Chapter 4

Somatic Embryogenesis in *Eucalyptus* — An Update to 2009

Gloria Pinto[1*], Yill-Sung Park[2], Joao Loureiro[3], Lucinda Neves[4], Clara Araujo[4], Sonia Silva[1] and Conceicao Santos[1]

[1]CESAM & Department of Biology, Laboratory of Biotechnology and Cytomics, University of Aveiro, 3810-193 Aveiro, Portugal
[2]Natural Resources Canada, Canadian Forestry Service - Canadian Wood Fibre Centre, P.O. Box 4000, Fredericton, NB, E3B 5P7 Canada
[3]Centre for Functional Ecology & Department of Botany of the Faculty of Sciences and Technology of the University of Coimbra, Calçada Martim de Freitas, 3001-455, Coimbra, Portugal
[4]Silvicaima SA Constância Sul, 2250-058 Constância, Portugal

SUMMARY

Eucalyptus species are among the most widely planted trees in the world. 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.

Although high rates of plant propagation from axillary shoots proliferation can be easily achieved in many *Eucalyptus* species, there are successful cases in other tree species that indicate much higher multiplication rates through somatic embryogenesis (SE). The clonal propagation of *Eucalyptus* through SE 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.

In this book chapter an update of the most important aspects of the SE process will be presented with particular emphasis on the state of the art of SE in *Eucalyptus* genus.

Studies considering the genetic control, as well as the influence of different factors in the SE process, from the induction to plant acclimatization, will be updated at two different levels: primary and secondary SE. The genetic stability of the process by assessing DNA ploidy levels using flow cytometry will also be discussed.

Keywords: Clonal propagation, *Eucalyptus*, micropropagation, review, somatic embryogenesis.

Corresponding author: gpinto@ua.pt

1. 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 and they occur over a wide range of environmental conditions (Williams and Woinarski 1997).

The genus *Eucalyptus* is a member of the Myrtaceae family composed by more than 700 species (Brooker 2000). It is divided in eight subgenera, with the subgenus *Symphyomyrthus* containing the majority of the species in the genus (Poke *et al*., 2005). Eldridge *et al*. (1993) ranked the ten most important *Eucalyptus* species around the world as follows: *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 species are by far the most important, with *E. globulus* being the first species to be used outside Australia as an ornamental and plantation tree. Potts *et al*, (2004) report that for pulp production and increasingly for solid wood *E. grandis, E. urophylla* and their hybrids are the most favoured in tropical and subtropical regions with *E. globulus* favoured in temperate regions.

Eucalypts are renowned for their fast growth rate, straight form and growth ability in a wide variety of climates and soils, and for their high value in both solid wood and pulp production. In the pulp industry, *Eucalyptus* pulps are preferred due to their low production cost and excellent bulk, softness, flexibility, opacity and porosity, which make them particularly suitable for printing and writing grades of paper. In comparison with other *Eucalyptus* species, *E. globulus* has superior fiber morphology and due to its lower lignin content, it needs fewer chemicals to obtain the bleached pulp (Eldridge *et al.*, 1993, Doughty 2000).

For all these reasons and due to the ability that these species have in coping the worldwide demand for wood, pulp and fuel, the continuous expansion of the fast-growing eucalypts seems inevitable (MacRae and Van Staden 2000).

2. THE EUCALYPTUS GENOME

Eucalypts are diploid plants with a haploid chromosome number of 11 (Eldridge *et al.*, 1993, Potts and Wiltshire 1997). So far there are no known polyploids in the genus. Grattapaglia and Bradshaw (1994) estimated, by flow cytometry, the genome size of several eucalypt species and their hybrids. Using chicken erythrocytes as standard, the monoploid genome size of these species ranged from 370 to 710 million base pairs (Mbp). Species from the subgenus *Symphyomyrtus* (that include the most planted species over the world) had on average a monoploid genome size of 650 Mbp with *E. globulus* and *E. dunnii* being at the lower end of the scale (530 Mbp), while *E. saligna* presented the highest genome size value (710 Mbp). Also, *Corymbia* species were reported to have a monoploid genome size of approximately 380 Mbp, which is a much smaller value than those reported for the other eucalypts. No *Angophora* species have been studied so far (Poke *et al.*, 2005).

More recently, the genome size of *E. globulus* has been re-estimated using propidium iodide flow cytometry (Pinto *et al.*, 2004). The authors reported a nuclear DNA content of 644 Mbp which is larger than that estimated by Grattapaglia and Bradshaw (1994). This re-estimation was based for the first time on the use of a plant internal control, *Solanum lycopersicum*¸ with

the authors following several best practices to assure that the known cytosolic compounds of *Eucalyptus* leaves did not interfere with the quantitative staining of DNA.

These data suggest that eucalypts present, in general, a small genome (according with the categories of Soltis and Soltis, 1995), which together with its enormous commercial interest, may place them among the interesting candidates for complete genome sequencing. In comparison with the plant species which had, recently, their genomes sequenced – *Arabidopsis thaliana* (125 Mbp), *Oryza sativa* (420 Mbp – 466 Mbp) and *Populus trichocarpa* (473 Mbp) – the genome size of *Eucalyptus* species is only slightly larger and its sequencing is in progress (for a complete review concerning the genomics of *Eucalyptus* see Poke *et al.,* 2005)

3. CLONAL FORESTRY OF EUCALYPTUS

Before the recent progress with mass vegetative propagation, all the *Eucalyptus* plantations were originated from seeds with varying degrees of establishment and competitive success (Watt *et al*., 2003a).

Although industrial needs demand an increase in *Eucalyptus* forest productivity, in most species we are still in an early stage of domestication. For example, in *Eucalyptus globulus*, 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. Therefore, most of the breeding programs are now moving to control-pollinated assessment, which will allow more accurate estimations of the genetic parameters and the separation of additive from non-additive genetic effects (Silva *et al.,* 2004).

Vegetative propagation is a widely used technique in tree breeding programs to efficiently manage populations (Eldrige *et al.,* 1993) and to produce improved plant stock (clonal forestry) faster than the conventional seed orchard procedures (Mullin and Park 1992). A major advantage of clonal forestry is the complete use of the genetic potential of desired genotypes. Another benefit is the short- term ability to quickly capture a greater proportion of additive and non-additive genetic variation. Clonal propagation enables the retention of most of the genetic potential of elite selected plantations, including the non-additive components of genetic variance of the new generation. Besides, in sexual propagation, non-additive combinations are mostly lost due to genetic combinations (Mullin and Park 1922). However, 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 propagation of *Eucalyptus* must be accompanied by an intensive breeding program.

