Development of a protocol for the micropropagation of mature *Eucalyptus grandis* clones through somatic embryogenesis

By

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PREFACE

The experimental work described in this dissertation was conducted in the School of Life and Environmental Sciences, University of Natal, Durban, under the supervision of Prof. Paula Watt and Mrs Danile Macdonald.

These investigations represent original work by the author and have not been submitted in any form for any diploma or degree to any other Technikon or University. Where use was made of the work of others it has been duly acknowledged in the text.

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ABSTRACT

Biotechnology techniques such as micropropagation via somatic embryogenesis offer potential significant advances in the improvement of forest species, which could sustain forest production in South Africa, as well as globally, without increased use of land. In order to apply such techniques to commercial breeding and clonal programmes of *E. grandis* species, it is necessary to develop reliable and efficient protocols applicable to explants of proven superior genotypes. Most of the research on *E. grandis* somatic embryogenesis has used the genetically variable embryos or seedlings as explant sources, which results in the propagation of material of unproven genetic value. In order to exploit somatic embryogenesis maximally for cloning of superior trees, somatic embryos have to be induced from highly selected and, hence, mature trees. The aim of this investigation was to develop such a protocol for *E. grandis* and to test its applicability to various *E. grandis* hybrids.

Somatic embryos were induced from buds, stems, leaves and petioles, with petioles and buds giving the best results. Thus, these were selected for further studies which involved testing the effect of medium composition on embryogenic callus induction. Media used for this purpose contained MS or B5 nutrients, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$ glutamine, 0.5 $g.l^{-1}$ casein hydrolysate, 4 $g.l^{-1}$ Gelrite and 30 or 50 $g.l^{-1}$ sucrose. All the media tested were able to support induction of embryogenic callus, although the number of explants producing embryogenic calli was affected significantly by the media composition (10-91%). Callus induction media with B5 nutrients seemed to have a significant effect onn the developmental stage of embryos in the callus induction medium. Presence of 50 $g.l^{-1}$ sucrose in the callus induction medium reduced the embryo yield, but the progress of embryo development was enhanced. The callus induction medium containing B5, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$ glutamine, 0.5 $g.l^{-1}$ casein hydrolysate, 4 $g.l^{-1}$ Gelrite and 30 $g.l^{-1}$ sucrose was chosen for subsequent studies. Of all the media tested for embryo development, the medium with B5, 2.5 $mg.l^{-1}$ 2iP, 0.5 $g.l^{-1}$ glutamine, 0.5 $g.l^{-1}$ casein hydrolysate, 4 $g.l^{-1}$ Gelrite and 50 $g.l^{-1}$ sucrose was found to be the most suitable for embryo development to the cotyledonary stage. Experiments involving incorporation of both ABA and 2iP aiming at maturation of *E. grandis* somatic embryos led to an increase in size of the cotyledonary embryos formed but not to germination. Although

several media were tested for germination of the cotyledonary embryos, low plantlet regeneration (3.3%) was only achieved on media containing MS nutrients, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite and 1 or 4 $g.l^{-1}$ BAP. Similarly, cotyledonary embryos chilled at 8°C for one and two weeks, and those dried for 24 hours in the laminar flow cabinet did not germinate. In these studies the potential (albeit low) for *E. grandis* somatic embryos to develop into whole plants has been demonstrated.

The developed protocol was tested on various genotypes (GN1, GN107, GN121, NH0, NH69, GU21, GU151 and GU297). Although all clones produced embryogenic callus the embryo yield was very low. The percentages of explants that produced callus were significantly affected by the genotype, with callus production ranging from 48% (GU21, GU151) to 96% (GU297). On the embryo development medium, calli with cotyledonary embryos failed to germinate on the germination medium with BAP.

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ABA	abscisic acid
AC	activated charcoal
BAP	benzylamino purine
B5	Gamborg's (1968) nutrient formulation
°C	degrees Celsius
CaOH	calcium hypochlorite
CH	casein hydrolysate
cm	centimetre
CM	coconut milk
2,4-D	2,4-dichlorophenoxyacetic acid
DSE	direct somatic embryogenesis
Fig	figure
g	gram
GA_3	giberellic acid
HCl	hydrochloric acid
IAA	indole-3-acetic acid
IEDCs	induced embryogenically determined cells
ISE	indirect somatic embryogenesis
2iP	isopentenyl adenine
Kin	kinetin
l	litre
µE	microEinstein
µm	micrometre
M	Molar
M	metre
ml	millilitre
mm	millimetre
MS	Murashige and Skoog (1962) nutrient formulation
NAA	1-naphthalene acetic acid
NaOH	sodium hydroxide
%	percent/ percentage
PEDCs	pre-embryogenic determined cells

PGR	plant growth regulator
pH	hydrogen ion concentration
s	second
Tween 20	polyoxyethylene sorbitan monolaurate

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 The genus *Eucalyptus*

The genus *Eucalyptus*, which belongs to the Myrtaceae family is among the world's widely planted trees (Zacharin, 1978). It is indigenous to Australia where it is confined to areas with 75 cm or more rainfall per annum (McComb & Bennett, 1986). Presence of a fused calyx and/or corolla into an operculum is the most distinguishing taxonomic feature of this genus. The genus name is derived from the Greek *eu* meaning "well" and *kalyptos* meaning "covered", referring to the operculum or cap which covers the flowers before they open (Holliday, 1973).

Eucalyptus trees are divided into three subgenera, *Symphomyrtus* whose species are associated with higher soil fertility, *Monocalyptus* and *Corymbia* which grow in areas of lower nutrient soils (McComb & Bennett, 1986). The genus *Eucalyptus* evolved under isolation into many species (Mueller-Dombois, 1992) and about 450-700 *Eucalyptus* species are recognised (Muralidharan & Mascarenhas, 1995) and these range from straight-trunked forest trees up to 90 m tall, to multiple-stemmed, shrubby mallees.

In Europe eucalypts started as botanical curiosities and were much in demand for botanic gardens and these became the centres for the secondary dispersal of eucalypts to various parts of the world (Turnbull, 1991). Later in the nineteenth century travelers, traders, botanists, goldminers, priests and soldiers were responsible for the spread of eucalypts to other parts of the world. *Eucalyptus* trees grow under various altitudes, soil types (including phosphorus and other nutrient-deficient soils) and agroclimates and are found in arid regions and in areas where the annual rainfall exceeds 100 cm (Gupta & Mascarenhas, 1987). They are adapted to occasional drought and most have evolved to cope with periodic fires (Mueller-Dombois, 1992). Thus, eucalypts can be generally considered hardy species. Because of their fast growth rates, wide adaptability and useful products, eucalypts, commonly called gum trees, are regarded as one of the most productive forest crops (Turnbull & Boland, 1984). These

characteristics have made *Eucalyptus* popular as an exotic species throughout the world where it is cultivated as invader plantations (Zacharin, 1978).

1.1.1 Global importance of *Eucalyptus* species

Eucalyptus trees are grown mainly for fuelwood, poles, shelter, timber, as raw material for the pulp and paper industry (Van Wyk, 1990; Le Roux & Van Staden, 1991) and for oil extraction from leaves (Gupta & Mascarenhas, 1987). Fuelwood plantations have been established in Malawi, Mozambique (Turnbull, 1991), Tanzania and Kenya (Mwangi, pers. comm.) using eucalypts because of their fast growth and ability to coppice. Rural landscapes in China, India, Ethiopia and Peru are often dominated by eucalypts used extensively for fuelwood (Turnbull, 1991). In Argentina and particularly Brazil, iron smelting is based on commercial charcoal produced from eucalypt wood (Turnbull, 1991). When eucalypts were initially planted in Brazil the purpose was to provide fuel for railways and today there still exist large plantations to provide charcoal for the iron and steel industry. The ability of eucalypts to dry up swampy ground reduces the incidence of malaria hence the plantation of these in Europe (Zacharin, 1978).

In some countries such as Australia, South Africa and Zimbabwe, selected species are grown exclusively for their leaf oils which are used for medicinal, industrial, or perfumery purposes (Penfold & Willis, 1961). Most of this oil production is in China, Chile, Portugal and Spain where there are extensive plantations of *E. globulus.* Many *Eucalyptus* species have abundant nectar and pollen, which allows production of honey with distinctive flavours making the trees important to apiarists (McComb & Bennett, 1986). In this regard, the most favoured are *E. grandis, E. eugenioides* which all produce nectar and pollen, and *E. citriodora, E. cladocalyx* and *E. lehmanni* which yield large amounts of nectar but little pollen (du Toit, 1973). In China, eucalypt plantations were established in the 1950's mainly to meet a demand for poles, especially for the mining industry and in 1980 the Chinese government changed its policy giving priority to growth of eucalypts for pulp and paper (Turnbull, 1991).

The use of eucalypts for pulp and paper originated in Portugal with eucalypt sulfite pulp produced from *E. globulus* (Rolo, 1988) and since then eucalypt pulp production has expanded greatly. In Brazil, Spain, Portugal, South Africa and Morocco most of eucalypt pulp is produced by the kraft process from *E. globulus, E. grandis* or *E. camaldulensis* (Sidaway, 1988). The excellent properties of the eucalypt pulp for papermaking have contributed to increase its share of the world pulp market. The uniform material obtained from plantation-grown wood has good opacity, high brightness and bulk which makes the pulp very preferable for production of printing, writing and tissue papers (Turnbull, 1991). However, not all eucalypt wood is suitable for pulping as wood properties can differ significantly depending on the genotype and the environment in which the tree is grown (Zobel, 1993). The best raw material for chemical pulping is mid to low density wood which pulps readily and is relatively easy to bleach (Turnbull, 1991). Different species produce pulps with different physical properties, for example, *E. grandis* pulp has good strength properties, *E. globulus* pulp has good porosity and bulk, and that of *E. camaldulensis* has good bulk and opacity (Sidaway, 1988).

It is not surprising, therefore, that there are many *Eucalyptus* genetic improvement programmes to increase both the quality and yield of various species. The global interest in the genus has been increased by the relative ease with which several eucalypts can be cloned thereby providing opportunities for improving both their productivity and quality. This is necessary due to the increasing world population, decreasing availability of arable land and demand for implementation of sound ecological practices.

1.1.2 *Eucalyptus* trees in South Africa

In the nineteenth century, the realization that indigenous forests in South Africa could never make a significant contribution to the timber requirements of the country made the South African forestry industry enter a field that was relatively unknown in silvicuture, that of establishing and managing exotic tree species. The planting of *Eucalyptus* for commercial wood began in 1876 (Lückhoff, 1973) and immediately after the First World War mining

timber contractors were felling every available windbreak or plantation in the vicinity of Witwatersrand and local supplies were soon depleted. The main sources of supply were wattle plantations, gum windbreaks and firebreaks. It was at this time that commercial plantations of *Eucalyptus* (mainly *E. grandis*) were first established on the eastern escarpment for the purpose of producing timber for the mines (Immelman, 1973). Towards the end of the twentieth century about 40% of the total plantation resource was *Eucalyptus* as compared with 51% softwood pine plantations (Graz & Von Gadow, 1990; Anon., 1996). Seventy five percent of this eucalypt plantation resource is now *Eucalyptus grandis* (Pierce & Verryn, 2000). South Africa has therefore grown from a country that is poor in natural forests and timber resources to a world leader in plantation forestry (Anon., 1996a) and is estimated to have approximately 524 000 hectares of eucalypt plantations. Commercial products that have been produced in South Africa include sawlogs, poles, pulpwood, mining timber, tan bark and veneer logs (Anon., 1996b). *E. grandis and E. saligna,* with lesser amounts of *E. fastigata* and *E. diversicolor,* dominate the South African hardwood sawn timber industry (Immelman, 1973). In net terms, this country is an exporter of wood products such as high quality timber, art papers and certain blending pulps (Van der Zel, 1989).

E. grandis is mainly used for pulpwood, mining timber, chip production and sawn timber. The large demand for *E. grandis* stems from its fast growth and general suitability of its timber for a range of products as well as its good form and pulping qualities (Gupta and Mascarenhas, 1987; Balmelli, 2000). In addition to its use for light constructional work, furniture, joinery, paneling and flooring this tree makes a fairly strong telephone or transmission pole (Poynton, 1973). It is the most important timber species grown in South Africa with the main centres of afforestation being in Zululand and the eastern and northern Transvaal (Anon., 1973). The species stands a certain amount of drought as well as frost, tolerates exposure to sea air within limits and thrives on both sandy and clayey soils if they are deep and adequately moist (Poynton, 1973). The trees used in pulping are grown on an eight year rotation period and this is important since premature harvesting could lead to significant decreases in yield, quality and strength properties (Denison & Kietzka, 1993b).

Hybrids between *E. grandis* and other *Eucalyptus* species have been developed over the past eight years in South Africa and are becoming increasingly important for specific climatic regions of the country. Examples include *E. grandis x E. camaldulensis, E. grandis x E. europhylla,* and *E. grandis x E. tereticornis*, for the subtropical regions, and *E. grandis X E. nitens* for more temperate areas (Denison & Kietzka, 1993a). Hybrid development using *E. grandis* has added considerable flexibility to the species. Hybrid vigour (heterosis) is usually the result and this hybrid development is of great benefit to the productivity of the forest industry. Other advantages of hybrid development are that growing sites can be extended to include drier and hotter areas, colder and more frost susceptible locations can be planted, greater disease resistance can be obtained, wood properties for specific end products can be improved (Denison & Quaile, 1987).

1.2 Propagation methods applied to *Eucalyptus* trees

In 1993 it was predicted that in order to meet the anticipated demand of forest products in South Africa, wood and fibre production need to double by the year 2005 (Denison & Kietzka, 1993a). Careful management of commercial forestry can help to meet this demand while also preserving indigenous forests, grasslands and floral biodiversity. However, because of the decreasing availability of arable land and demand for implementation of sound ecological practices, more productive, rather than extensive, commercial forests with economically important trees such as *Eucalyptus* are needed. This species can be naturally propagated from seed, but the time to flowering is quite long and heterozygosity may lead to great variations between individual trees (Lakshmi Sita, 1986; Watt *et al.*, 1991). Natural propagation from seed takes place when stands of trees are left to re-seed the cleared area after felling, or it can be achieved by aerially sowing the seeds or planting with the nursery grown seedlings (McComb & Bennett, 1986).

