, Stauber T, Wilhelm E (2002) Assessment of age-related polyploidy in *Quercus robur* L. somatic embryos and regenerated plants using DNA flow cytometry. Biol Plant 44:339-345

Fourré J-L, Berger P, Niquet L, Andre P (1997) Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. Theor Appl Genet 94:159–169

Gilly C, Rohr R, Chamel A (1997) Ultrastructure and radiolabelling of leaf cuticles from ivy (*Hedera helix* L.) plants in vitro and during ex vitro acclimatization. Ann Bot 80:139–145

Grout B, Aston MJ (1977) Transplanting of cauliflower plants regenerated from meristem culture. I. Water loss and water transfer related to changes in leaf wax and to xylem regeneration. Horticultural Research 17:107- 112

Hazarika BN (2003) Acclimatization of tissue-culture plants. Curr Sci 85 (12): 1704- 1712

Hazarika BN (2006) Morpho- physsiologycal disorders in in vitro culture of plants. Sci Hortic 108:105- 120

Hogberg K-A, Bozhkov PV, Gronroos R, Von Arnold S (2001) Critical factors affecting Ex vitro performance of somatic embryos plants of *Picea abies*. Scand J For Res 16:295-304

James SA, Smith WK, Vogelmann TC (1999) Ontogenetic differences in mesophyll structure and chlorophyll distribution *in Eucalyptus globulus* ssp. *globulus* (Myrtaceae) Amer J Bot 86 (2): 198-207

Jones NB, Drennan PM, Van Staden J (1993) Leaf anatomy, chloroplast organization and photosynthetic rate of hyperhydric *Eucalyptus saligna* Sm. material. S Afric J Bot 59:551- 555

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology 43: 179-188

Kessler F, Schnell D, Blobel G (1999) Identification of proteins associated with plastoglobules isolated from pea (*Pisum sativum* L.) chloroplasts. Planta 208:107–113

Larkin PJ, Scowcroft WR (1981) Somaclonal variation- a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Loureiro J, Pinto G, Lopes T, Santos C (2005). Assessment of ploidy stability of the somatic embryogenesis process in *Quercus suber* L. plants using flow cytometry. Planta 221:815-822

Louro RP (1994) Análise ultraestrutural e imunocitoquimica do híbrido *Eucalyptus grandis* x *Eucalyptus urophylla* cultivado in vitro e ex vitro. PhD thesis. Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

Louro RP, Dos Santos AV, Machado RD (1999) Ultrastructure of *Eucalyptus grandis* X Eucalyptus *urophylla*. I. Shoots cultivated in vitro in multiplication and elongation-rooting medium. Int J Plant Sci 160 (2):217- 227

Louro RP, Santiagi LJM, Dos Santos AV, Machado RD (2003) Ultrastructure of *Eucalyptus grandis* x *Eucalyptus urophylla* plants cultivated ex vitro in greenhouse and field conditions. Trees 17:11- 22

Muralidharan EM, Gupta PK, Mascarenhas AF (1989) Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus citriodora.* Plant Cell Rep 8:41–43

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

Pereira JS, Tenhunen JD, Lange OL (1987) Stomatal control of photosynthesis of *Eucalyptus globulus* Labill. trees under field conditions in Portugal. J Exp Bot 38:1678–1688

Pinto G, Loureiro J, Lopes T, Santos CV (2004a) Analysis of genetic stability of *Eucalyptus globulus* Labill. somatic embryos by flow cytometry. Theor Appl Genet 109: 580- 587

Pinto G, Loureiro J, Silva S, Araújo C, Santos C, Neves L (2004c). Somatic embryogenesis in zygotic embryos of *Eucalyptus globulus*: "Where are we standing?". In Book of abstracts and proceedings of COST 814 International Meeting pp 13-14.

Pinto G, Santos C, Neves L, C Araújo (2002a) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. Plant Cell Rep 21:208-213

Pinto G, Silva S, Santos C, Neves L, Araujo C (2004b). Somatic embryogenesis of *Eucalyptus globulus* Labill. and assessment of genetic stability. International IUFRO Conference of the WP2.08.03 on silviculture and improvement of *Eucalyptus*, Aveiro, Portugal pp 543-544

Pinto G, Valentim H, Costa A, Santos C (2002b). Somatic embryogenesis in leaf callus from mature *Quercus suber* L. tree. In Vitro Cell Dev Biol Plant 38:569- 572

Piqueras A, Van Huylenbroeck JM, Han BH, Debergh (1998) Carbohydrate partitioning and metabolism during acclimatization of micropropagated Calathea. Plant Growth Regul 26:25- 31

Pospisilova J, Ticha I, Kadlecek P, Haisel D, Plzakova S (1999) Acclimatization of micropropagated plants to ex vitro conditions. Biol Plant 42 (4):481- 497

Santos C, Loureiro J, Lopes T, Pinto G (2007) Genetic fidelity analyses of in vitro propagated cork oak (*Quercus suber* L). In Protocols for micropropagation of woody trees and fruits (eds. S. Mohan Jain and H. Häggman) Springer, Dordrecht, The Netherlands (in press)

Tremblay L, Levasseur C, Tremblay FM (1999) Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) rerived from somatic embryogenesis and identification of same factors involved in genetic istability. Am J Bot 86 (10):1373–1381