In *Eucalyptus*, the preferred method for vegetative propagation consists of rooted cuttings, a strategy already used with success in several clonal propagation programs (e.g. Celbi and Raiz in Portugal, for a review see Watt *et al.,* 2003a). Nevertheless, this strategy is limited by the heterogeneous response in rotting ability among clones and the decrease of rooting potential with the aging of parent plants (Eldridge *et al.,* 1993, Watt *et al.,* 2003a).

Clonal propagation through *in vitro* approaches can provide alternative vegetative multiplication methods to overcome some of the difficulties referred above.

4. MICROPROPAGATION

Micropropagation, the first approach to *in vitro* propagate desired clones of *Eucalyptus* spp., has the potential to provide very high multiplication rates of selected tree genotypes, with short-term silviculture gains. Some of the earlier reports date back to the 1960s and over the last decade some progress has been made in the development of complete plant regeneration protocols. In their review on *Eucalyptus* micropropagation, Le Roux and Van Staden (1991) reported that between 1968 and 1991 only 30 out of 204 publications in the genus included protocols for plant regeneration. Since then, 29 out of 65 new publications reported plant regeneration, which represents a substantial increase on the interest of applying such approach. So far, *Eucalyptus* aseptic cultures have been established from a wide range of explant sources (e.g. seeds, seedlings, shoots, flowers and lignotubers), either from juvenile or mature trees (for a review see Watt *et al.,* 2003a).

Micropropagation through axillary proliferation and adventitious shoot proliferation on nodal explants has been successfully applied in some *Eucalyptus* species (e.g., Cid *et al.,* 1999, Glocke *et al.,* 2006). The most common culture medium for shoot multiplication is the MS medium (Murashige and Skoog 1962) with a low auxin/cytokinin ratio (Watt *et al.,* 2003a, b). Nevertheless, other salt media compositions have been reported as appropriate, such as WPM (Lloyd and McCown 1981) used in the micropropagation of the ornamental *Eucalyptus* cv. 'Urrbrae Gem' (Glocke *et al.,* 2006) and JADS medium used in *E. grandis* × *E. urophylla* (Correia *et al*., 1995). The stimulation of shoot elongation has been achieved in many cases by the addition of gibberellic acid to the media (Cid *et al.,* 1999, Glocke *et al.,* 2006). Several media composition have been used for the *in vitro* rooting; a few days-pulse of indole-3-butyric acid (IBA) followed by subculture in IBA-free medium is one of the most common procedures. Finally, there are already several reports where regenerated plants have already been transferred to the field with apparent success (Azmi *et al.,* 1997, Glocke *et al.,* 2006). However, problems such as hyperhydricity and shoot senescence are still a reality in many *Eucalyptus in vitro* cultures (Louro *et al.,* 1999, Whitehouse *et al.,* 2002). Despite that, large-scale micropropagation of selected *Eucalyptus* trees via axillary bud proliferation is being currently employed in numerous research and commercial laboratories with considerable success (Watt *et al.,* 2003a, b).

Plant regeneration from indirect organogenesis, somatic embryogenesis (SE) and protoplasts have been also used with some success in the *in vitro* propagation of some *Eucalyptus* species (for reviews see Le Roux and van Standen 1991, Watt *et al.,* 1999, 2003a, Hajari *et al.,* 2006). However, many of the protocols developed so far still need to be optimized not only in what concerns plant regeneration but also for the application of genetic engineering strategies, such as genetic transformation (Tournier *et al.,* 2003, Poke *et al.,* 2005). In fact, the main reason why genetic transformation is not yet being used more widely at the industrial level is because it lacks well developed (reliable and low cost) *in vitro* plant regeneration protocols, as it has been already reported for other softwood trees (MacKay *et al.,* 2006).

5. APPLICATIONS OF SOMATIC EMBRYOGENESIS IN CLONAL FORESTRY

Although high rates of plant propagation from axillary shoots proliferation are easily achieved in *Eucalyptus* spp., the successful cases observed 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). This approach presents several positive aspects for its application in clonal forestry: amenability to a high-throughput production, necessary to reduce costs; embryogenic cultures may be maintained through cryopreservation, essential for the field-testing and further selection of the best clones; flexibility to rapidly deploy suitable clones, in case the breeding aims and/or environmental conditions have changed; ability to manage genetic diversity and genetic gain in the plantation. Besides, SE also allows the mass production of selected clones from relatively small quantities of seeds from controlled crosses. This advantage may be particularly useful in the propagation of outstanding parents that have problems in flowering and/or that produce small quantities of seeds. Furthermore, SE is considered a good strategy to speed up the deployment of outstanding families identified in progeny trials, while integrated in tree breeding programs – multi-varietal forestry (for a review see Högberg *et al.*, 1998, Park *et al.*, 1998, Park 2002, Park *et al.*, 2006). Despite all these advantages, clonal forestry is highly dependent of an efficient plant propagation/regeneration system that can mass produce genetically tested material.

5.1 General Features of SE

Somatic embryogenesis has been defined as a non-sexual developmental process that produces a bipolar embryo (presenting both shoot and root meristems) from somatic tissue (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 (Zimmerman 1993, Von Arnold *et al.*, 2002).

Different patterns of 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 embryogenic 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 enter cell division and go through embryogenesis. By contrast, indirect embryogenesis requires redetermination of differentiated cells, callus proliferation and the development of the embryogenically determined state. Growth regulators are also required, but in this case, not only for the re-entry of cells into mitosis but also for the determination of the embryogenic state (Thorpe 2000). It has been proposed that both processes are extremes of one continuous developmental pathway, with the distinction between direct and indirect SE being considered difficult, and with both processes seen 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. With some exceptions (e.g., Canhoto and Cruz 1996, Canhoto *et al.*, 1999), in

Myrtaceous species in general, and in *Eucalyptus* in particular, there is a notorious paucity of cytological, histological and ultrastructural information on the different aspects associated with the induction and development of somatic embryos from explant tissues. In *Eucalyptus*, most works described that somatic embryos showed morphological resemblances with zygotic ones at various developmental stages (e.g. Muralidharan *et al.,* 1989, Watt *et al.,* 1999), although in some of them a clear definition of the different developmental phases was lacking. According to Watt *et al.,* (1991), the embryogenic cells of *E. nitens* present the typical characteristics of other embryogenic systems: dense cytoplasm, small volume, prominent nucleus and small vacuole. In this study, histological analyses of somatic embryos at different development stages are presented, although no further details were given on embryo histology or origin. 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 and compared them with mature zygotic embryos and highlight the similarities between both structures. Further clues were given by Arruda *et al*. (2000) that demonstrated, at morphological and histological levels, the role of calcium in favoring the morphogenic route for SE in *E. urophylla*.