1.2.1 Conventional methods of vegetative propagation

Although conventional plant breeding has resulted in progress in the improvement of crop and forest plants, this process is slow, time-consuming, labour-intensive and requires cultivable land and financial resources (Watt *et al.*, 1991). In the case of *E. grandis*, existing conventional breeding programmes are limited by long life cycle of the forest trees, difficulty of carrying out controlled crosses in large numbers and the inability of the breeder to always distinguish between genotypic expression and environmental effects (Watt *et al.*, 1999). Therefore, it takes a long time for breeders to select for superior genotypes.

Alternatively, selected genotypes can be propagated vegetatively, but selection of superior trees can be done only when they are mature, which therefore requires that vegetative propagation be from adult trees. While sexual reproduction involves the fusion of gametes to form a single cell (zygote) which germinates into a new plant, in vegetative (asexual) propagation, spontaneously or artificially excised parts that bear buds may be induced to form roots and grow into new plants. This means that in the former the resultant plant arises from a single cell with a new genotype, whereas in the latter the new plant has the same genetic make up as its mother plant. Thus, in sexual reproduction there is a new individual from the beginning and in vegetative propagation there is an isolated part of an established plant (Haccius, 1978).

Vegetative propagation does not improve trees or create anything new; it only facilitates the mass propagation of trees already improved in a conventional breeding program (Zobel & Talbert, 1984). The use of vegetative propagation allows replication and preservation of exact genotypes without sexual recombination (Namkoog, 1986; 1989; Millar, 1993). Using this approach it is possible to transfer the complete genetic potential of a selected tree, the donor tree or ortet, to its asexually reproduced progeny (Monteuuis, 1988). Thus, cloning of superior genotypes or "elite" individuals by vegetative propagation methods allows exploitation of maximum genetic gain available (Bonga, 1982).

Various types of vegetative propagation exist and these include air layering, cuttings, grafting and micropropagation by means of tissue culture. Kleinschmit *et al.* (1993) provided evidence on air-layering of fruit trees using containers with soil in the crowns of the trees, and grafting of *Ficus* clones over a period of about 3 000 years. However, eucalypts are very slow to root when layered and air layering is labour-intensive. Few reports of successfully layered young trees exist, and mature trees are even more difficult (Hartney, 1980). Grafting done on genetically variable seedling rootstocks was found to produce trees with aboveground clonal uniformity that was similar to that of clones grown from rooted cuttings (Kleinschmit *et al.,* 1993). Grafting can be used for many important timber species like *Eucalyptus* to establish seed nurseries and to 'rejuvenate' shoots for subsequent use, either as cuttings or explants for tissue culture, but the procedure is costly and labour-intensive (McComb & Bennet, 1986). Moreover, grafting from adult trees often fails due to rejection, and rooting often fails because of the production of inhibitors (Paton & Willing 1973).

Cloning trees by vegetative propagation from rooted cuttings started more than 500 years ago with the conifer sugi (*Cryptomeria japonica*), and with poplars and willows by planting unrooted cuttings as mentioned by Zsuffa *et al.* (1993). In South Africa, *Eucalyptus* clonal programs based on rooted cuttings were successfully introduced in 1983 (Denison & Quaile, 1987; Denison & Kietzka, 1993b). Prior to field trials, cuttings are rooted and multiplied vegetatively and then screened for adaptability, good growth, disease resistance, ability to produce wood of high yield and quality (Denison & Quaile, 1987). Following screening those clones that perform well are then used for commercial production. The required number of plants to begin commercial production is produced after three years. However, there are difficulties associated with vegetative propagation from cuttings. These are the lack of a standard universal method due to the large variability among and within species, and, for many tree species and clones, including eucalypts, cuttings from mature plants do not root (Hammatt, 1992; Watt *et al.*, 1999). The rooting potential is reduced as the mother tree ages making it more difficult to propagate from conventional cuttings (Rancillac *et al.*, 1996). To improve rooting, individuals with high rooting ability can be selected from cuttings taken from coppice shoots after the parent trees are felled (McComb & Bennet, 1986). It has also

been observed that *E. grandis* cuttings will root only if taken from juvenile tissue below node 15, that is, from seedlings less than 30 cm high (Paton *et al.*, 1970; Paton & Willing, 1973).

1.2.2 *In vitro* propagation of *Eucalyptus* trees

With application of biotechnology techniques, such as *in vitro* micropropagation, there is potential to improve the speed of vegetative propagation of selected clones. Micropropagation refers to *in vitro* vegetative multiplication and maintenance of selected plant genotypes under sterile conditions (Nashar, 1989). It involves removing pieces of material from the donor plants and culturing them aseptically on a nutrient medium that will induce cell division resulting in either callus formation or direct shoot formation (Minocha & Minocha, 1995). In the latter, the morphological integrity of the starting material is maintained. The composition of the medium can be manipulated to stimulate the regeneration of whole plants from the cultures based on the property of totipotency, which was hypothesised by Haberlandt (1902).

Haberlandt (1902) first attempted *in vitro* culture of plants. Driven by the interest to know whether plant cells could continue to grow after isolation from the intact plant he grew and maintained alive isolated cells in Knop`s solution with 1 to 5 % (w/v) sucrose. Since then much work has been done on micropropagation and further studies on plant hormones allowed a better understanding of plant growth, hence more progress in the field of tissue culture. Micropropagation is advantageous in that it is often more rapid than other traditional clonal propagation methods and when it is difficult or impossible to propagate species by conventional techniques (George, 1993).

Micropropagation may be achieved via one of many regeneration systems including axillary bud explant multiplication (organogenesis), adventitious budding (organogenesis) or somatic embryogenesis. Generally, a particular cell will follow either regeneration pathway depending on its response to appropriate culture conditions (Laparra *et al.*, 1997).

Organogenesis refers to *in vitro* culturing of plant tissues aseptically in conditions that will allow them to differentiate to organs such as shoots, roots and flowers (Thorpe, 1980). In organogenesis axillary buds are induced to shoot on a nutrient medium containing growth regulators, usually at least one cytokinin, followed by multiplication of the shoots, which are then rooted and planted out (George, 1993). Shoot multiplication is a result of axillary buds that grow out to form side branches that can be rooted or transferred to fresh multiplication medium in which the axillary buds continue to grow out to produce a number of shoots (Hammatt, 1992). Normally, due to apical dominance bud proliferation to form branches from axillary meristems is limited and the degree of apical dominance varies according to the plant type and can be further modified by environmental factors, particularly, light intensity and nitrogen supply (Hussey, 1978). Adventitious budding occurs either directly on the explant or from callus cultures. Direct adventitious meristems arise from many different sites either spontaneously or more often as a result of isolation and treatment of the organ as a cutting (Hussey, 1978).

In contrast, somatic embryogenesis is the development of embryo-like structures from somatic or asexual cells through an orderly series of characteristic morphological stages (Emons, 1994). Somatic embryos may arise directly from the explant or from an intervening callus (Williams & Maheswaran, 1986). The term "embryogenic cells" is given to the somatic cells that give rise to embryos. A somatic embryo is a new individual or independent bipolar structure arising from a single cell and, with no vascular connection with the tissue of origin, it develops and germinates into a plant (Haccius, 1978). The bipolar structure contains both the shoot and root meristems.

Considerable work has been done over years with regard to micropropagation of *Eucalyptus* species (De Fossard *et al.*, 1974; De Fossard *et al.,* 1977; Durand–Creswell & Nitsch, 1977; Franclet & Boulay, 1982; Gupta *et al.,* 1983; Furze & Cresswell, 1985) but, with very few exceptions, the reported protocols involve the organogenic route of axillary bud proliferation. A list of 28 species was produced by Le Roux & van Staden (1991) for which entire protocols for plantlet regeneration from axillary buds were established, including *E. grandis,*

E. dunnii, and *E. citriodora.* Plantlet regeneration via indirect organogenesis was reported for 14 species and embryogenic callus was produced in only five of these species (Le Roux & van Staden, 1991), of which regeneration was obtained only in three. Some of the work done since then on organogenesis is shown Table1.1.

Plant regeneration by somatic embryogenesis was first demonstrated from cultured cells of *Daucus carota* (Steward *et al.*, 1958), *Santalum album* (Rao, 1965), *Ilex aquifolium* (Sussex, 1972) and *Picea abies* (Hakman & von Arnold, 1985; Hakman *et al.*, 1985). Since then there has been dramatic progress in induction of somatic embryogenesis and production of embryo-derived plantlets of both conifers and hardwoods (Merkle, 1995). This is illustrated in the books published by Jain *et al.* (1995) and Jain (1999) with reports on embryogenic culture generation for almost every commercially important genus of forest trees, including eucalypts. However, in *Eucalyptus*, only low frequencies of plantlet regeneration via somatic embryogenesis in *Eucalyptus* species have been reported. The exception is the work on *E. citriodora* (Muralidharan & Mascarenhas, 1987; Muralidharan *et al.*, 1989; Muralidharan & Mascarenhas, 1995) and *E. dunnii* (Termignoni *et al.*, 1998). Furthermore, only the protocol for *E. dunnii* is appropriate for adult trees; other published protocols used seedling explants. Table 1.2 gives a list of *Eucalyptus* species from which somatic embryogenesis was induced with or without plantlet regeneration.

Table 1.1: Examples of published reports on the micropropagation of *Eucalyptus* species via organogenesis.

Species	Explant	Results	Reference
E. botyroides	Seedling shoot tips	Shoot regeneration and rooting	Ito *et al.*, 1996
E. calmadulensis	Seedling shoot tips	Shoot regeneration and rooting	Ito *et al.*, 1996
E. deglupta	Seedling shoot tips	Shoot regeneration and rooting	Ito *et al.*, 1996
E. globulus	Seedling hypocotyls and cotyledons	Callus growth, shoot regeneration and rooting	Bandyopadhyay *et al.*, 1999
E. grandis	Seedling shoot tips	Shoot regeneration and rooting	Ito *et al.*, 1996
E. grandis x E. urophylla	Nodes from juvenile material	Axillary bud proliferation	Jones & van Staden, 1994
E. grandis x E. urophylla	Seedling hypocotyls, cotyledons and cotyledonary nodes	Callus, shoot growth and rooting	Barrueto Cid *et al.*, 1999
E. nitens	Seedling hypocotyls and cotyledons	Callus growth, shoot regeneration and rooting	Bandyopadhyay *et al.*, 1999
E. regnans	Nodes	Axillary bud proliferation, shoots and rooting	Blomstedt *et al.*, 1991
E. teriticornis	Nodes from coppice shoots	Bud sprouting, shoot multiplication and rooting	Patil & Kuruvinashetti, 1998
E. teriticornis	Nodes from mature shoots	Bud sprouting, shoot multiplication and rooting	Patil & Kuruvinashetti, 1998
E. urophylla	Seedling hypocotyls	Callus growth, shoot regeneration and rooting	Tibok *et al.*, 1995

Table 1.2: A list of *Eucalyptus* species for which somatic embryogenesis has been induced with or without successful plantlet development, by either direct or indirect somatic embryogenesis (DSE or ISE, respectively). Unless stated otherwise the yield of plantlets was low.

Species	Explant	Results	Reference
E. botyroides	Hypocotyls, cotyledons and leaves from seedlings	Embryo-like structures and shoots via DSE	Qin-Chang-Le & Kirby,1990
E. citriodora	Mature seed	DSE, high yield of regenerated plantlets	Muralidharan *et al.*, 1989
E. dunnii	Hypocotyls, cotyledons and leaves from seedlings	Embryo-like structures and shoots via DSE	Qin-Chang-Le & Kirby,1990
E. grandis	Nodes from mature material	Callus with embryos that failed to develop into plantlets	Lakshmi Sita, 1986
E. grandis	Hypocotyl, cotyledons, leaves and young leaves from shoots of adult trees	Embryo-like structures	Qin-Chang-Le & Kirby,1990
E. grandis	Seedling leaf	ISE with plantlet regeneration	Watt *et al.*, 1991
E. gunii	Hypocotyl and internodes	ISE	Boulay, 1987

1.3 Somatic embryogenesis in dicotyledonous trees and factors affecting this morphogenic pathway

The ordered expression of morphogenic stimuli is needed for the formation of somatic embryos via the complex, highly conserved embryogenic developmental events (Altman *et al.*, 1990). Such morphogenic stimuli include endogenous (cellular and genotype-specific) factors and various culture conditions (medium composition and the physical environment) (Watt *et al.*, 1991; George, 1993). As physical and chemical factors greatly affect micropropagation, they can be manipulated for successful growth and development of the plant material resulting in different tissue culture systems. Consequently, the development of somatic embryos can be achieved by using the appropriate explant, culture media and environmental conditions.

1.3.1 Choice and preparation of explant

The research objective and explant availability dictate the choice of the explant. Callus production can be induced in almost any part of a plant if given the right stimulus (Gamborg & Shyluk, 1981; Konar & Nagmani, 1973), but for any given species or variety a particular explant may be more appropriate for successful plant regeneration. Different cell populations often exist in the cultured explant (starting material) and these respond differentially to similar growth conditions (Jain, 1999). Therefore, not all the cells of the explant will result in developing somatic embryos and those that do are usually known as competent (to embryogenesis) cells (Emons, 1994). The ability of competent cells to express their embryogenic character is influenced by the tissue culture environment such as hormone balance, osmotic condition, sucrose, amino acid and salt concentration (Armstrong & Green, 1985; Rhodes *et al.*, 1986; George, 1993). Use of explants of relatively great mass with larger cell numbers increases the chances of getting a viable culture with more competent cells (Evans *et al.*, 1981).