Van Huylenbroeck JM, Debergh PC (1996) Impact of sugar concentration in vitro on photosynthesis and carbon metabolism during ex vitro acclimatization of *Spatiphyllum* plantlets. Physiol Plant 96:298- 304

Wetzstein HY, Sommer HE (1982) Leaf anatomy of tissue cultured *Liquidambar styraciflua* (Hamamelidaceae) during acclimatization. Am J Bot 69:1579–1586

Ytterberg A, Peltier J, van Wijk K (2006) Protein Profiling of Plastoglobules in Chloroplasts and Chromoplasts. A Surprising Site for Differential Accumulation of Metabolic Enzymes. Plant Physiol 140:984–997

Zimmerman RH (1981) Micropropagation of fruit plants. Acta Horticulturae 120: 217-222

Zobayed SMA, Afreen- Zobayed F, Kubota C, Kozai T (2000) Mass propagation of *Eucalyptus camaldulensis* in a scaled-up vessel under in vitro photoautotrophic condition. Ann Bot 85:587- 592

Chapter IV

Concluding Remarks

Concluding Remarks

This PhD Thesis reports relevant advances in somatic embryogenesis of *E. globulus* from somatic embryo induction to plant acclimatization. Emphasis was given to identify, and eventually overcome the current bottlenecks and devise a successful strategy for the establishment of a somatic embryogenesis system in this economically important forest species.

Primary Somatic Embryogenesis

SE Induction

Concerning primary somatic embryogenesis, a reproducible protocol was developed using mature zygotic embryos as primary explants. Conversion of somatic embryos to plants was successfully obtained. MS basal medium was the best medium tested for inducing SE in this species. Results also showed that the addition of antioxidants in the protocol currently in use for this species had a negative effect on SE and therefore their addition is not recommended. Finally, the mineral composition of zygotic embryos was compared with the salt composition of several media previously used for somatic embryo induction and it was found that zygotic embryos have different mineral proportions when compared to all basal media used, including the MS, but this last medium has, however, the mineral proportion closest to zygotic embryos.

Genetic control of SE induction

The importance of the year of seed production and of genotype were clearly demonstrated in this thesis for *Eucalyptus globulus*. An incomplete diallel cross design gave clear evidence that somatic embryogenesis induction is under additive genetic control and that the character is amenable to breeding, opening the possibility to introgress the character in genotypes that have been selected by other important economic characters. These aspects have particular importance for implementation of SE in *Eucalyptus* breeding programs.

Histological and ultrastructural characterization of SE induction

Histological and ultrastructural characterization of *E. globulus* somatic embryogenesis also contributed to fundamental knowledge concerning the SE process in this species in particular and in *Eucalyptus* genus in general. Histological and ultrastructural analyses showed that cotyledons from both zygotic and primary somatic embryos differ in lipid, protein and carbohydrate reserve patterns, and the influence of these differences may be involved in germination/convertion differences, requiring further studies.

Secondary Somatic Embryogenesis

Maintenance of embryogenic competence

Concerning secondary somatic embryogenesis in *E. globulus*, significant advances have been achieved and optimal culture conditions were defined to induce and maintain repetitive cycles of secondary SE in *E. globulus*. The efficient protocol defined for repetitive SE produced a larger number of somatic embryos than primary SE, and therefore, increased the plant's potential for regeneration. Another significant result from this investigation is the possibility of maintaining embryogenic competence in a plant growth regulator -free medium. Besides, improvements were achieved by adding cytokinins during embling regeneration. By maintaining secondary SE, researchers can now access a permanent source of material over a long period. These emblings apparently showed no morphological differences, nor ploidy changes.

Emblings acclimatization

Forestry use of SE requires that embling acclimatization procedures are optimized. Acclimatization may however be better understood and controlled if fundamental research on leaf morphology and histocytology are performed during this period. This work demonstrated that *Eucalyptus globulus* emblings transferred from in vitro to ex vitro conditions suffered substantial changes in leaf morphology and anatomy in epidermal characteristics, leaf thickness, and differentiation of leaf mesophyll, chloroplast number and structure. In fact, control of embling acclimatization is a critical step before the use of somatic embryogenesis deployment strategies can be used in *Eucalyptus* clonal forestry.

Challenges for the future

Despite of the large amount of research conducted during the last years, knowledge is still vague in regards to the mechanisms involved in the regulation of SE and additional research is needed to optimize different steps of SE and to perform an efficient plant delivery system that could be used in an industrial way.

Also, with respect to the SE protocols developed in this Thesis, further studies could lead to optimization of the SE process leading to large number of low cost emblings, with effective impact for industry.

Some of the aspects that may require further research in the near future are, most of them, focused on the need to overcome the still low-frequency of embryo induction and poor conversion. For example,

-We demonstrated that the frequency of somatic embryogenesis response can be successfully enhanced through breeding, opening perspectives for the integration of SE in breeding programs.

- Secondary somatic embryogenesis can easily overcome the problems found in genotypes with low somatic embryogenic induction rates. Nevertheless, more attention should be given to repetitive embryogenic systems ensuring the reproductivity of the protocol.

- On the other hand, the final evaluation of the SE process will only be achieved when emblings´ physiology and productivity are evaluated in the field.

Besides, the strategies developed in this Thesis may be used in further research covering several fields from *e.g.* agronomy to functional genomics to study the genes involved in the different SE stages.