Indeed, 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, histo-differentiation, maturation and germination/conversion phases (e.g. Merkle *et al.,* 1995). The characteristic phases of somatic embryos are usually considered similar to those present in zygotic embryos, i.e. globular, heart-shaped, torpedo-shaped and cotyledonar stages (Jiménez 2005).

The success of any propagation system is visible in the quantity (survival) and quality (growth rate, genetic stability) of the final product, i.e. the regenerated plants. From a strictly academic point of view, the success of a SE protocol is materialized in the form of the emblings. However, any applied research, i.e. with commercial and industrial goals, requires the large-scale production of such structures and demands for efforts directed to the acclimatization phase, where analyses of performance under *ex vitro* conditions and of genetic fidelity assume particular importance.

5.2. Factors Influencing SE Induction

The following section will provide a general idea of the different factors that influence SE, with particular emphasis on those related with *Eucalyptus*.

The successful establishment of a SE system is dependent on the correct choice of the plant material, i.e., which explants are the most appropriate source of competent cells (considering the genotype, age and type of the explants), and on the selection of the best culture conditions, i.e., both the physical and chemical factors (media composition, light, temperature, pH, humidity, among others) that may lead to the embryogenic development pathway (Merkle *et al.,* 1995, Thorpe 2000, Phillips 2004). The interaction between these factors is the key for the successful induction and expression of a specific mode of cell differentiation and development (Gaj 2004).

5.3. Plant Material

The effect of the genotype is currently considered a crucial factor in micropropagation and, in particular, in the induction of SE in *Eucalyptus*. However, up to the moment few research has been performed that clearly investigates the magnitude of the genetic influence in this genus (Pinto *et al.,* 2008c).

In other species, the genetic influence during the SE process is well known (Merkle *et al.,* 1995), and understanding the genetic control has been considered an important aspect in the improvement of the SE process (Park *et al.,* 1998). Actually, such genotypic variability in embryogenic capacity may reflect differences in the ability to activate key elements of the embryogenic pathway (Merkle *et al.,* 1995). Thus, depending on the type and magnitude of genetic variation, an ameliorated SE initiation procedure may be applied to recalcitrant genotypes (Park *et al.,* 1998, Park 2002).

Pinto *et al.* (2008c) reported an extensive study to clarify the genetic influence in *E. globulus* SE process. The embryogenic capacities and the variability in yearly production among 13 open pollinated families were studied. Also, the degree of genetic control during SE was examined using full-sib families. Such results have the potential to be applied in the improvement of the SE process and further integration into breeding programs.

Most woody species have marked phase changes that result in a decline of their potential for micropropagation and in particular for SE (Bonga and Von Aderkas 1992). Although adult material is desirable as explant source, in most of the SE induction experiments in *Eucalyptus*, juvenile material was preferred. In fact this has been a common procedure in woody species, and in Myrtaceous species, in particular (for a review see Canhoto *et al.,* 1999). The need for juvenile material still represents the major limitation for the propagation of woody species using SE, as the quality of the adult trees to be formed is still unknown.

Somatic embryogenesis can be induced in cultures 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 plus the intact cotyledon. In *E. grandis*, Watt *et al.* (1991) used leaves from *in vitro* propagated shoots, while in *E. dunnii* 3-day-old seedlings were used (Termignoni *et al.,* 1996). Furthermore, according to Watt *et al.* (2003a), a successful protocol of SE with explants of mature trees were alredy obtained although no details are given.

Concerning *E. globulus*, Trindade (1996) tested explants in different phases of development and found that partially germinated seeds were more suitable for SE than cotyledons or leaves from micropropagated plants. Bandyopathyay *et al.* (1999) observed organized structures resembling somatic embryos obtained from mature seeds, but their evolution was not followed. Later on, Nugent *et al.* (2001) was able to use, with considerable success, cotyledons from mature seeds as source material for inducing SE. Nonetheless, in any of these studies complete somatic embryogenesis structures, i.e., having well-defined shoot and root poles, were obtained. A considerable progress was achieved, when Pinto *et al.* (2002) tested several types of explants (mature zygotic embryos, isolated cotyledons, hypocotyls, leaves and stems) to induce SE and were able to develop a successful protocol for plant regeneration from mature zygotic embryos

(Pinto *et al.*, 2002). The authors also attempted to induce SE from mature plant material (floral buds), from several families and in different stages of development, but up to this moment, only non-embryogenic callus was observed (unpublished data, 2005).

One of the initial steps of any induction protocol is the surface sterilization of the source material. Several procedures can already be found on the literature and vary according with the species of *Eucalyptus* and type of material to be disinfected. The most common disinfectants used so far include alcohol, NaOCl and $HgCl_2$ (Le Roux and Van Satden 1991), usually with the addition of a few drops of detergent (either Teepol or Tween 20). Muralidharan and Mascarenhas (1995) washed the *E. citriodora* seeds with water and a few drops of Teepol, and after three washes with distilled water, seeds were treated with 0.05% $HgCl_2$ (0.05%; w/v) for 5 min. and rinsed with sterile water. Mercuric chloride (2 min. treatment; 0.1 %) was also used in *E. tereticornis* after immersion of the seeds in 70% (v/v) of ethanol for 2 min. (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 in the disinfection of explants obtained from mature trees (Watt *et al.*, 1995). Recently, Pinto *et al.* (2008a, b and c) reported the successful use of hydrogen peroxide for disinfecting seeds of *E. globulus*.

5.4. Culture Conditions

The nutrient medium composition is undoubtedly one of the most important factors 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 for embryo growth. However in the excised zygotic embryos, this must be achieved by an artificial medium. Thus, a comparative analysis of the initial medium composition and of the nutritional composition of the developing seed can provide relevant information for optimizing the nutrient requirements for SE induction/expression (Pullman *et al.*, 2003). Such approach requires a complex complete factorial design, being seldom applied in woody species (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 and amino acids supplemented with a carbon source (Bonga and Anderkas 1992, Ramage and Williams 2002). The most extensively used medium for the induction of SE in woody angiosperms is the nitrogen-rich medium Murashige and Skoog (MS) (Dunstan *et al.*, 1995). This medium has also been extensively used in the micropropagation of several species of *Eucalyptus* (Le Roux and Van Staden 1991, Watt *et al.*, 2003a). In 2008, Pinto and co-workers analyzed the effectiveness of several media (MS, ½ MS, B5, WPM, DKW and JADS) during SE induction and expression in *E. globulus*. The results showed that MS and B5 were the best media for SE induction and for emblings regeneration (Pinto *et al.*, 2008a).