In addition to size and shape, the explant source, genotype, physiological age and culture medium influence callus induction and plant regeneration in somatic embryogenesis (Grewal *et al.*, 1980; George, 1993; Rancillac *et al.*, 1996; von Arnold *et al.*, 1996; McLean & Nowak, 1998). Successful callus initiation within a given plant is influenced by the explant source, as various explant sources have various culture medium requirements due to phenotypic differences in their physiology (Evans *et al.*, 1981). In general, the frequency of plant regeneration of most species, including woody species, tends to decline with the age of the explant source (Sankara Rao & Venkateswara, 1985). Consequently, juvenile tissues serve as better explant sources for micropropagation than mature ones (Gamborg & Shyluk, 1981). Certain plant groups or genotypes, even within a species, as well as different tissue sources, tend to respond more readily in culture than others (Ammirato, 1986). This variation can be overcome by manipulating culture conditions. Due to the differences from cell to cell and from organ to organ within a plant, in response to induction of somatic embryogenesis, certain tissues seem to have more potential to grow and divide in culture. They are the embryonic, meristematic and reproductive tissues (Ammirato, 1986; Cohen, 1986; Thorpe *et al.*, 1991). Explant orientation and contact with the nutrient medium may be important for some woody plants such as *Quercus rubra* and *E. globulus* (Sutter & Barker; 1985; Mackay & Kitto,1988; Rancillac *et al.*, 1996; Bandyopadhyay *et al.*, 1999).

Somatic embryogenesis has been induced from various explants such as seeds and embryos (Vasil & Vasil, 1982; Jain *et al.*, 1989), hypocotyls, cotyledons (Parra & Amo-Marco, 1999) and female gametophytes (Vasil & Vasil, 1982; Nagmani & Bonga, 1985; Gupta & Durzan, 1987; Becwar *et al.*, 1988; 1990). In *Eucalyptus* species, the most successful explants for initiation of cultures are mature embryos or seedling parts (Le Roux & van Staden, 1991; Watt *et al.*, 1991; 1996; Blakeway *et al.*, 1993; Muralidharan & Mascarenhas, 1995). This is because, as mentioned, vegetative explants from mature trees are often recalcitrant in culture (Thorpe *et al.*, 1991). However, the need in the industry is for the propagation of mature, highly selected plants of good wood quality, which has proven difficult to do via somatic embryogenesis. As a result, in woody species, other than eucalyptus, strategies have been developed to overcome or minimize this difficulty. These include obtaining, from *Quercus*

ilex mature trees, leaf fragments that in turn generate somatic embryos (Feraud-Keller & Espagnac, 1989), leaf blades or petiole tissues from micropropagated shoots derived from mature trees of *Populus* and *Olea* (Michler & Bauer, 1991; Park & Son, 1989; Rugini & Caricato, 1995) and floral and inflorescence tissue of *Aesculus hippocastanum* (Jorgensen, 1993) and *Theobroma cacao* (Lopez-Baez *et al.*, 1993). Such approaches have yet to be successful with *Eucalyptus* (Lakshmi Sita, 1981; Watt *et al.*, 1999).

Contamination of *in vitro* grown cultures is often a major problem in tissue culture activities. Explants may be superficially contaminated by bacteria or fungi or these contaminants may be carried endogenously making it difficult to eliminate by simple sterilization procedures (De Fossard *et al.*, 1977; Watt *et al.*, 1996). Many woody species such as *Eucalyptus*, with long life cycles, are more prone to endogenous contamination because they are exposed for long periods of time to the soil microorganisms. In such cases, treatment of explants with fungicides and antibiotics before or during the first culture stage, together with sterilization of explants, is necessary for explants to survive and grow since the tissue culture media used will support growth of common saprophytic bacteria and fungi. The sterilants used should not severely damage the explant tissue as to cause serious injury or retard growth (Hussey, 1978) as is the case with some fungicides such as benomyl and chlorothalonil which were found to be phytotoxic on *E. grandis* (Watt *et al.*, 1996). Various sterilizing solutions such as sodium hypochlorite, calcium hypochlorite, mercuric chloride are used routinely for surface sterilization of *Eucalyptus* tissue with the concentration and exposure time of the explant to the sterilant depending on the type and size of the explant (McComb & Bennett, 1986; Leifert & Waites, 1990; Watt *et al.*, 1999).

1.3.2 Dedifferentiation of explant cells

Plant cells as found in explant tissues have the ability to divide, differentiate and develop into organs and complete plants (Murashige, 1974; Reinert & Bajaj, 1977; Sharp *et al.*, 1980). Somatic embryos originate from somatic cells that are more or less differentiated (Ovečka *et al.*, 1997). Therefore the process of somatic embryogenesis involves an initial stage known as

dedifferentiation of the somatic cells which results in their complete reprogramming (Dudits *et al.*, 1995). Dedifferentiation is the reinitiation of cell division and often it can be achieved by application of high auxin concentrations (Emons, 1994). Dedifferentiated cells are cytologically distinct from the differentiated ones and have a high nucleo-cytoplasmic ratio (Laparra *et al.*, 1997).

Somatic embryo formation from embryogenic cells may occur directly from pro-embryonic-determined cells (PEDC), which are immediately able to produce embryos, or indirectly from a mass of unorganised tissue (callus) called induced embryogenic-determined cells (IEDC) (Minocha & Minocha, 1995). In indirect somatic embryogenesis differentiated cells are redetermined to the embryogenic pattern of development and produce callus, followed by differentiation of the IEDCs (Evans *et al.*, 1981). In most cases somatic embryogenesis occurs indirectly from IEDCs (George, 1996). Merkle (1995) suggested that in mature trees embryogenesis occurs through IEDCs because tissues have long been differentiated from those of the embryo and this route is very difficult to induce due to the presence of the differentiated vegetative cells that must undergo drastic epigenetic changes to initiate embryo production. According to Evans *et al.* (1981), for PEDCs to undergo mitosis and embryogenic development, either an inducer substance must be synthesised or an inhibitory substance be removed for mitotic activity to start. In contrast, IEDCs require a mitogenic substance to enter the mitotic cell cycle and/or exposure to certain concentrations of growth regulators (Evans *et al.*, 1981). Pro-embryonic-determined cells are present in the nucellar tissue of varieties of *Citrus, Mangifera* and carrot cultures (Evans *et al.*, 1981; Jain *et al.*, 1995) and in hypocotyl cells of *Trifolium repens* (Maheswaran & Williams, 1985; 1986) and *Eucalyptus nitens* (Ruaud *et al.*, 1997). A number of examples of IEDCs exist in the literature and these include cells of carrots and leaf tissue of *Coffea arabica* (Sondahl *et al.*, 1979; Evans *et al.*, 1981).

Callus (with IEDCs) is produced as a wound reaction from the excised point (point of induction) on the explant where cell division is triggered by the release of endogenous growth factors, particularly auxin (Hussey, 1978; Evans *et al.*, 1981). Chemical stimuli,

including synthetic and natural auxins and cytokinins can induce callus which when subcultured, grows and enlarges but does not differentiate into the organs of the plant body (Konar & Nagmani, 1973). The cells of a callus occur as a mass of unorganized, dividing and enlarging cells derived from the different tissues of the explant, have large vacuoles and are irregularly interspersed with areas of smaller meristematic cells (Hussey, 1978). In *Eucalyptus*, as in other species, embryogenic calli are usually white or pale yellow, soft and friable while non-embryogenic calli are often yellow, compact and translucent (Blakeway *et al.*, 1993). These two calli populations are often seen to cluster together.

Once dedifferentiation has begun, highly cytoplasmic cells present in the explant, together with their daughter cells, remain attached to each other and from these the competent cells undergo the two prerequisites for embryo development, i.e. cell division and the formation of multicellular structures (Emons, 1994). When subjected to high auxin concentrations or frequent subcultures, the small, highly cytoplasmic cells disappear from culture due to further disruption and cell elongation as the embryo grows (Emons, 1994). In suspension cultures, embryogenic cells are those that can still divide and adhere to each other, and have escaped the influence of prolonged auxin application. In contrast, non-embryogenic cells do not divide, they only elongate and adhere to each other loosely or occur freely in the medium (Emons, 1994). The tendency of cells to separate from each other (friability) can be increased by lowering the cytokinin concentration and/or addition of giberellin to the medium (Lance *et al.*, 1976). In suspension cultures, somatic embryos can develop as single structures while in agar-solidified medium they are attached to each other via callus tissue (Emons, 1994).

1.3.3 Somatic embryo production

The development of embryos through the process of somatic embryogenesis is analogous to that of zygotic embryos with embryos developing from embryogenic cells through the distinct structural stages of globular, heart, torpedo and cotyledonary shaped structures (Ammirato, 1986; Watt *et al.*, 1991; George, 1993; Emons, 1994). Such is the case with *Eucalyptus* species (Watt *et al.*, 1991).

1.3.3.1 Initiation of somatic embryogenic cultures

As mentioned earlier, the explant and its associated physiological traits are the most significant determinants of embryo initiation and the *in vitro* environment mainly acts to enhance or repress the embryogenic process. The reason for this is that it is the predetermined cells that undergo embryo initiation and their subsequent exposure to exogenous growth regulators merely allows embryogenesis to take place (Tisserat, 1979).
In most species, including *Eucalyptus*, a single hormonal signal is required in the induction of somatic embryogenesis for the induction of the bipolar structure that can grow into a complete plant in the development medium (George, 1996). Consequently, induction of somatic embryos in most species requires addition of a high auxin concentration, usually 2,4-dichlorophenoxyacetic acid (2,4-D), together with a source of reduced nitrogen such as ammonium to the culture medium (Ammirato, 1986; George, 1993). In indirect somatic embryogenesis an explant is cultured in an auxin-enriched medium where the callus proliferates to form pro-embryos as the auxin levels in the medium decrease (Ammirato, 1987; George, 1996; Watt *et al.*, 1991).

Most common growth regulators used for initiation of *Eucalyptus* embryogenic cultures are 2,4-D and naphthalene acetic acid (NAA), but, in some species such as *Abies*, these auxins have been inhibitory and needed addition of a cytokinin, kinetin or benzylaminopurine (BAP) (Jain *et al.*, 1995). For initiation of embryogenic cultures in *Picea* species both cytokinin and auxin are required (Jain & Ishii, 1997). Other conditions or molecules completely different from plant growth regulators, such as a change of carbon source in citrus (Gavish *et al.*, 1991) and pH changes in carrot (Smith & Krikorian, 1990) were found to induce somatic embryos without any hormones.

Cell polarity and asymmetric division are involved in the initiation of somatic embryogenesis (Stage I) (Dodeman *et al.*, 1997). Maheswaran & Williams (1985) observed that the first sign of induction of embryogenic cells in the direct mode of somatic embryogenesis in plant tissue, including that of *Eucalyptus*, is a shift from the normal division pattern to division

planes in atypical directions, which are not necessarily asymmetric. In indirect somatic embryogenesis, the first prerequisite for embryo development is a cell that is capable of dividing and producing daughter cells that adhere to each other as is the case with the small cytoplasm-rich cells (Emons, 1994). However, whether somatic embryos develop directly or indirectly, the requirement for an embryogenic pathway from a somatic cell is the presence of a densely cytoplasmic cell which, through repeated cell division not followed by cell growth, forms a globular multicellular structure commonly referred to as pro-embryo (Stage I) (Emons, 1994). These proembryos go through the globular, heart, torpedo and then fully formed cotyledonary embryos as with zygotic embryo develpoment, after the callus is transferred to an auxin-free medium (Evans *et al.*, 1981; Ammirato, 1987; Qin-Chang-Le & Kirby, 1990; Watt *et al.*, 1991; Muralidharan & Mascarenhas, 1995).

Proliferation of proembryos from cultures of various *Eucalyptus* species has been achieved using a variety of explants most of which developed further in media with little or no auxin. Initiation of such cultures from *E. grandis* was achieved by researchers who conducted pioneer work on this species and induction of somatic embryos has been successful since then (Lakshmi Sita, 1981; 1986; Ouyang *et al.*, 1980; 1981; Watt *et al.*, 1991; 1999).

1.3.3.2 Maturation and germination of somatic embryos

The maturation stage (Stage II) is the most critical stage because fully developed somatic embryos that are capable of germinating must be produced at this stage. In many species, such as *Eucalyptus* trees, *Pinus nigra* and horse chestnut, transfer of callus to media with reduced or no auxin supply leads to embryo development beyond the globular stage and transfer to abscisic acid (ABA) leads to more synchronous embryo development and then maturation (Ammirato, 1983; Allavena, 1984; Muralidharan & Mascarenhas, 1995; Capuana & Debergh, 1997; Cvikrová *et al.*, 1998; Salajova *et al.*, 1999). High levels of auxin inhibit embryo maturation resulting in delayed embryo germination and plantlet production (Ammirato, 1983).

Depending on the species, low levels of hormones in the embryo development medium may be desirable to promote normal embryo development. In dicotyledonous species, once somatic embryos acquire polarity, two meristems, a root and a shoot meristem, eventually result at opposite ends of the somatic embryo structure (Emons, 1994). From the bipolar structure of the dicotyledonous species, including *E. grandis* and *Euonymus europaes* trees, a shoot from which cotyledons develop forms at one end and a root at the other (Ammirato, 1986; Bonneau *et al.*, 1994; Dodeman *et al.*, 1997). The connection of the shoot and root tissues by a solid procambium gives rise to a vascular tissue as seen in carrot cultures and *Acanthopanax senticosus* (Gui *et al.*, 1991).