Carbohydrates are included in all tissue culture media and may be involved in multiple roles during somatic embryogenesis, as the type and concentration of sugar used is known to influence SE. In general, sugars mainly act as: (1) sources of carbon and energy; (2) osmotica; (3) stress protectants; and (4) signal molecules (Lipavska and Dova 2004). In particular, 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 by several authors (for a review see Lipavska and Dova (2004).

Sucrose has been the sugar compound most frequently employed to induce SE in different plant species. In *Eucalyptus*, the recommended concentrations of sucrose for SE induction vary in different cultures, but usually it range from 2 to 5% (Table 1). In other embryogenic systems other carbohydrates (e.g., glucose, fructose, maltose, lactose, cellobiose, mannitol, sorbitol, myo-inositol) were tested, as well (e.g. Canhoto *et al.,* 1999, Pinto *et al.,* 2002, Lipavska and Dova 2004). For example, the addition of mannitol to the induction medium inhibited the formation of callus in the surface of the explants during SE induction of *E. globulus* (Pinto *et al.,* 2002).

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

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 frequently used to reactivate the cell cycle and initiate embryo formation. In some woody species, the initiation of embryogenic cultures as well as the persistent production of secondary embryos requires the continuous exposure to auxin (or in some cases, to other PGRs); in others, a short pulse of auxin (e.g. few days) is sufficient to induce secondary embryogenesis that may 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 for the initiation of somatic embryo formation in some woody species (Dunstan *et al.,* 1995), being usually supplied in combination with an auxin in the induction medium. However, in some culture systems, PGRs, as abscisic acid (ABA), ethylene, gibberellic acid (GA_3), among others, also play regulatory roles that shouldn't be ignored (Jiménez 2005). The PGR used to induce SE in *Eucalyptus* are summarized in the Table 1 and will be described latter in this review.

It is now 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 the environment of the original tissue and placed on synthetic media in artificial conditions. In fact, the wounding itself may be a significant signal for the induction and dedifferentiation (Fehér *et al.,* 2003). Embryogenic competence of *in vitro* cultured somatic cells can be stimulated by factors, such as osmotic pressure, pH, low or high temperature, starvation, mechanical wounding of explants or high auxin level. The molecular mechanisms involved in this stimulatory/regulatory effect of the stress treatment on cell differentiation and morphogenesis remain unclear (for a review see Fehér *et al.,* 2003, Gaj 2004).

6. THE SOMATIC EMBRYOGENESIS PROCESS IN *EUCALYPTUS*

After some more general considerations about the SE process, this section intends to make an up to date review of all the relevant findings related with both the primary and the secondary SE process in *Eucalyptus*. An overview of the subsequent stages of this micropropagation process will also be presented.

Table 1: Summary of somatic embryogenesis induction in *Eucalyptus* genus.

References (By year)	Species	Explant type	BM	Culture conditions	Sucrorse	PGR	Response	Emblings
Ouyang *et al.,* 1980*	*"E. × leichow"*	*In vitro* seedlings, callus	nd	Light	nd	nd	Embryoids	**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	**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	**Yes**
Chang-Le and Kirby 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	**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

Table 1: Contd.

References (By year)	Species	Explant type	BM	Culture conditions	Sucrorse	PGR	Response	Emblings
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. BAP for SE formation	Somatic embryos	**Yes**
Pinto *et al.,* 2008a	*E. globulus*	Mature ZE	Several media	Dark/24ºC	3%	NAA	Somatic embryos	**Yes**
Pinto *et al.,* 2008c	*E. globulus*	Mature ZE (several genotypes)	MS	Dark/24ºC	3%	NAA	Somatic embryos	**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)

6.1. Primary Somatic Embryogenesis

In *Eucalyptus* literature there are several reports on the induction of SE (for a summary see Table 1). Somatic embryogenesis and plant regeneration was reported for the first time from callus of seedlings of "*E.* × Liechow" (Ouyang *et al.*, 1980, 1981, according to Le Roux and Van Staden 1991). Boulay (1987, according to Le Roux and Van Staden 1991) achieved SE from hypocotyl and internode *calli* derived from seedlings of *E. gunnii* using two different media and a variety of PGR concentrations. Also, Chang-Le and Kirby (1990, according to Le Roux and Van Staden 1991) were able to induce embryo-like structures in cultures of hypocotyls, cotyledons, and leaves of young seedlings 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. The authors used a sequential culture technique in a medium containing Murashige and Skoog salts, RV vitamins (reference not given) and amino acids. Slow growing green protuberances developed from cut surfaces of explants after 2 weeks in culture on a medium containing 1.1 mg L^{-1} 2,4-D. These developed into adventitious shoots and embryo-like structures when transferred to medium with 1.l mg L^{-1} BAP.

Somatic embryogenesis has also been reported on callus derived from shoots of 4-year-old trees of *E. grandis* on MS medium supplemented with 0.1 mg 1^{-1} NAA and 5 mg L^{-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 L^{-1} of BAP, KIN, NAA and 2.4-D each. In *E. citriodora*, somatic embryos were obtained from zygotic embryos grown on B5 medium with 3 mg L^{-1} NAA and 5% sucrose (Muralidharan and Mascarenhas 1987, Muralidharan *et al.*, 1989). Recently, Prakash and Gurumurthi (2005) reported SE and plant regeneration in *E. tereticornis* from embryogenic calli obtained from mature zygotic embryos. When calli were transferred to the respective induction medium (MS or B5 with 2,4-D or NAA) also containing BAP, somatic embryos developed after 1-2 weeks. Somatic embryos were successfully germinated and converted in MS PGR-free medium, and rooted plants were effectively acclimatized (Prakash and Gurumurthi 2005).

In the case of *Eucalyptus globulus*, a protocol for inducing SE was first presented by Trindade (1996). Later, Bandyopadhyay *et al.* (1999) and Nugent *et al.* (2001) also reported SE induction and embryo formation although with very low reproducibility. Pinto *et al.* (2002) reported the regeneration of emblings and studied the effect of the explant, the PGRs and time of exposure on the induction process of SE. These authors were able to induce SE in the presence of NAA from callus derived from cotyledon explants and from mature zygotic embryos. Oller *et al.* (2004) were capable to reach the embryogenic callus phase from leaf callus in a basal medium with IBA, but further progress is unknown. Later on, Pinto *et al.* (2008a) investigated the importance of different culture media in the induction phase. The results that they obtained revealed that MS and B5 were the best media for inducting SE in this species. These authors also studied the addition of anti-browning compounds (ascorbic acid, charcoal, dithioerythritol, dithiothreitol, polivinylpirrolidone, polyvinylpolypyrrolidone and silver nitrate) to the induction and expression media (MS) to control tissue oxidation in *E. globulus* SE process. However, the results were discouraging as with any of the tested compounds there was a decrease in SE potential on expression medium, with only DTE, charcoal and silver nitrate reducing the explants'

browning when added to the expression medium. On the other hand, when added only during the induction period, anti-browning agents reduced accumulation of phenolics but also severely reduced the potential to SE. The continuous exposure to anti-oxidants completely inhibited the SE response (Pinto *et al.,* 2008a).

Finally, the genetic control of the SE induction process was investigated, using 13 open-pollinates families that were analyzed over 3 consecutive years in a diallele mating design with five parents (Pinto *et al.,* 2008c). The results showed that the SE induction varies among families and over the years of seed pollination. Furthermore, it was proved that SE was under the control of additive genetic effects.

6.2. Secondary Somatic Embryogenesis

In contrast to primary SE induced from explant cells, repetitive, secondary or recurrent SE may also occur from somatic embryos in culture, either directly or through callus (for a review see Raemakers *et al.,* 1995, Merlke *et al.,* 1995). This phenomenon is of potential importance both for mass clonal propagation and for the gene transfer technology (Thorpe 2000). A much higher efficiency of secondary SE over primary SE is also found in many plant species (Raemakers *et al.,* 1995, Akula *et al.,* 2000, Vasic *et al.,* 2001, Nair and Gupta 2006). In many cases, the cultures are able to retain their competence for secondary embryogenesis for many years and thus constitute a very useful material for an array of different studies (e.g., *Vitis rupestris*, Martinelli *et al.,* 2001).

Similarly to what was described for the induction stage, the proliferation of embryogenic cells may take a number of forms and is influenced by a variety of factors. In general, the embryogenic callus is maintained on a medium similar to that used for induction, being the use of a liquid medium preferred for large-scale propagation (Von Arnold *et al.,* 2002). From all the known reports concerning SE in *Eucalyptus* just three reports repetitive SE (Table 2).

Boulay (1987, according to Le Roux and Van Staden 1991) reported the occurrence of secondary SE in *E. gunnii* after the subculture of embryogenic calli. In *E. citriodora*, Muralidharan *et al.,* (1989) and Muralidharan and Mascarenhas (1995) were able to develop a highly efficient protocol of secondary SE that has allowed the large-scale propagation of *E. citriodora*. According to Muralidharan *et al.,* (1989), the embryogenic potential was maintained for a period over 3 years in the dark, on B5 medium containing 5 mg L^{-1} NAA, casein hydrolysate (500 mg L^{-1}), glutamine (500 mg L^{-1}) and 30 gL^{-1} of sucrose. Later on, the authors found that the addition of inositol was crucial for the maintenance of long term embryogenic competence (up to 9 years) 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).

In *E. globulus*, the occurrence of repetitive SE was first reported in 2004 by Pinto *et al.* (2004a, b; Figure 1). The authors developed a protocol where somatic embryos were cultured in MS medium with 3 mg L^{-1} NAA, 30 g L^{-1} of sucrose and maintained at 24ºC in the dark. In order to evaluated the genetic stability and the true-to-type propagation of *Eucalyptus globulus* via repetitive SE, eight month old somatic embryos was investigated using flow cytometry and no major ploidy changes were detected between somatic embryos and mother plants (Pinto *et al.,* 2004b).

Table 2: Repetitive somatic embryogenesis in *Eucalyptus* genus.

Specie	Reference	Explant	BM	Culture conditions	PGR and other supplements	Response Variation	Somaclonal	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.,* 2004b Pinto *et al.,* 2006	Somatic embryos	MS	Dark/ 24ºC	3mgl^{-1} NAA	secondary somatic embryos	No**	8 months	**Yes**
E. globulus	Pinto *et al.,* 2008b	Somatic embryos	MS and B5	Dark and Ligth 24ºC	several conditions	secondary somatic embryos maintenance and germination	nd	24 month	nd

* According to Le Roux and Van Staden. (1991)
** Flow cytometry and microssatellites
(BM: basal medium, EC: embryogenic callus; CH: Casein hydrolisade; nd: not described)