The various treatments applied to callus prior to embryo formation have an influence on embryo conversion and consequently, germination and plantlet development (George, 1996). Germination of somatic embryos still remains a big problem in many tree species, including *E. grandis*, probably because of incomplete embryo maturation (Watt *et al.*, 1999). In *Eucalyptus* species it has been observed that hormones effective in inducing plantlet regeneration from callus produced by juvenile explants may not have the same effect on callus obtained from mature explants (McComb & Bennett, 1986). This is evidenced by work done by Watt *et al.* (1991; 1999) where media used for successful, albeit low yield, of embryo germination and plantlet regeneration from *E. grandis* seedling explants, have not been appropriate for mature explants of the same species. For successful germination, conditions experienced by zygotic embryos leading to germination may have to be applied in somatic embryos and these include subjecting the immature embryo to high levels of ABA, low temperatures, high osmotica or physical desiccation (Merkle, 1995).

1.3.3.3 Chemical and environmental factors affecting somatic embryogenesis

a) Composition of culture media

To increase the frequency of somatic embryogenesis growth regulator concentrations, particularly auxins, nitrogen concentration and source and concentrations of other growth

additives can be manipulated. Since there is no single common mineral formulation to support the growth and development of all plant species, various basal media and concentrations and combinations of plant growth regulators have been employed for callus culture of *Eucalyptus* and other tree species. Basic nutrient media contain a salt mixture that supplies macro- and micro-elements, a carbon source, plant hormones and vitamins (Anandarajah & McKersie, 1990). The ingredients of culture media can be classified into the following categories: inorganic salts, organic compounds, complex natural preparations, and inert supportive materials.

A number of different salt mixtures have been formulated to optimise needs of specific plants and explants and the most extensively used of these is that of Murashige & Skoog (1962). In *Eucalyptus*, as well as in many other species, the Murashige & Skoog (1962) medium is frequently used when morphogenesis is required, while the B5 medium, often in combination with 2,4-D, is used when rapid cell proliferation is desirable (Muralidharan & Mascarenhas, 1995).

To establish *in vitro* cultures from excised plant tissues and organs it is necessary to supply them with organic compounds in addition to the inorganic salts (Kumar & Thorpe, 1991). Organic compounds comprise of carbohydrates, hormones, vitamins as well as amino acids or their amides, certain purines and pyrimidines, hexitols and organic acids (Huang & Murashige, 1977).

The most widely used carbon source is sucrose but glucose, fructose, maltose as well as starch have also been incorporated into the medium for some species (Hildebrandt & Riker, 1953; Nickell & Maretzki, 1970). The choice and concentration of the sugar depends on the explant type and on the purpose of the research (George, 1993). In addition to being a carbon source, sucrose also acts as an osmoticum affecting water potential which in turn influences water availability in cultures and morphogenesis (Ziv, 1986). Generally, about 2-3% (w/v) of sucrose is added to the medium (Huang & Murashige, 1977; Hussey, 1978; Kumar & Thorpe, 1991).

Hussey (1978) reported that although isolated plant cells and tissues require simple nutrients and are capable of growing on sterile media consisting of salts, sugar and vitamins, for many species, addition of the empirically determined hormones and various growth factors aids in controlling tissue growth together with organ initiation and development. This is also true for *Eucalyptus* trees and, in this regard, plant growth regulators are critical for the expression of embryogenesis as well as organogenesis. In some plant cells such as excised embryos of orange, establishment of culture and plant regeneration was achieved without the influence of exogenous plant growth regulators (Vardi *et al.*, 1975).

Hormones are endogenous substances that regulate developmental processes in plants by inducing mitosis in plant cells and tissues and therefore play a key role in cell division and cell differentiation *in vitro* and *in vivo* (Barendse & Peeters, 1995). External hormonal stimuli such as amount and duration of synthetic auxin application are very important in somatic embryogenesis (Nomura & Komamine, 1985; Komamine *et al.*, 1990; Vlašínová & Havel, 1999). Further, it may be necessary to reduce hormone levels or optimise the ratio of phytohormones in the medium at critical times to allow for continuous differentiation (Kochba *et al.*, 1978; Ammirato, 1983). This was found to be true for *E. grandis* embryoids that developed in medium containing hormones but failed to develop into plantlets because auxins were not removed (Lakshmi Sita, 1986).

Growth regulators and other nutritional factors play a role in cell size regulation (Evans *et al.*, 1981). It is known that all plant hormones act on the orientation of cortical microtubules and affect growth through the cytoskeletal system (Shibaoka, 1991). It is not just one growth regulator that regulates morphogenesis but interactions of all, with exogenous regulators supplementing the endogenous levels (Ammirato, 1983). However, auxins and cytokinins are the critical hormones and for *Eucalyptus* and other woody species their ratio in the medium may determine the route of morphogenesis. According to Zimmerman (1982) there exists a differential sensitivity to the type and concentration of cytokinin and auxin and to the ratio between these, which is particularly important in the callus induction, embryo development and maturation stages. High auxin:cytokinin promotes abundant cell proliferation with

subsequent callus formation while the reverse promotes shoot morphogenesis (Hussey, 1978).

Auxins and giberellins promote an orientation of the cortical microtubules perpendicular to the cell axis thereby promoting growth in length (Bergfield *et al.*, 1988; Shibaoka, 1991). Giberellic acid (GA_3) has been used for rapid growth of shoot apices into plants (Hu & Wang, 1983) and for conversion of somatic embryos into plants (Ammirato, 1983). In *E. citriodora,* GA_3 promoted embryogenic callus production in two weeks (Muralidharan & Mascarenhas, 1995). Cytokinins, ABA and ethylene lead to a decline in growth and cell elongation by promoting orientation of the cortical microtubules in a direction of the long axis of the cell.

The naturally occurring plant growth regulator, abscisic acid (ABA), acts as a growth inhibitor and is important in somatic embryo development by selectively inhibiting callusing of the developing somatic embryo, including formation of accessory embryos along the axis, and it does so when added at moderately inhibitory levels to young embryos that are growing into mature embryos (Ammirato, 1986; George, 1996). In the same manner, it has been reported that ABA also prevents aberrant cotyledon formation and germination or conversion into plantlets, but it does not affect normal development of the embryo (Ammirato, 1986; Cvikrová *et al.*, 1998). Its selective inhibition results in maturation of a large number of embryos with two cotyledons on axes free of accessory embryos (Ammirato, 1986). Such was the case with addition of ABA to *E. citriodora* cultures which led to a decrease in the growth of embryogenic masses and the number of embryos while there was an increased number of mature embryos (Muralidharan & Mascarenhas, 1995). Thus, for development of artificial seeds from somatic embryos, ABA seems to be essential. It is important to note that, in woody angiosperms, ABA does not trigger somatic embryo development as it does in many conifers (Cvikrová *et al.*, 1998). In *E. grandis*, ABA and PEG treatments induced new cycles of cell division and secondary embryogenesis leading to callus proliferation and still no regeneration (Watt *et al.*, 1999). In sandalwood, addition of ABA led to improved desiccation tolerance (Jain & Ishii, 1997).

Vitamins that are commonly added to culture media include thiamine, pyridoxine, nicotinic acid, biotin and calcium pantothenate. Of these, thiamine is the most commonly used and it is important for plant growth while the others are used because they may stimulate specific growth processes thus enhancing growth in some systems (Huang & Murashige, 1977; Evans *et al.*, 1981). Addition of thiamine, pyridoxine and nicotinic acid to liquid media on which calli from callus induction media were transferred greatly enhanced active cell division in cell suspension cultures of *E. grandis* (Blakeway *et al.*, 1993).

Amino acids and their amides are common sources of organic nitrogen and, in somatic embryogenesis, these are generally used only during callus induction. Amino acids or amides that have frequently proved to be beneficial for many *Eucalyptus* species are the L-forms of glutamine, glutamic acid, arginine, aspartic acid, asparagine and proline. In some cases L-glutamine may replace casein hydrolysate as a source of organic nitrogen (Gamborg & Shyluk, 1981). In dicotyledons, amino acids have been shown to promote or even be essential for embryogenesis in a medium that already has ammonium ions (George, 1996). Some amino acids accelerate the rate of callus proliferation and enhance the development of somatic embryos (George, 1996). In *E. citriodora*, when glutamine and casein hydrolysate were added together to the callus induction medium the total number of embryos and the number of mature embryos was higher than when they were added individually (Muralidharan & Mascarenhas, 1995). Purines and pyrimidines, although not limiting factors to growth, may be beneficial for morphogenesis, while hexitols, especially inositol, may have stimulating effects (Pollard *et al.*, 1961).

Sometimes, when the desired level of growth and development is not obtained from a fully defined medium, certain plant extracts or undefined additives may have to be added to increase cell growth. These include casein hydrolysate, yeast extract, malt extract, orange juice and coconut milk (Gamborg & Shyluk, 1981; Kumar & Thorpe, 1991; Muralidharan & Mascarenhas, 1995). Frequently, most of these extracts, except for casein hydrolysate (which provides additional amino acids) and coconut milk (serving as a cytokinin source), are used as a last resort (Huang & Murashige, 1977; Gamborg & Shyluk, 1981; George, 1993).

Depending on the species and research needs, explants can be cultured under different conditions. They can be inoculated on semi-solid media, immersed in liquid media and incubated on rotary shakers or placed on filter paper bridges over liquid media (Kumar & Thorpe, 1991). If a solid medium is to be used for culture establishment, gelling agents are added as supportive materials before autoclaving. Although agar is mostly employed as an excellent supportive agent for making the nutrient medium a moist gel, it is not physiologically inert. It contributes varying amounts of growth stimulators or inhibitors hence the use of other gelling agents such as Gelrite, silica gel and polyacrylamide by many researchers. Gelling agents are generally added to media used for *Eucalyptus* cultures after adjustment of the pH medium to a range of 5.0-5.8. The influence of pH on plant growth regulator uptake was demonstrated by Kaiser and Hartung (1981). The medium must be firm enough such that tissues are held in place and not too hard as this may inhibit tissue contact with the medium used, especially after drying out (Hussey, 1978). In liquid media, glass wool and filter paper bridges may be used to provide supportive growth surfaces to the tissue above the liquid (Huang & Murashige, 1977; Hussey, 1978).

Somatic embryogenic cultures of *Eucalyptus* species can be maintained on a liquid or semi-solid medium similar to the initiation medium (Muralidharan & Mascarenhas, 1995; George, 1996; Jain, 1999). It is often necessary to subculture somatic embryos onto fresh medium to ensure their continued growth and to prevent the callus from overgrowing the embryos, which would eventually be lost (George, 1996). Furthermore, subculturing is done to prevent accumulation of harmful metabolites and drying out of the medium (George, 1993).

Sometimes antioxidants such as citric acid, ascorbic acid and diethyl-dithiocarbamate may have to be added to prevent or reduce tissue browning, i.e. phenolic production (Reynolds & Murashige, 1979; Kumar & Thorpe, 1991). When culturing woody plant species activated charcoal is usually added as it promotes growth of tissues by adsorbing adversely affecting metabolites that are released by many tissues into the medium (Huang & Murashige, 1977). It also provides a degree of darkness in *in vitro* cultures thereby imitating soil conditions (Proskauer & Bernman, 1970; Pan & van Staden, 1998). Activated charcoal has been added

to absorb medium constituents such as vitamins (Weatherhead *et al.*, 1978), cytokinins and auxins (Fridborg *et al.*, 1978) and ascorbic acid (Weatherhead *et al.*, 1978; Scholl *et al.*, 1981). The effect of activated charcoal on *in vitro* cultures depends on the plant species cultured and the effect may be beneficial or adverse on growth and development, depending on the explant type, the medium and the research objective (Pan & van Staden, 1998). In *E. grandis* cultures, supplemented with activated charcoal, Watt *et al.* (1991) observed a decrease in the time taken by somatic embryos to germinate and an increase in the number of calli that regenerated plantlets.

b) Light and temperature

The presence or absence of light strongly influences the organised development *in vitro*, including somatic embryogenesis (Ammirato, 1986; Pan & van Staden, 1998). Light was observed to have both promoting and inhibitory effects on somatic embryogenesis (Baker *et al.*, 1994). In *E. grandis*, cultures are normally incubated in darkness for embryo initiation and development and in light for the embryo to mature and germinate into plantlets (Lakshmi Sita, 1986; Watt *et al.*, 1991; 1999). Fluorescent light tubes and/or incandescent light bulbs are used to supply the required light intensities (Kumar & Thorpe, 1991). A combination of light intensity, daily light period and quality of light affect tissue cultures and influences growth and development (Pan & van Staden, 1998). The daylight used seems to affect optimum light intensity (Hussey, 1978). Muralidharan & Mascarenhas (1995) have recommended use of natural light as a source of illumination that helps in the repair of the photosynthetic system thereby improving the vigor of plantlets. Light reduction or complete elimination may affect the activity and/or stability of plant growth regulators *in vitro* (Pan & van Staden, 1998).

Among the published work on environmental control of micropropagation, very little is found on the effects of temperature on somatic embryogenesis in *E. grandis* and other trees. For many species the temperature range used in somatic embryogenesis is maintained between 20-30°C (Kumar & Thorpe, 1991; George, 1996). Different plant species may show

different temperature optima and suitable temperature ranges and normal temperature requirements give guide to temperatures required by cultured plant tissues *in vitro*. Explant tissues of the subtropical *E. grandis* grew well at a temperature range of 24-27°C (Blakeway *et al.*, 1993).

1.3.4 Hardening off and field trials of somatic seedlings

Hardening of plants is done after germination of somatic embryos and plantlet establishment (occur *in vitro*). Plantlets obtained from conversion of somatic embryos (emblings) are placed on a solid substrate such as sterilised soil, free draining compost or vermiculite and exposed to reduced relative humidity, and an environment that will affect the shoot system without disturbing or injuring the delicate root system (Ziv, 1986; George, 1996). In this way, plantlets are transferred from the aseptic *in vitro* environment to the greenhouse.