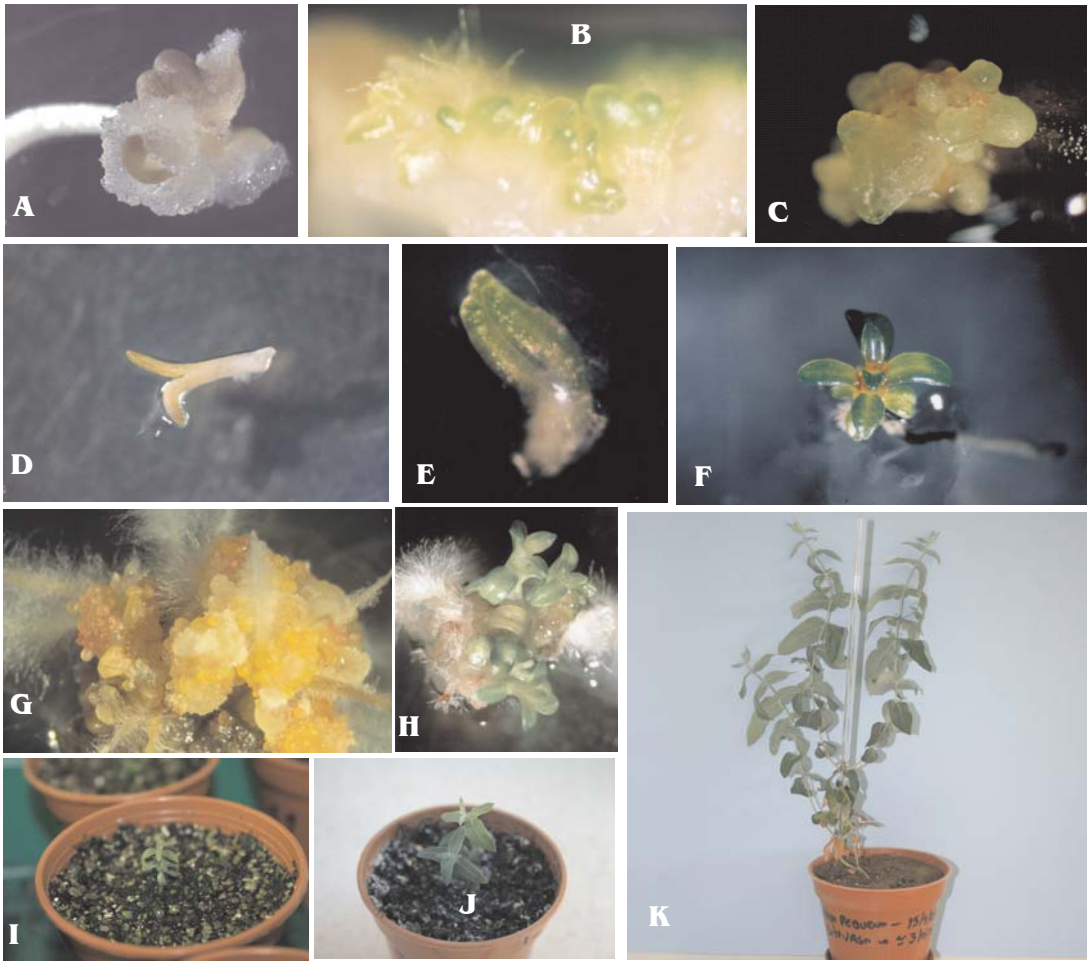

Fig. 1A-K: Somatic embryogenesis and plant regeneration in *Eucalyptus globulus*. **(A)** Aspect of the explant three weeks after induction **(B)** Embryogenic callus with primary somatic embryos produced on a cotyledon of a zygotic embryo explant. **(C)** Cluster of primary somatic embryos. **(D)** and **(E)** Examples of Cotyledon- stage primary somatic embryos. **(F)** Emblings from primary somatic embryo conversion. **(G)** Cluster of secondary somatic embryos. **(H)** Shoot elongation. **(I), (J)** and **(K)** Emblings from secondary somatic embryos in different steps of acclimatization.

6.3. Maturation, Germination and Conversion Somatic Embryos

Only the mature embryos that have accumulated enough storage materials and have acquired desiccation tolerance at the end of the maturation stage, seem able 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., by adding polyethylene glycol), desiccation and low temperature exposure may allow the entering into the latter phases of SE by stimulating maturation and inhibiting precocious germination (Merkle 1995, Watt *et al.*, 1999, Jiménez 2005).

Even when large quantities of somatic embryos are obtained, a common bottleneck for large-scale propagation is the conversion of these somatic embryos in plants. On a culture medium lacking PGRs, somatic embryos usually develop into small emblings comparable to seedlings. However, there are cases where auxin and cytokinin may be important to stimulate germination/conversion. Furthermore, a significant change in the composition of the basal medium is often required. Even, in some species, it is required to include extra compounds like glutamine and casein hydrolysate (for a review see Von Arnold *et al.*, 2002). Of course, all the factors that contribute to the success of this step may also be important for a better performance of emblings in the later acclimatization to *ex vitro* conditions.

In *Eucalyptus*, mature somatic embryos usually do not develop in the presence of auxin. Also, plant regeneration (emblings) has been usually achieved in auxin free medium or, occasionally, in media containing cytokinins and/or gibberellic acid (GA_3) (Table 3). In *E. citriodora*, mature embryos germinated easily when transferred to an auxin-free medium (Muralidharan and Mascarenhas 1995). After isolation in a liquid medium, individual mature somatic embryos were transferred to germination medium (B5 medium with 20g l^{-1} sucrose), after which 52% of the embryos germinated and developed healthy shoot and root systems. In this species the addition of ABA had a negative effect on the growth of embryogenic masses, with embryos getting moribund with increasing concentrations of this PGR (Muralidharan and Mascarenhas 1995). In *E. dunnii* and in *E. grandis*, embryo maturation and subsequent germination were also achieved, despite that at low success rates (Watt *et al.*, 1995). In *E. grandis*, the addition of ABA and PEG (alone or in combination), and 3 hours of dissection did not show any success, with the rate of emblings regeneration being low or absent according with the explant source (Watt *et al.*, 1995). It is consensual that the regeneration of viable emblings is still a problem in many species (Merlke 1995), and *Eucalyptus* is no exception.

In *E. globulus* the influence of the culture medium (MS and B5), PGRs (auxins and cytokinins), and light, on secondary SE was tested (Pinto *et al.*, 2008b, Figure 1). These authors reported that MS medium without growth regulators (MSWH) was more efficient for cotyledonary embryo formation and germination than B5 medium. Besides, reducing the levels of auxin (NAA) increased the proliferation of globular somatic embryos and allowed the maintenance of SE competence maintained on medium free of PGRs. The addition of two cytokinins (BAP and KIN) to the MS medium did not improve proliferation of globular secondary embryos, but was crucial during later stages of the SE process (germination and conversion). Data also show that depending on the SE stage, light may also play an important role, influencing the quality of the process (Pinto *et al.*, 2008b).

6.4. Acclimatization and Genetic Fidelity of the Emblings

The ultimate goal of the application of SE to micropropagate selected individuals is only achieved with the successful acclimatization of a large number of plants to field conditions. Also, as the in vitro propagated plants should be true-to-type, i.e., with the interest characteristics (e.g., high yield, uniform quality, shorter rotation period) of the donor genotypes, somaclonal variation is regarded as undesirable, at least at this stage. Therefore it is essential to verify and follow 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).

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

Species	References	Explant	BM	PGR or other supplements	Light conditions	Soma-clonal	Response Variation	Acclimati-zation	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. tereticornis	Prakash and Gurumurthi 2005	Primary somatic embryos	MS	PGR free	16h L	nd	Emblings	**Yes**	-
E. globulus	Pinto *et al,.* 2008b	Secondary somatic embryo	MS and B5	PGR free, BAP, Kinetin	Dark and L	nd	germination	no	-

* According to Watt *et al.,* (1995)
(nd: not defined; L: light; EC: embryogenic callus; BM: basal medium)

In general, the *in vitro* culture procedures as well as the environment conditions, genotype, and age of explants in culture are often associated with the occurrence of somaclonal variation (Rani and Raina 2000). Morphological markers, chromosome analysis, breeding behavior, isoenzymes or DNA markers may be used to detect somaclonal variation. The early assessment of genetic fidelity at various culture stages may help to identify which culture condition(s) is inducing the observed variation (Rani and Raina 2000). Flow cytometry (FCM), a high throughput and reliable methods, has been increasingly chosen for analysing of major ploidy changes during genetic variation assays. However, up to this moment, very few reports have used this technique to assay somaclonal variation in woody plants (Santos *et al.,* 2007).

Also the preservation of somatic embryos, for example through encapsulation procedures, although important, has only been reported for *E. citriodora* (Muralidharan and Mascarenhas 1995). The application of this preservation method together with cryopreservation is still in its infancy in this genus, but its success strongly depends on the development of reliable SE protocols.

The propagation of plants through in vitro culture 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 documented, 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 included a gradual reduction of the environmental relative humidity and the transfer to soil substrates (peat, perlite or sand). However, in those works, emblings' performance was only measured as survival rates. Up to this moment, no studies are known that have explored important aspects, as histocytology, physiology, or genetics, during the emblings acclimatization in *Eucalyptus* genus.

In conclusion, this chapter reviews the most relevant recent advances on the SE process in *Eucalyptus,* from the somatic embryo induction to the plant acclimatization. Nevertheless, despite of the large amount of research conducted during the last years, there is still a gap on the knowledge of the mechanisms involved in the regulation of SE. Besides, additional research is also needed to identify, and eventually overcome some of the current bottlenecks and, so, devise a successful strategy in this economically important forest species to efficiently establish a SE system at the industrial level.

ACKNOWLEDGEMENTS

Authors thank Celbi for providing the material used in this study and for supporting part of the project. Thanks are also due to Armando Costa for technical assistance. The Portuguese Foundation for Science and Technology (FCT) supported the fellowship of Sónia Silva (SFRH/BD/32257/2006).

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, S.C.C., Souza, G.M., Almeida, M. and Gonçalves, A.N. (2000). Anatomical and biochemical characterization of calcium effect on *Eucalyptus urophylla* callus morphogenesis in vitro. *Plant Cell Tiss. Organ. Cult.* **63:** 143-54.

Azmi, A., Noin, M., Landré, P., Proteau, M., Boudet, A.M. and 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. and Hamill, J.D. (1999). Efficient plant regeneration from seedlings explants of two commercially important temperate eucalyptus species- *Eucalyptus nitens* and *E. globulus*. *Plant Science*. **140:** 189-198.

Bogunic, F.E.M., Brown, S.C. and 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, J.M. and Von Aderkas, P. (1992). *In vitro* culture of trees. Kluwer Academic Publishers, Dordrecht, 236 p.

Brooker, M.I.H. (2000). A new classification of the genus *Eucalyptus* L'Her. (Myrtaceae). *Aust. Syst. Bot.* **13:** 79-148.

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

Canhoto, J.M., Lopes, M.L. and Cruz, G.S. (1999). Somatic embryogenesis in myrtaceous plants. In Somatic embryogenesis in woody plants Vol. 4. (Eds: Jain, S.M., Gupta, P.K., Newton, R.J.) Kluwer Academic Publishers, pp 293-338.

Cid, L.P.B., Machado, A.C.M.G., Carvalheira, S.B.R.C. and Brasileiro, A.C.M. (1999). Plant regeneration from seedlings explants of *Eucalyptus grandis* x *E. urophylla*. *Plant Cell Tiss. Organ. Cult.* **56(1):** 17-23.

Correia, D., Gonçalves, N.A., Couto, H.Y.Z. and Ribeiro, M.C. (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.

Dodeman, V.L., Ducreux, G. and Kreis, M. (1997). Zygotic embryogenesis versus somatic embryogenesis. *J. Exp. Bot.* **48(313):** 1493-1509.

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

Dunstan, D.I., Tautorus, T.E. and Thorpe, T.A. (1995). Somatic embryogenesis in woody plants. *In:* Thorpe, T.A. (ed.). *In vitro* embyogensis in plants, Kluwer Academic, pp 471-538.

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

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

Gaj, M.D. (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. and 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. and Bradshaw, H.D. (1994). Nuclear DNA content of commercially important *Eucalyptus* species and hybrids. *Can. J. For. Res.* **24:** 1074-1078.

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

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

Högberg, K-A., Ekberg, I., Norell, L. and 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.

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

Kaeppler, S.M., Kaeppler, H.F. and Rhee, Y. (2000). Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology* **43:** 179-188.

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

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

Lloyd, G. and McCown, B. (1980). Commercially- feasible micropropagation of mountain Laurel, Kalmia latifolia by use of shoot- tip culure. *Com. Proc. Int. Plant Propagator´s Soc.* **30:** 421-427.

Lopez, G.A., Potts, B.M., Dutkowski, G.W., Apiolaza, L.A. and 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, R.P., Dos Santos, A.V. and Machado, R.D. (1999). Ultrastructure of *Eucalyptus grandis* X Eucalyptus *urophylla*. I. Shoots cultivated *in vitro* in multiplication and elongation- rooting medium. *Int. J. Plant Sci.* **160:** 217-227.

MacKay, J.J., Becwar, M.R., Park, Y.S., Corderro, J.P., and Pullman, G.S. (2006). Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. *Tree Genet. Genom.* **2:** 1-9.

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

Martinelli, L., Candioli, E., Costa, D. and 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. and Nairn, J.C. (2005). Hardwood tree biotechnology. *In Vitro Cell Dev. Biol. Plant* **41:** 602-619.

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

Merkle, S.A., Parrott, W.A. and Flinn, B.S. (1995). Morphogenic aspects of somatic embryogenesis. *In:* Thorpe, T.A. (ed.). In vitro embryogenesis in plants, pp. 155-203.

Mullin, T.J. and Park, Y.S. (1992). Estimating genetic gains from breeding strategies for clonal forestry. *Can. J. For. R.* **22:** 14-23.

Muralidharan, E.M., Gupta, P.K. and Mascarenhas, A.F. (1989). Plantlet production trough high frequency somatic embryogenesis im long term cultures of *Eucalyptus citriodora*. *Plant Cell Rep.*. **8:** 41-43.