Although various methods of hardening plants exist, they are based on the principle of gradually reducing humidity around plantlets and changing plant metabolism from partial dependence to complete independence of an external carbohydrate source (Le Roux & van Staden, 1991; George, 1996). Plantlets are often transferred when the developing leaves are able to initiate photosynthesis and support themselves (Hussey, 1978). It is important to prevent leaf desiccation resulting from the lack of a complete cuticle covering the leaves produced *in vitro* (McComb & Bennett, 1986). The actual conditions of plant hardening off will depend on either climatic conditions of the region and season, or availability of controlled light and humidity facilities (Le Roux & van Staden, 1991; George, 1996). Hardening off of plants helps to modify their response to the stressful conditions encountered after transplanting to the soil where the plantlets are grown into maturity. For *Eucalyptus* spp. the quality of the *in vitro* plant affects the success of acclimatization in the greenhouse (Poissonnier *et al.*, 1984). The quality of the *in vitro* plant can be modified or improved by *in vitro* acclimatization before transfer to the greenhouse and this may save time, space, and labour required during conventional greenhouse hardening (Ziv, 1986).

Emblings that survive in the greenhouse are then transferred to the field. Due to low germination rate of somatic embryos induced from *Eucalyptus* and other forest trees, few have undergone field trials (Jain & Ishii, 1997). Reports on *Eucalyptus* plantlets that have undergone field trials are limited to those produced from axillary bud proliferation and these exhibited more uniformity and superior survival, with better growth rates than plants raised from the normal macrocutting system (Gupta *et al.*, 1981; Denison & Kietzka, 1993b; Watt *et al.*, 1995). Somatic seedlings produced from leaf explants of *E. grandis* started flowering a season before those generated from seeds and field trials have not yet been analysed (Watt *et al.*, 1997). From fifty emblings of *E. citriodora* planted in the field, only five survived and these had no abnormalities in terms of morphology and growth rate when compared with the control seedlings (Muralidharan & Mascarenhas, 1995).

1.4 Potential applications of micropropagation via somatic embryogenesis

Somatic embryogenesis used in conjuction with conventional methods, can contribute markedly to clonal multiplication of commercially valuable genotypes (Jain *et al.*,1995; Capuana & Debergh, 1997; Timmis, 1998). Somatic embryogenesis offers a number of improvements over micropropagation via organogenesis (Haccius, 1978; Schwendiman *et al.*, 1990; Emons, 1994). One is the presence of both shoot and root meristems in the same unit, meaning that, whereas the rooting system produced via the organogenic route is adventitious, seedlings developed from somatic embryos have well-developed tap-root systems (Lakshmi Sita, 1981; Ostrolucka & Pretova, 1991; Watt *et al.*,1991).

Other advantages are the large numbers of regenerated plantlets (Ammirato, 1987; Jain *et al.*, 1995) since every cell in the explant or callus has the potential to regenerate into an embryo (Ammirato, 1989; Attree & Fowke, 1993; Rout *et al.*, 1995); the potential for dormancy induction and long term storage (Watt *et al.*,1991; 1997; Jain & Ishii, 1997); the possibilities of encapsulation (Lutz *et al.*, 1985; Watt *et al.*,1997) and other methods of packaging and/ or direct delivery systems, whether as naked embryos or young plantlets (Ammirato, 1987); the need for fewer culture stages, thus less labour and expense (Watt *et al.*,1999); and quick and

easy scale-up in liquid culture (Merkle *et al.*, 1990). Moreover, analysis using RAPD molecular markers has confirmed that somatic embryogenesis is useful for large-scale clonal propagation of plantlets by keeping intact their genetic fidelity (Jain & Ishii, 1997; Watt *et al.*,1991). Somatic embryos can be used for genetic transformation to produce transgenic somatic seedlings, thus making somatic embryogenesis an effective tool for genetic engineering (Warrag *et al.*, 1990; Jain & Ishii, 1997; Timmis, 1998). It is also a useful protocol to study plant differentiation and the mechanisms of cell totipotency (Nomura & Komamine, 1986a; 1986b; Thorpe, 1988).

In view of the applications of somatic embryogenesis to forest species, it is believed that this technique has the potential to offer significant advances in rapid propagation of the commercially important *E. grandis* as well as other *Eucalyptus* species and hybrids. However, in order to be of value to clonal and breeding programmes the material used to initiate embryogenic cultures must be from mature trees whose response to environmental factors as well as quality traits are known (Watt *et al.*, 1997). This, in fact, has been the major barrier to the application of somatic embryogenesis to commercially important forest trees such as *E. grandis*. As mentioned, to date embryogenic cultures reported for *E. grandis* have relied on explants from seeds or seedlings for induction of somatic embryogenesis, which means that the material being propagated is of unproven genetic value. Therefore, an efficient plant regeneration system via somatic embryogenesis for vegetative explants of mature *E. grandis* trees, needs to be developed.

1.5 Aims of this investigation

The primary aim of this project is to develop a protocol for propagation of mature *E. grandis* clones through somatic embryogenesis, with investigations focusing on establishment of the most appropriate explant, media and treatments for high yields of plantlet regeneration. The second aim is to test such a protocol on various clones of *E. grandis* hybrids to determine its wide range applicability to *Eucalyptus* genotypes.

CHAPTER 2: MATERIALS AND METHODS

2.1 Source of plant material and its maintenance

Potted plants of mature *E. grandis* and the hybrids *E. grandis* x *nitens* (clones GN1, GN107, GN121, NH0 and NH69) and *E. grandis* x *urophylla* (clones GU21, GU151 and GU297) were obtained from Mountain Home Estates, Mondi Forests, Hilton (KwaZulu-Natal). These plants were then maintained in the greenhouses at the University of Natal, Durban (29°C 52' S, 30°C 59'E; 25°C day/18°C night). Plants in the greenhouse were watered daily and sprayed weekly with fungicides and fertilizers. Fungicides used were mixtures of 2 $g.l^{-1}$ mancozeb (Dithane; Efekto, South Africa) and 1 $ml.l^{-1}$ chlorothalonil (Bravo; Shell, South Africa) applied every Monday to the leaves in the form of a spray, and 1 $g.l^{-1}$ prochloraz manganese chloride (Sporgon; Hoechst Schering AgrErvo, South Africa) and 1.25 $ml.l^{-1}$ tebuconazole (Folicur; Bayer, South Africa) applied every Friday as a soil spray. The fertilizers applied on Wedenesdays, alternately, were 1 $g.l^{-1}$ Mondi Orange 1N-2P-1K (Harvest Chemicals, South Africa) applied as a soil spray and 2.5 $ml.l^{-1}$ trace element solution (per liter: 18g Fe, 4g Cu, 2g Zn, 1g B and 0.4g Mo) (Trelmix; Hubers, South Africa) applied as a foliar spray. To stimulate coppice growth the parent plants were cut back every 3-4 weeks depending on the state of the plant and the need for experimental material.

2.2 Production of aseptic explants

Surface sterilization of *E. grandis* branches (10-15 cm long) was done by immersing them into 0.2 $g.l^{-1}$ $HgCl_2$ mixed with a drop of Tween 20 for five minutes, then rinsed with sterile water 5-6 times. Branches were then placed in 10 $g.l^{-1}$ calcium hypochlorite containing a drop of Tween 20 for five minutes and then rinsed with sterile water 5-6 times. The sterilization procedure used for GN107 and GN121 was similar to that of *E. grandis* except that branches were left for 10 minutes rather than 5 minutes, on 0.2 $g.l^{-1}$ $HgCl_2$ with Tween 20 and in 10 $g.l^{-1}$ calcium hypochlorite containing Tween 20. Branches of NH0, NH69, GU21, GU151 and GU297 were sterilized in fungicide cocktail (1 $g.l^{-1}$ Benlate, 1 $g.l^{-1}$ boric

acid and 0.5 ml.l^{-1} Bravo) containing a drop of Tween 20 for 15 minutes, rinsed 5-6 times and soaked for 3 minutes in 10 g.l^{-1} calcium hypochlorite mixed with Tween 20 and finally rinsed for 5-6 times.

After sterilization the plant material was dissected into the required explant types, which were then placed in callus induction media in 90 mm Petri dishes sealed with Parafilm. Four to five explants were placed in each Petri dish. The pH of all the media tested for this study was adjusted with 0.1 N HCl or NaOH to the range between 5.6-5.8 prior to autoclaving.

2.3 Embryogenic callus induction and somatic embryo initiation

2.3.1 Selection of explant type

Buds, leaves, stems and petioles were used as explants in this study. They were cultured on two media CI-1 and CI-2 (Table 2.1). The former, CI-1 contained MS with vitamins, 1 mg.l^{-1} 2,4-D, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite (Watt *et al.*, 1991), while CI-2 had B5 nutrients, 1 mg.l^{-1} 2,4-D, 0.5 g.l^{-1} glutamine, 0.5 g.l^{-1} casein hydrolysate, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite (Muralidharan & Mascarenhas, 1995). Explants were cultured in these media for six weeks in the dark at 24-25°C. Explants were assessed weekly for embryogenic callus production. The best explant was determined by assessing the amount and type of callus produced on the two callus induction media. The best explant was the one that produced the greatest amount of embryogenic callus as assessed by microscopy. Also, the stage of somatic embryos on the callus as well as the extent of microbial contamination on each of the explants was determined.

2.3.2 Selection of callus induction media

The callus induction stage was further investigated by testing various culture media formulations (Table 2.1) to investigate the effects of the medium composition on callus proliferation and embryo development. Bud and petiole explants were cultured on the various

callus induction media for six weeks in the dark at 24-25°C. The effect of replacing 30 $g.l^{-1}$ of sucrose on the selected CI-2 medium, after four weeks, by 50 $g.l^{-1}$sucrose for two weeks was also tested using buds and petioles.

Table 2.1: **Nutrient composition of callus induction media tested for embryogenic callus production from bud and petiole explants.** Nutrients (B5 or MS) with 30 or 50 $g.l^{-1}$ sucrose were added to the media, which also contained 1 $mg.l^{-1}$ 2,4-D, 4 $g.l^{-1}$ Gelrite and, except for medium CI-1, 0.5 $g.l^{-1}$ glutamine and 0.5 $g.l^{-1}$ casein hydrolysate.

Medium code	Medium nutrients	Sucrose ($g.l^{-1}$)
CI-1	MS	30
CI-2	B5	30
CI-3	B5	50
CI-4	MS	50
CI-5	MS	30

2.3.3 Introduction of an organogenesis stage prior to callus induction stage

Bud and petiole explants were cultured for four weeks on a medium capable of inducing organogenesis through callus formation. The organogenesis medium used contained MS salts with vitamins, 0.2 $g.l^{-1}$ glutamine, 0.1 $g.l^{-1}$ coconut milk, 4 $mg.l^{-1}$ NAA, 0.5 $mg.l^{-1}$ kinetin, 45 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Callus produced was then transferred to the selected callus induction medium CI-2.

2.4 Embryo development and maturation

2.4.1 Selection of embryo development medium

After six weeks on callus induction media embryogenic calli were transferred to different

embryo development media on 90 mm Petri dishes. Culture conditions were the same as in callus induction (Section 2.3.1). The effect of inclusion of glutamine and/or casein hydrolysate on embryo development was tested (Table 2.2).

Table 2.2: **Inclusion of glutamine and casein hydrolysate on embryo development and maturation media.** Bud, stem, leaf and petiole explants were cultured on media that contained B5 nutrients, 2.5 $mg.l^{-1}$2iP, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$Gelrite with and without glutamine and/ or casein hydrolysate.

Medium code	Additive ($g.l^{-1}$)
ED1	(0) Glu + (0) CH
ED2	(0.5) CH
ED3	(0.5) Glu + (0.5) CH
ED4	(0.5) Glu

In one investigation the effect of culturing embryogenic calli on a medium containing B5 nutrients, 0.5 $g.l^{-1}$ glutamine, 0.5 $g.l^{-1}$ casein hydrolysate, 50 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite supplemented with 2.5 $mg.l^{-1}$ 2iP for four weeks and then decreasing the 2iP concentration to 1 $mg.l^{-1}$ 2iP for two weeks was tested using calli produced from bud, leaf, stem and petiole explants on CI-1 and CI-2 media. Further investigations involved testing various concentrations of plant growth regulators singly and in combinations (Table 2.3).

2.4.2 Selection of embryo maturation medium

To improve embryo development and maturation the effect of culturing somatic embryos on embryo development medium ED8 (Table 2.3) for four weeks followed by transferring the resultant torpedo shaped embryos to medium ED12 containing B5 nutrients, 2.5 $mg.l^{-1}$ 2iP combined with 10 $mg.l^{-1}$ ABA, 0.5 $g.l^{-1}$ glutamine, 0.5 $g.l^{-1}$ casein hydrolysate, 50 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite was tested. Media formulations ED8 and ED12 described here are listed in Table 2.3. The control ED14 to which embryos from ED8 were also transferred, had

B5 nutrients, 0.5 $g.l^{-1}$ glutamine, 0.5 $g.l^{-1}$ casein hydrolysate, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite and 10 $mg.l^{-1}$ ABA but no 2iP. Embryos from ED12 were then sampled after two, four and six weeks for desiccation treatments. Desiccation treatments were carried out by placing embryos in empty, closed but unsealed Petri dishes in the laminar flow cabinet for 24 hours after which they were transferred to embryo germination media.

Table 2.3: **Plant growth regulator combinations and concentrations tested for embryo development from petiole explants**. Explants were cultured on B5 nutrients, 0.5 $g.l^{-1}$ glutamine, 0.5 $g.l^{-1}$ casein hydrolysate, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite supplemented with various plant growth regulators (PGR's).

Medium code	PGR ($mg.l^{-1}$)
ED5	–
ED6	(0.5) 2iP
ED7	(1.0) 2iP
ED8	(2.5) 2iP
ED9	(4.0) 2iP
ED10	(2.5) 2iP+ (2) ABA
ED11	(2.5) 2iP+ (5) ABA
ED12	(2.5) 2iP+ 10 ABA
ED13	(5.0) ABA

2.5 Embryo germination

Calli that had produced cotyledonary embryos from embryo development media were used for this study. Different media were tested for their ability to promote germination of the cotyledonary embryos of *E. grandis*. Media contained MS or B5 nutrients with 30 or 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite supplemented with various additives (Tables 2.4 and 2.5) and plant growth regulators (Table 2.6). Calli were cultured on germination media and maintained in

light conditions at 24-25°C and a 16 h light and 8 h dark photoperiod at a light intensity of 67μE/m^2/s for up to 12 weeks.