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

Muralidharan, E.M. and Mascarenhas, A.F. (1995). Somatic embryogenesis in *Eucalyptus*. *In:* Somatic embryogenesis in woody plants, Vol. 2. Angiosperms, Jain, S.M., Gupta, P.K., Newton, R.J. (eds.). Kluwer Academic Publishers, Dordrecht, pp. 23-40.

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

Nair, R.R. and Gupta, S.D. (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, S.F., Whitemn, P. and Stevenson, T. (2001). Somatic embryogenesis in *Eucalyptus globulus*. *Plant Cell Tiss. Organ. Cul.* **67:** 85-88.

Oller, J., Toribio, M., Celestino, C. and 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.

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

Park, Y.S., Barrett, J.D. and Bonga, J.M. (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, Y.S., Lelu-Walter, M.A., Harvengt, L., Trontin, J.F., MacEacheron, I., Klimaszewska, K. and Bonga, J.M. (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. and 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. and Araújo, C. (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. and 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. and Araújo, C. (2004a). Somatic embryogenesis of *Eucalyptus globulus* Labill. and assessement of genetic stability. *In* "*Eucalyptus* in a changing world" (Eds. Borralho, N.M.G., Pereira, J.S., Marques, C., Coutinho, J., Madeira, M. and Tomé, M.) pg 543-544. Proc. IUFRO Conf. Aveiro, 11-15 Oct.

Pinto, G., Silva, S., Araújo, C., Neves, L., Park, Y-S. and Santos, C. (2008a). Factors influencing somatic embryogenesis induction in *Eucalyptus globulus* Labill.: basal medium and anti-browning agents. *Plant Cell Tiss. and Organ. Cult.* **95:** 79-88.

Pinto, G., Park, Y-S., Silva, S., Araújo, C., Neves, L., Santos, C. (2008b). Factors affecting maintenance, proliferation, and germination of secondary somatic embryos of *Eucalyptus globulus* Labill. *Plant Cell Tiss. and Organ Cult.* **95:** 69-78.

Pinto, G., Park, Y-S., Neves, L., Araujo, C. and Santos, C. (2008c). Genetic control of somatic embryogenesis in *Eucalyptus globulus* Labill. *Plant Cell Rep.* **27:** 1093-1101.

Poke, F.S., Vaillancourt, R.E., Potts, B.M. and Reis, J.B. (2005). Genomic research in *Eucalyptus*. *Genética,* **125:** 79-101.

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

Potts, B.M. and Wiltshire, R.J.E. (1997). Eucalypt genetics and genecology. *In:* Williams, J.E. and Woinarski, J.C.Z. (eds.). Eucalypt ecology: individuals to ecosystems. University Press, Cambridge, UK pp. 56-91.

Potts, B.M. and Dungey, H.S. (2004). Interspecific hybridization of eucalypts: key issues for breeders and geneticists. **New For. 27:** 115-138.

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

Pullman, G.S., Montello, P., Cairney, J., Xu, N. and 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, C.J.J.M., Jacobsen, E. and Visser, R.G.F. (1995). Secondary somatic embryogenesis and aplications in plant breeding. *Euphytica.* **81:** 93-107.

Ramage, C.M. and Williams, R.R. (2002). Mineral nutrition and plant morphogenesis. *In Vitro Cell Dev. Biol. Plant* **38:** 116-124.

Rani, V. and Raina, S.N. (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. and Pepper, S. (1997). Somatic embryogenesis initiation in *Eucalyptus nitens*. *Acta Horticulture*, **447:** 185-186.

Santos, C., Loureiro, J., Lopes, T. and 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. Mohan Jain, S. and Häggman, H.) Springer, Dordrecht pp 63-87.

Silva, J.C., Borralho, N.M.G. and Potts, B.M. (2004). Additive and non-additive genetic parameters from clonally replicated and seedling progenies of *Eucalyptus globulus*. *Theor. Appl. Genet.* **108:** 1113-1119.

Soltis, D.E. and Soltis, P.S. (1995). The dynamic nature of polyploidy genomes. *PNAS.* **92:** 8089-8091.

Termignoni, R.R., Wang, P.J. and HU, C.Y. (1996). Somatic embryo induction in *Eucalyptus dunnii*. *Plant Cell Tiss. Organ. Cult.* **45:** 129-132.

Thorpe, T.A. (2000). Somatic embryogenesis: morphogenesis, physiology, biochemistry 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. and Teuliéres, C. (2003). An efficient procedure to stably introduce genes into an economically important pulp tree (*Eucalyptus grandis* x *Eucalyptus uroplylla*). *Transgenic Res.* **12(4):** 403-411.

Tremblay, L., Levasseur, C. and Tremblay, F.M. (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. *Am. 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. and 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. and Filanova, L. (2002). Developmental pathways of somatic embryogenesis. *Plant Cell Tiss. Organ. Cult.* **69:** 233-249.

Watt, M.P., Berjak, P., Aneliswa, M. and Blakway, F. (2003a). *In vitro* field collection techniques for *Eucalyptus* micropropagation. *Plant Cell Tiss. Organ. Cult.* **75:** 233-240.

Watt, M.P., Blakeway, F.C., MEO Mekotedi, M.E.O. and Jain, S.M. (2003b). Micropropagation of *Eucalyptus*. In: SM Jain and K Ishii (Eds.), Micropropagation of woody trees and fruits, Kluwer Academic Publishers, Dordrecht, pp. 217–244.

Watt, M.P., Blakway, F., Cresswell, C.F. and Herman, B. (1991). Somatic embryogenesis in *Eucalyptus grandis*. *South African Forestry J.* **157:** 59-65.

Watt, M.P., Blakway, F.C., Termignoni, R. and Jain, S.M. (1995). Somatic embryogenesis in *Eucalyptus grandis* and *E. dunnii*. *In:* Jain, S.M., Gupta P.K. and Newton, R.J. (Eds.). Somatic embryogenesis in woody plants, vol 5, Kluwer Academic Publishers, Dordrecht, pp. 1-16.

Whitehouse, A.B., Timothy, R.M. and Glyn, A.E. (2002). Control of hyperhydricity in *Eucalyptus* axillary shoots cultures grown in liquid medium. *Plant Cell Tiss. Organ. Cult.* **71:** 245-252.

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

Williams, J.E. and Woinarski, J.C.Z. (1997). Eucalypt Ecology: Individuals to Ecosystems. Cambridge University Press, Cambridge, UK.

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