Table 2.4: **Addition of activated charcoal on germination media containing cotyledonary embryos from petiole explants.** Calli with cotyledonary embryos were produced from embryo development media ED8 (Table 2.3) for six weeks and then transferred to embryo germination media.

Medium code	Medium salt	Sucrose ($g.l^{-1}$)	Activated charcoal ($g.l^{-1}$)
EG 9a	MS	50	4
EG9b	MS	30	4
EG9c	B5	50	10

Table 2.5: **Inclusion of glutamine and casein hydrolysate on embryo germination.** Calli with cotyledonary embryos were cultured on media containing B5 nutrients, 2.5 $mg.l^{-1}$ 2iP, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite, with and without glutamine and/ or casein hydrolysate.

Medium code	Additive ($g.l^{-1}$)
EG1	(0.5) Glu, (0.5) CH
EG2	(0) Glu, (0) CH
EG3	(0.5) Glu
EG 11a	(0.5) Glu, (1) CH
EG 11b	(0.5) Glu, (2.5) CH
EG 11c	(0.5) Glu, (4) CH
EG 11d	(0.5) Glu, (5) CH

To test the influence of light on plantlet production calli on germination media EG1, EG2 and EG3 (Table 2.5) were cultured at 24-25°C both in light (16 h light and 8 h dark photoperiod, light intensity of 67μE/m^2/s) and in darkness for up to 12 weeks. Calli on germination media EG1-3, EG11a-d (Table 2.5) and EG5a-c (Table 2.6) were also subjected to cold treatment (chilling) in the cold room at 8-9°C for one, two and four weeks.

Table 2.6: **Plant growth regulator combinations tested for embryo germination.** Culture media contained MS or B5 nutrients, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite supplemented with different plant growth regulators.

Medium code	Medium salt	PGR ($mg.l^{-1}$)
EG4a	MS	(1) Kin
EG4b	MS	(2) Kin
EG4c	MS	(4)Kin
EG4d	MS	(8) Kin
EG4e	MS	(10) Kin
EG5a	MS	(1) BAP
EG5b	MS	(2) BAP
EG5c	MS	(4) BAP
EG5d	MS	(8) BAP
EG5e	MS	(10) BAP
EG6	MS	–
EG10a	MS	(1.0) BAP+ (0.1) NAA
EG10b	MS	(0.5) BAP+(0.05) NAA
EG10c	MS	(0.2) BAP+ (0.02)NAA
EG10d	MS	(0.5) BAP+(0) NAA
EG10e	MS	(0) BAP+ (0.05) NAA
EG7a	B5	(1) BAP
EG7b	B5	(2) BAP
EG7c	B5	(4) BAP
EG7d	B5	(8) BAP
EG7e	B5	(10) BAP
EG8a	B5	(1) Kin
EG8b	B5	(2) Kin
EG8c	B5	(4) Kin
EG8d	B5	(8) Kin
EG8e	B5	(10) Kin

2.6 Application of the developed protocol to various genotypes

Hybrids tested were five genotypes of *E. grandis* x *nitens* hybrid (GN1, GN107, GN121, NH0 and NH69) and three genotypes of *E. grandis* x *urophylla* (GU21, GU151 and GU297). Pure *E. grandis* served as a control. This investigation was carried out using petiole explants only for all the hybrids tested. Callus was induced from medium CI-2 which contains B5 nutrients, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$glutamine, 0.5 $g.l^{-1}$ casein hydrolysate, 30 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Somatic embryos were then transferred to an embryo development medium ED8 which has B5 nutrients, 2.5 $mg.l^{-1}$2iP, 0.5 $mg.l^{-1}$l glutamine, 0.5 $mg.l^{-1}$l casein hydrolysate, 50 $g.l^{-1}$sucrose and 4 $g.l^{-1}$ Gelrite and then to germination media EG5a or EG5c.

2.7 Microscopy and photography

Calli and embryos produced were examined microscopically using a Wild M3 stereomicroscope and were recorded photographically using a Wild photoautomat MPS 55 system. Photographs showing gross morphology of calli with embryos at various stages leading to plantlet regeneration were also taken using a Nikon FM2 camera fitted with a 60 mm Mikro Nikkor macro lens.

2.8 Data analysis

Scores (+, ++, +++) were used to indicate the amount of callus and embryos at the different stages of development. To compare the response of different explants and the success of the different media used in all stages leading to plantlet regeneration average values with standard error were recorded for percentages of callus formation, callus producing plantlets and, where appropriate, percentage of contamination. Differences in the obtained mean values (variables investigated) were assessed using a one way analysis of variance (ANOVA) (SAS, 1982). This statistical analysis assigns alphabetical values to the mean values recorded per treatment. Mean values that did not share the same letter were recognised as being significantly different. All statistical analyses were performed at a 5% level of significance.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Description and identification of embryogenic stages

Somatic embryogenesis involves differentiation of somatic cells through characteristic embryonal stages to produce somatic embryos. These develop from somatic cells into early globular, globular, heart, torpedo and cotyledonary-shaped embryos in a manner similar to zygotic embryos (Ammirato, 1986; Watt *et al.*, 1991; George, 1993; Emons, 1994). In this study, formation and development of somatic embryos from four types of explants (leaves, buds, stems and petioles) was investigated as described below. Callus (Fig. 3.1) was induced from the cut surface of the explants tested and callus proliferation was preceeded by tissue swelling at the cut surface. The process described here involved indirect production of somatic embryos from callus cells produced by the explant tissues (Sharp *et al.*, 1980; Minocha & Minocha, 1995).

Calli produced on media containing 1 $mg.l^{-1}$ 2,4-D were mainly nodular, compact and yellow and brown with clusters of compact embryogenic cells interspersed with soft, transparent non-embryogenic cells (Fig 3.1). The presence of non-embryogenic cells was not surprising since, according to Street (1979) and Ammirato (1986), not all cells of a given explant respond to culture conditions by forming somatic embryos. Early globular (proembryos) globular embryos and, sometimes, few heart-shaped embryos (Fig. 3.2) were produced on the callus induction media. When viewed with a light microscope, embryogenic cells appeared smaller and regular in shape in contrast to the large, irregularly shaped non-embryogenic cells that had large-vacuoles, as discussed by Blakeway *et al.* (1993) (results not shown). After six weeks in callus induction media, calli were transferred to embryo development medium containing a cytokinin but lacking auxins, where embryos developed further. In this medium, embryos developed from the heart through to the torpedo and cotyledonary stages (Fig. 3.3). The heart, torpedo and cotyledonary embryos were bipolar with apical meristems at the opposite ends of the embryogenic axis (Fig. 3.2B and 3.3) In the torpedo stage and the fully formed cotyledonary embryo, two, and occasionally more than two, cotyledons

differentiated and these were often partially or completely fused together. This fusion of cotyledons is a sign of abnormal embryo formation (Wetzstein & Baker, 1993) which depends on the efficieny by which proembryonic masses release separate embryos (Karlsson & Vasil, 1986) and on the size of the proembryo (Ammirato, 1987). Most germination media tested were unsuccessful, except for media which contained MS nutrients supplemented with 1 $mg.l^{-1}$ or 4 $mg.l^{-1}$ BAP, respectively, as will be discussed later.

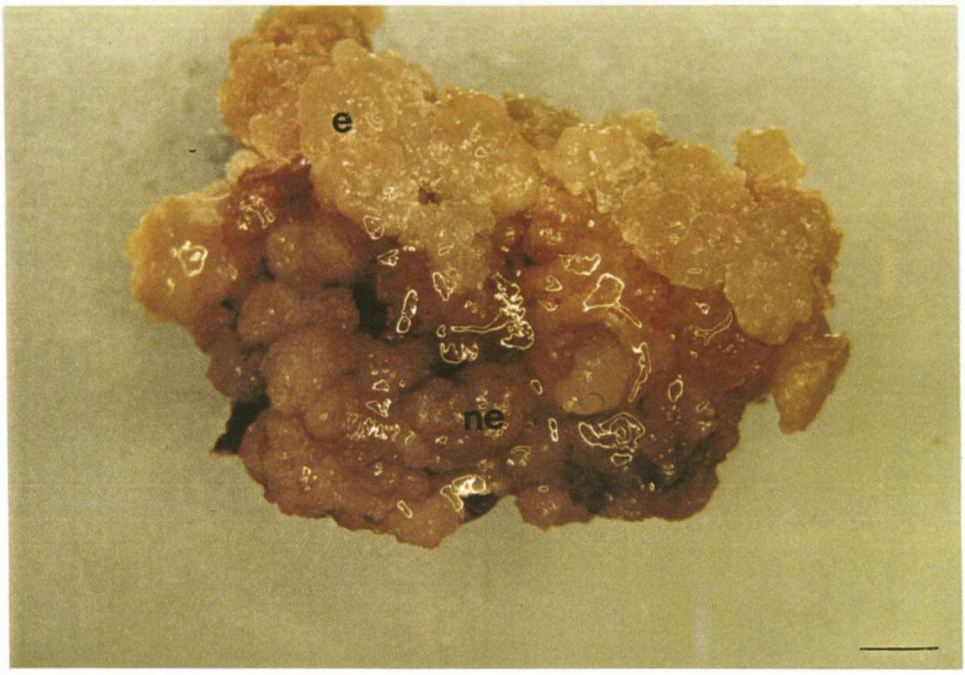

Figure 3.1: **Callus obtained from petiole explants of *E. grandis*.** Embryogenic (e) areas appear nodular, compact and yellow and brown in colour, while non-embryogenic (ne) callus appears soft, transparent and gelatinous. Calli were initiated on medium containing B5, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$ casein hydrolysate, 0.5 $g.l^{-1}$ glutamine, 30 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Bar = 1 mm.

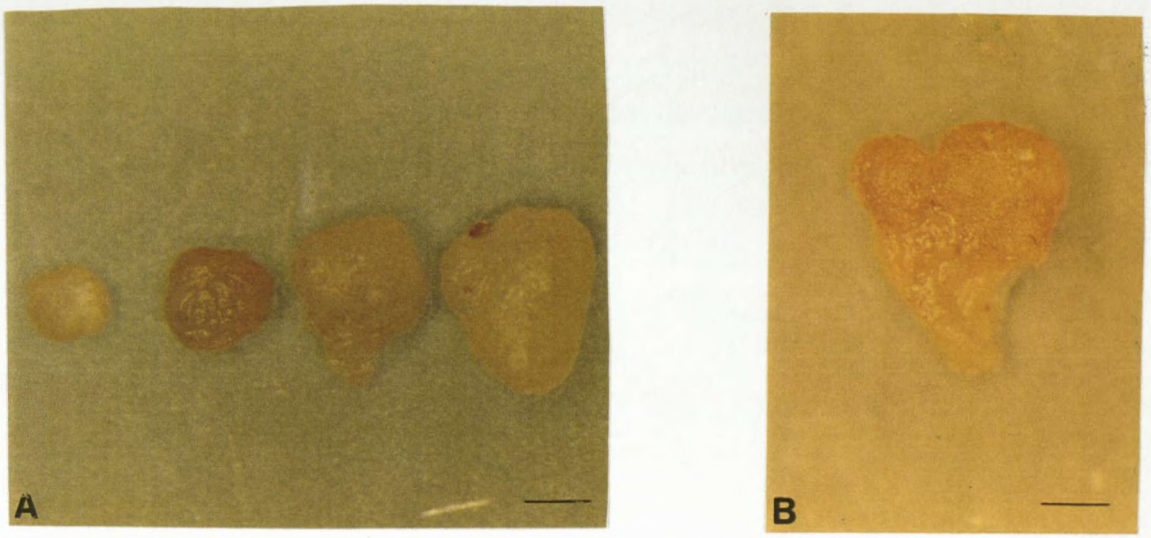

Figure 3.2: **Early stages of development of somatic embryos of *E. grandis* from early globular to the heart-shaped stage on the callus induction medium containing B5, 1 mg.l^{-1} 2,4-D, 0.5 g.l^{-1} casein hydrolysate, 0.5 g.l^{-1} glutamine, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite.** (A) Somatic embryo development to the globular stage. (B) Heart-shaped embryo of *E.grandis*. Bar = 0.5 mm.

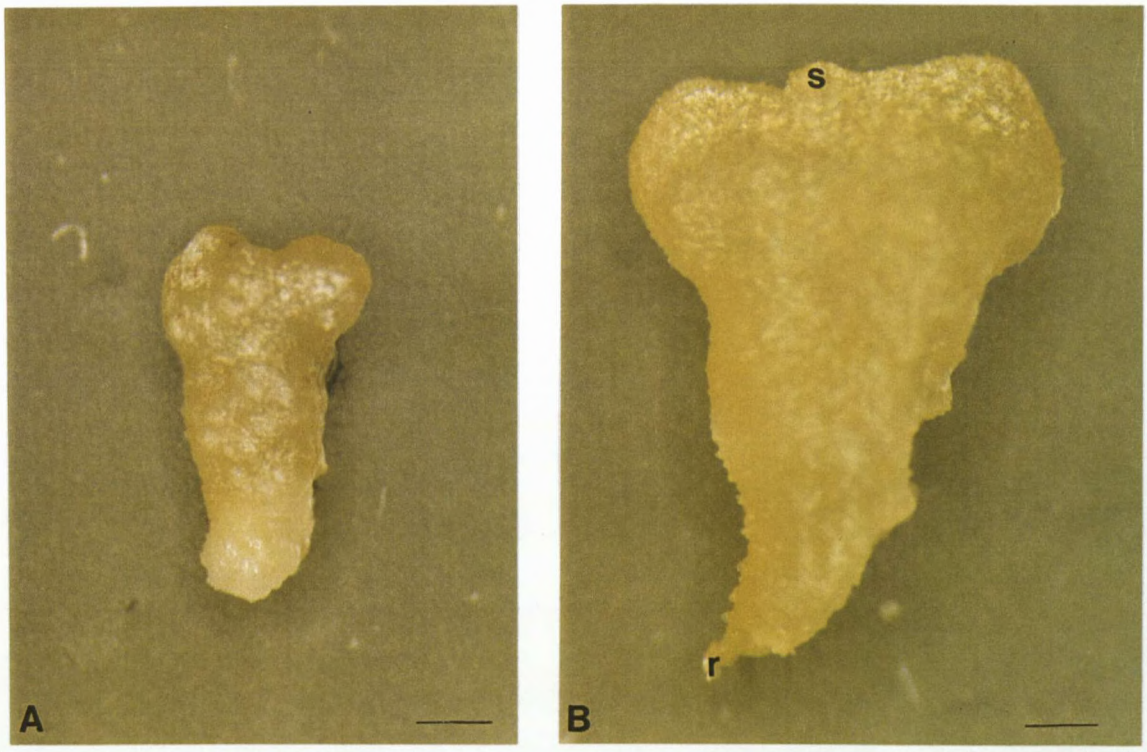

Figure 3.3: **Late stages of somatic embryo development from the torpedo (A) to the fully formed cotyledonary stage (B) with the shoot (s) and root (r) meristem.** Bar = 0.5 mm.

3.2 Development of a working protocol

As discussed in section 3.1, the first objective of this work was to develop a protocol for the mass production of somatic embryos that would develop and germinate into plantlets. The whole process involves the initiation of somatic embryos from an appropriate primary explant, proliferation of embryogenic cultures, maturation of somatic embryos, plant regeneration from the somatic embryos, transfer to the greenhouse and hardening-off (George, 1993). Hence, in order to develop such a protocol for *E. grandis,* the appropriate explants and optimum culture conditions for somatic embryogenesis were investigated.

3.2.1 Embryogenic callus induction and somatic embryo initiation

As mentioned earlier, the process of indirect somatic embryogenesis in *E. grandis* begins after the explant is cultured on the callus induction medium to obtain embryogenic callus (Fig. 3.1) (Tisserat, 1985; Watt *et al.*, 1991; Muralidharan & Mascarenhas, 1995). As reported in the literature, successful callus induction in somatic embyogenesis is measured by the yield of somatic embryos that can be obtained during the callus induction stage and this assessment was followed in the present study.

a) Selection of explant type

According to Tisserat *et al.* (1979) the explant and certain of its associated physiological qualities are the most important determinants of embryo initiation, while the *in vitro* environment is mainly involved in enhancing or repressing the embryogenic process. Since various plant tissues or organs rich in meristematic or parenchymatic cells can be grown *in vitro* (Ammirato, 1986; Cohen, 1986; Thorpe *et al.*, 1991) four types of explants, *viz.* buds, leaves, stems and petioles were tested in this preliminary study. Initially, explants were cultured on two types of media, CI-1 (MS, 1 $mg.l^{-1}$ 2,4-D, 30 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite) and CI-2 (B5, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$ casein hydrolysate, 0.5 $g.l^{-1}$ glutamine, 30 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite). Callus proliferation from the explant cut surface was observed after 21-28

days on callus induction media regardless of the explant type (Table 3.1). Callus proliferation was a result of successive cell divisions undergone by dedifferentiated cells that took place in no particular orientation. Explants of some woody plants take longer to proliferate in culture than non-woody species. This is exacerbated when explants are taken from mature trees and this delay is associated with the necessity for adapting the explants to the culture conditions (Zimmerman, 1982). In the case of *E. grandis,* callus from all explants types continued to proliferate for up to six weeks in the dark after which no more callus growth was observed (Fig. 3.1). To the naked eye the rest of the explant maintained its normal organization.

Successive cell divisions in all directions gave rise to the small clusters resembling embryogenic masses (Fig. 3.1), which continued to grow to globular and early globular embryos in six weeks. The explants that did not produce embryogenic callus (non-competent) had minute non-embryogenic callus on the cut surface which, in competent explants, was followed by continuous cell divisions to produce more callus. The initial cell division on the cut surface is triggered by the release of endogenous growth factors, especially auxins (Hussey, 1978; Evans *et al.*, 1981). The 2,4-D auxin exogenously supplied to the initiation medium sustained continuous cell divisions in the competent explants which, therefore continued to proliferate. Occasionally, explants that failed to produce callus just turned brown, probably due to heat-damage during the sterile manipulations for culture initiation.

With the exception of leaf explants, bud, stem and petiole explants showed no significant difference regarding the degree of embryogenic callus production on both types of media tested (Table 3.1). In bud, stem and petiole explants which all gave high proportion of embryogenic calli, the percentage of explants per explant type that produced embryogenic calli and the amount of calli on the various explant types on the two media tested were not significantly different (Table 3.1). On the other hand, the percentage of leaf explants which produced embryogenic callus as well as the estimated amount of callus per explant were sugnificantly lower than the other explants on both types of media employed (Table 3.1).

Data obtained from the two types of media used, which differed in their salt formulation, indicated that there were no significant differences in the effect of the salt media formulation on callus induction from the different explant types. However, the developmental stage of somatic embryos was affected by the salt media formulation. On the CI-2 medium embryos from bud, petiole and leaf explants, developed into the globular stage while on medium with MS nutrients many embryos remained in the early globular stage of development (Table 3.1). Calli produced from stem explants had globular somatic embryos on both media. Somatic embryos did not show any apparent morphological differences associated with the type of explant used. Moreover, no repetitive embryogenesis was observed in any of the callus induction media used. The total embryo yield in both callus induction media was estimated as 450-550 embryos/ gram fresh weight of callus. This was determined, after six weeks, by taking a few samples of calli and counting the number of somatic embryos per callus sample. The average number of embryos was then calculated which was the estimated number of embryos.

The variation in terms of embryogenic callus production observed between leaves and the other three explant types in culture agrees with the view that within a given explant, the explant source or type determines the success of callus initiation since the different explants differ in their nutrient requirements as a consequence of phenotypic differences in physiology (Evans *et al.*, 1981, Jain, 1999). Such differences in response among explants as well as different areas from the same explant may be closely paralleled by the intrinsic hormonal gradient (Bandyopadhyay *et al.*, 1999). Such was the case with the mesocarp and cotyledon tissue of *Persea.* The former did not require cytokinin addition while the latter did. According to Becher *et al.* (1992) segments closest to the seedling axis show much higher callus growth frequencies than more distant ones which may explain the low callus production frequency observed in this study when leaf explants were used.

Although stem explants responded well in terms of callus yield they were very susceptible to microbial contamination which made them unsuitable for further investigations (Table 3.1).

Table 3.1: **Effect of explant type on embryogenic callus production and developmental stage of proembryos or embryo structures after six weeks in culture.** Two different callus induction media CI-1 (MS, 1 mg.l^{-1} 2,4-D, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite) and CI-2 (B5, 1 mg.l^{-1} 2,4-D, 0.5 g.l^{-1} casein hydrolysate, 0.5 g.l^{-1} glutamine, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite) were used. n=40-45.

Proportion of callus and embryos:

+ small
++ moderate
+++ large

Explant type	Media	Explant forming callus (%)	Amount of callus	Contamination (%)	Estimated amount and developmenntal stage of embryos	
					Early globular	Globular
Buds	CI-1	86.69±4.71^{a}	+++	0^{a}	++	-
	CI-2	91.25±6.39^{a}	+++	1.11±1.11^{a}	-	++
Stems	CI-1	91.11±4.84^{a}	+++	13.33±4.72^{b}	-	++
	CI-2	100^{a}	+++	7.06±5.93^{ab}	-	++
Leaves	CI-1	71.84±5.75^{b}	++	4.44±2.94^{a}	++	-
	CI-2	40.00±15.92^{c}	++	18.82±7.76^{b}	-	++
Petioles	CI-1	93.33±4.71^{a}	+++	2.22± 2.22^{a}	+++	-
	CI-2	90.00±5.35^{a}	+++	1.18±1.18^{a}	-	++

Microbial contamination was observed mainly from the cut surfaces on the explants which is attributed to presence of microbes within or superficially on the explant, which were not eradicated by the sterilization procedure used. It is difficult to determine the sources of contaminated cultures but Leifert & Waites (1994) have identified explants, laboratory environments, the operator, ineffective sterilization techniques and mites to be the usual cause. It has also been suggested that the explant type employed may have an influence on the survival of microorganisms after surface sterilization has been carried out (Tisserat, 1985) and this can be seen from the results. Differences in the meristematic activity of the different explants used may have also contributed to the differences in the degree of contamination.

In conclusion, there was no significant difference between the two media tested, except in the case of leaves. However, as the stage of embryo development appeared to be positively affected by CI-2 nutrients, this was investigated further, as discussed below.

b) The effect of medium composition

Having selected buds and petioles as explants of choice for further investigations, subsequent studies were to investigate the effects of the medium composition on callus proliferation and embryo development. Although various sugars such as maltose, glucose and fructose can be used in somatic embryogenesis, for *E. grandis* successful callus induction has been achieved by other researchers using 30 $g.l^{-1}$ sucrose as the carbon source. This concentration, as well as 50 $g.l^{-1}$, were tested here. In the previous investigation (selection of explant type) casein hydrolysate and glutamine were added to the medium with B5 nutrients (CI-2) but not to the MS medium (CI-1). The effect of these additives when combined with MS was therefore investigated here by adding 0.5 $g.l^{-1}$ casein hydrolysate and 0.5 $g.l^{-1}$ glutamine it. Therefore, the following media were tested: CI-2, CI-3 (B5, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$ casein hydrolysate, 0.5 $g.l^{-1}$ glutamine, 4 $g.l^{-1}$ Gelrite, 30 $g.l^{-1}$ or 50 $g.l^{-1}$ sucrose, respectively), CI-4 and CI-5 (MS, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$ casein hydrolysate, 0.5 $g.l^{-1}$ glutamine, 30 $g.l^{-1}$ or 50 $g.l^{-1}$ sucrose, respectively). In this way it was possible to compare the effect of B5 nutrients

versus MS nutrients and 30 $g.l^{-1}$ vs 50 $g.l^{-1}$ sucrose in the induction media. Among other components, MS and B5 nutrients were used to supply the required reduced nitrogen for somatic embryo initiation.

An auxin such as 2,4-D is required in combination with a source of reduced nitrogen, usually ammonium, for induction of embryogenic cells and continued proliferation (Ammirato, 1986). Among the various plant growth regulators tested for many species including *E. grandis*, the role of 2,4-D in somatic embryogensis induction and embryo development is well established (Lakshmi Sita, 1986; Watt *et al.*, 1991; Blakeway *et al.*, 1993; Dunstan *et al.*, 1995; Laparra *et al.*, 1997). Hence, 1 $mg.l^{-1}$ was used for callus induction based on published studies. Presence of early globular, globular and heart-shaped embryos (Fig. 3A, 3B) after six weeks in culture was attributed to utilization of the auxin 2,4-D, present in the medium, by the proembryogenic cells (Evans *et al.*, 1991). In *Eucalyptus globulus*, somatic embryogenesis could be induced on media lacking an auxin, however, the embryos formed failed to develop beyond the globular stage (Canhoto *et al.*, 1999).

All explants responded by producing embryogenic calli in the four callus induction media tested. Although the number of somatic embryos per embryogenic explant was not enumerated it was observed that this number was very variable among the media. In some cases a few embryos were obtained (<20) whereas in others more than a hundred embryos were produced. Of the four callus induction media tested, CI-2 and CI-3 (both contained B5 nutrients but different sucrose concentrations) gave the best results with the highest number of explants that produced embryogenic calli (Table 3.2). This rapid cell proliferation was due to the effect of B5 nutrients combined with 2,4-D (Muralidharan & Mascarenhas, 1995). On CI-4 and CI-5 the frequency of explants forming callus was significantly lower (Table3.2). Also, the amount of callus produced by explants on media containing B5 nutrients was greater than on media with MS nutrients (Table 3.2).

These observations show that B5 nutrients promoted more rapid embryo development than MS nutrients. These results differ with those of Bandyopadhyay *et al.* (1999) who obtained

Table 3.2: **The effect of nutrient composition of the callus induction media on embryogenic callus production and developmental stage of proembryos or embryo structures after six weeks in culture.** The callus induction media used contained MS or B5 nutrients, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$ casein hydrolysate, 0.5 $g.l^{-1}$ glutamine, 30 or 50 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. n=20-55.

Proportion of callus and embryos:
+ small
++ moderate
+++ large

Explant type	Media		Explant forming callus (%)	Amount of callus	Early globular/ globular embryos	Heart-shaped embryos
	Nutrients	Sucrose ($g.l^{-1}$)				
Buds	B5	30	91.25±6.39[a]	+++	+++	-
		50	87.69±4.82[a]	+++	+	++
	MS	30	52.00±16.25[b]	++	++	+++
		50	53.33±18.38[b]	+	+	-
Petioles	B5	30	90.00±5.34[a]	+++	++	-
		50	87.69±4.82[a]	+++	-	+++
	MS	30	43.00±18.91[b]	++	++	-
		50	10.00±4.47[c]	+	+	-

more vigorous callus production on media containing MS basal nutrients than on media with B5 basal nutrients. One possible explanation for the observed differences in the amount of callus produced on the various callus induction media used is that MS basal medium contains relatively higher concentrations of ammonium-nitrate than B5 and ammonium is known to be toxic to plant cells at high concentrations (Beevers & Hageman, 1980; Ota & Yamamoto, 1989; Komai *et al.*, 1996). Furthermore, according to McCown & Lloyd (1981) tissues of woody plants grow better on media with low salt content.

On media containing B5 nutrients (CI-2 and CI-3 media), the variation of sucrose concentration did not result in any differences with regard to the amount of embryogenic callus production from both explant types but embryos developed to the heart-shaped stage when 50 $g.l^{-1}$ sucrose was added. On media with MS nutrients (CI-4 and CI-5), the amount of callus formed was greater in the presence of 30 $g.l^{-1}$ than with 50 $g.l^{-1}$ sucrose and all embryos from buds and petioles remained at early globular and globular stages. Although both 30 $g.l^{-1}$ (CI-2) and 50 $g.l^{-1}$ (CI-3) sucrose on medium with B5 were found to be optimal for embryo initiation, only CI-2 was chosen for routine callus induction.

Addition of 20-30 $g.l^{-1}$ sucrose to a medium for somatic embryogenesis is done to provide a carbon source (Huang & Murashige, 1977; Hussey, 1978; Kumar & Thorpe, 1991) and osmoticum (Brown *et al.*, 1976). Often it is added to mimic conditions found in the liquid endosperm that bathes immature zygotic embryos and, in such cases, is of higher osmotic concentration than in most culture media (Ammirato, 1986). Increased osmotic levels restrict the expansion of embryonic cotyledons and axes thereby enabling the cells of the embryonic axis to retain their embryogenic or mesistematic activity (Ammirato, 1986). The beneficial effects of additional sucrose resulting in increased osmotic levels were observed by many researchers in species such as papaya (Litz & Conover, 1983), cacao (Pence *et al.*, 1981a; 1981b) and millet (Vasil & Vasil, 1982). Sucrose concentrations can be varied to control embryo development, maturation, desiccation tolerance and also to prevent precocious embryo germination as was done for maturation of carrot somatic embryos (Ammirato & Steward, 1971; Kitto & Janick, 1985; Kim & Janick, 1989). In the present study, 50 $g.l^{-1}$ sucrose had no positive effect on explants on MS medium while it seemed to improve the

embryo development stage on medium with B5 nutrients but decreased the embryo yield (Table 3.2).

Amino acids do play a role in somatic embryo morphology as reported for alfalfa (*Medicago sativa*) (Stuart & Strickland, 1984; Baker *et al.*, 1994). Pioneer studies on the effect of various amino acids and amides showed that 10^{-3} M glutamine was most effective and, when tested with other additives, the best callus growth was obtained with 2,4-D (Konar & Nagmani, 1973). Casein hydrolysate is added as a nitrogen source to provide the amino acids required for embryo development (Huang & Murashige, 1977). Casein hydrolysate is a mixture of organic nitrogen as the hydrolysate contains an undefined mixture of a number of amino acids (Klein & Klein, 1970). The effect of casein hydrolysate and glutamine was a positive one (Table 3.2) indicating that no competitive interactions took place between the combined amino acids in the medium causing growth inhibition. This effect of glutamine combined with casein hydrolysate and 2,4-D is similar to that reported by Konar (1972) when he tried to grow callus of some pine trees.

By contrast, bud and petiole explants cultured on medium with MS, 30 or 50 $g.l^{-1}$ sucrose (CI-5 and CI-4, respectively), glutamine and casein hydrolysate produced a dramatically lower amount of callus than those cultured on medium with B5 and the same additives, as well as on MS medium without glutamine and casein hydrolysate (CI-1) (Tables 3.1 and 3.2, section 3.2.1). It is possible that the increased supply of amino acids or reduced nitrogen source in the former medium became inhibitory or detrimental to the development of embryos. No structural improvement of somatic embryos was noticed in these studies. On the contrary, in alfalfa, the exogenously applied ammonium ion was reported to have a qualitative and quantitative effect on the morphogenesis pattern (Walker & Sato, 1981). Those authors observed that addition of glutamine improved the number of somatic embryos formed but this was not true in these investigations.

Carbohydrates, high molecular weight osmoticum and time in culture affect normal somatic embryo development as shown by the results in Table 3.3. Therefore in addition to

Table 3.3: **The effect of replacing 30 g.l^{-1} sucrose on the established callus induction medium with 50 g.l^{-1} sucrose after 4 weeks.** Embryogenic calli were produced on CI-2 (Table 2.1) and after 4 weeks, were transferred to ED1 with the same medium formulation but with 30 g.l^{-1} sucrose replaced by 50 g.l^{-1} sucrose for 2 weeks. n= 45-55.

Proportion of callus and embryos:
+ small
++ moderate
+++ largest

Explant type	Treatment	Explant forming callus (%)	Amount of callus	Early globular embryos	Globular embryos	Heart-shaped embryos
Buds	30 g.l^{-1} sucrose for 6 weeks	90.00±10.00[a]	+++	-	+++	-
	30 g.l^{-1} sucrose for 4 weeks, then 50 g.l^{-1} sucrose for 2 weeks		++	-	-	++
Petioles	30 g.l^{-1} sucrose for 6 weeks	97.22±2.778[a]	+++	-	+++	-
	30 g.l^{-1} sucrose for 4 weeks, then 50 g.l^{-1} sucrose for 2 weeks		++	-	-	++

concentration, timing of application of sucrose was also evaluated using bud and petiole explants. This was done with bud and petiole explants cultured on 30 $g.l^{-1}$ sucrose in the induction medium for four weeks and then transferred to medium with 50 $g.l^{-1}$ sucrose for two weeks, and then compared to culture on 30 $g.l^{-1}$ sucrose for six weeks. The number of somatic embryos produced was greatest when 30 $g.l^{-1}$ of sucrose was added to the callus induction medium for 6 weeks. Percent (%) callus formation from both explants on the medium with 30 $g.l^{-1}$ sucrose for six weeks was not significantly different from the medium in which 30 $g.l^{-1}$ sucrose was replaced with 50 $g.l^{-1}$ sucrose. Replacing 30 $g.l^{-1}$ sucrose with 50 $g.l^{-1}$ sucrose for two weeks reduced the embryo yield but not as dramatically and further promoted somatic embryo development to the heart-shaped stage (Table 3.3). After four weeks on 30 $g.l^{-1}$ sucrose embryos had developed to the early globular stage (results not shown) while on the control medium with 30 $g.l^{-1}$ sucrose left for six weeks, embryos reached the globular stage (Table 3.3). Thus the concentration and application time of sucrose in the basal medium affected embryo development. The increased levels of sucrose in the induction medium led to a decrease in the embryo yield.

These results compare favorably to those of Westcott & Henshaw (1976) on sycamore cells where elevated sucrose levels led to increased accumulation of phenolics in the growing embryos and therefore a reduction in the embryo yield. In future investigations it is recommended that the callus induction medium supplemented with 30 $g.l^{-1}$ sucrose be used as the higher embryo yield at this sucrose level is likely to result in higher numbers of regenerated plantlets until more work is undertaken to elucidate the effect of sucrose on the somatic embryogenesis process leading to plantlet regeneration in *E. grandis*.

Carbohydrates influence the morphogenic responses and their chemical nature greatly affects the morphogenesis process (Maataoui *et al.*, 1998; Salajova *et al.*, 1999). Perhaps further investigations should involve incorporation of carbon sources such as maltose on culture media to stimulate embryo germination. Further studies to evaluate osmotic effects of sucrose on somatic embryos could be beneficial in somatic embryogenesis as well as in zygotic embryogenesis.

Leaving calli for more than six weeks on induction media led to browning due to phenolic production, development ceased and the embryogenic potential was lost. Phenols are inhibitory substances and their accumulation in cultures can be prevented by transferring explants to fresh media. Delalonde *et al.* (1996) pointed out that phenols can interfere with the endogenous levels of auxin by affecting the activity of IAA oxidase and conditioning somatic embryo induction.

c) Introduction of an organogenesis stage prior to callus induction stage

Culturing explants in an organogenic callus induction medium was found beneficial by some investigators in significantly increasing the plantlet regeneration frequency in the later stages of development (Bandyopadhyay, 1999). For this reason, this approach was attempted. Bud and petiole explants were precultured on an organogenesis induction medium for six weeks and then transferred to the selected callus induction medium CI-2 where they were left for another six weeks. Initiating embryogenic callus on CI-2 medium containing an auxin (1 $mg.l^{-1}$ 2,4-D) was successful while, as expected, the organogenesis inducing medium introduced prior to culturing on CI-2 resulted in non-embryogenic callus production. Calli produced on the organogenesis induction medium were creamy-white and brown, soft to the touch and appeared nodular. Formation of non-embryogenic callus at this stage indicates that somatic embryogenesis induction is dependent on hormonal balance as the hormonal balance of this organogenesis induction medium differs from that of the selected CI-2 medium. The high cytokinin:auxin ratio in the organogenesis medium inhibited somatic embryo induction while promoting abundant cell proliferation with subsequent callus proliferation from the wounded surface.

When the non-embryogenic callus produced on the organogenesis medium was transferred to CI-2 for six weeks, heart-shaped embryos developed. The response of calli with regards to the stage of embryo development following transfer to this callus induction medium showed a similar pattern regardless of the explant type. On transfer to the embryo development medium ED8 the yield of heart-shaped embryos increased but further development was arrested (Table 3.4). Rather, embryos senesced after longer periods on culture. These results

Table 3.4: **Effect on callus production and subsequent embryo development of medium used to induce organogenesis**. Explants were cultured for four weeks on organogenesis medium (OC-1) containing MS salts and vitamins, 0.2 $g.l^{-1}$ glutamine, 0.1 $g.l^{-1}$ coconut milk, 4 $mg.l^{-1}$ NAA, 0.5 $mg.l^{-1}$ kinetin, 45 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Calli produced were then transferred to CI-2 (Table 2.1) and after 6 weeks, to embryo development medium ED8 (Table 2.3). Percent (%) explant forming callus and amount of callus on OC-1, amount and stage of embryos on the subsequent callus induction medium CI-2 and embryo development medium ED8 are given. n= 45-60.

Proportion of callus and embryos:
+ small
++ moderate
+++ largest

Explant type	Media	Explant forming callus (%)	Amount of callus	Estimated amount and stage of embryos per callus	
				Early globular/ globular	Heart-shaped
Buds	OC-1	66.67±11.55 [a]	++	-	-
	CI-2		++	-	++
	ED8		+++	-	+++
Petioles	OC-1	98.33±1.67 [b]	++	-	-
	CI-2		++	-	++
	ED8		++	-	++

confirm the theory of Ammirato (1986) that the specific sequence of regulators may be important, as transfer of the non-embryogenic callus to CI-2 medium induced somatic embryo production. In conclusion, culturing embryos in an organogenesis medium is not essential for *E. grandis* explants as this had no observed positive effects on further embryo development beyond the heart-shaped stage.

3.2.2 Establishment of somatic embryo development and maturation medium

a) Inclusion of glutamine and casein hydrolysate

In dicotyledonous trees, availability of amino acids in the culture medium appears to be essential for the initiation of embryogenesis and to enhance the development of somatic embryos (George, 1996). Further, they have been reported to modify embryo morphology (Baker *et al.* 1994). The need for glutamine and casein hydrolysate on embryo development and maturation and on improving embryo maturation in the later stages of development was, therefore, tested. To investigate this, media containing 0-0.5 $g.l^{-1}$ glutamine and/or 0-0.5 $g.l^{-1}$ casein hydrolysate, alone or in combination, were tested (Table 3.5). After a culture period of six weeks, bud and petiole explants responded differently to the presence of glutamine and casein hydrolysate. The former exhibited a requirement for these two additives for their development to the fully formed cotyledonary stage (Fig. 3.3B), while the latter did not (Table 3.5). The stage of embryo development after six weeks in culture showed that for buds only the combined casein hydrolysate and glutamine treatment had a significant stimulating effect on embryo development compared to media without or with glutamine or casein hydrolysate alone. Petioles, on the other hand, responded equally well to all treatments. Such differences may be explained by tissue organization in petiole explants. It may be possible that petiole explants have enough tissue to contain the necessary amino acids and plant hormones required for further development (Shu & Lo, 1991).

In this study, for each type of explant, little or no morphological differences were observed in media with and without glutamine. This is in contrast to reports by Stuart & Strickland (1984) and Arcioni *et al.* (1990) who found that the structural quality of embryos was

Table 3.5: **Qualitative effect of inclusion of glutamine and casein hydrolysate on embryo development and maturation after six weeks in culture.** Calli were produced from buds, stems, leaves and petiole explants on medium CI-2 (Table 2.1) and then transferred to embryo development media containing B5 nutrients, 2.5 $mg.l^{-1}$ 2iP, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite with and without glutamine and/or casein hydrolysate. Concentrations of the additives glutamine (Glu) and casein hydrolysate (CH) are given. n= 60-65

Proportion of callus and embryos:
+ small
++ moderate
+++ largest

Additive ($g.l^{-1}$)	Explant type	Early globular/Globular embryos	Heart-shaped embryos	Torpedo embryos	Cotyledonary embryos
(0.5) Glu + (0.5) CH	Buds	-	-	-	+++
	Petioles	-	-	-	+++
(0.5) CH	Buds	-	+	+	-
	Petioles	-	-	-	+++
(0.5) Glu	Buds	++	+	++	-
	Petioles	-	-	-	+++
(0) Glu + (0) CH	Buds	-	++	+	-
	Petioles	-	-	-	+++

enhanced when glutamine was included in the regeneration medium. In this regard, in alfalfa embryos the highest degree of polarity was obtained on addition of arginine and glutamine (Stuart & Strickland, 1984).

b) Effect of 2iP concentration

Cytokinins promote cell division and regulate growth and development of plant tissues and organs (Dodds & Roberts, 1985). Cytokinin-binding proteins regulate cytokinin availability by sequestering cytokinin in protein bodies, so as to control their effective concentrations during embryogenesis or to store them for further use during germination (Letham & Palni, 1983; Brinegar *et al.*, 1985; Brinegar & Fox, 1987). The cytokinin 2iP is among the most active cytokinins for woody plant tissue culture (Huang & Murashige, 1977). For this reason, the effect of 2iP on embryo development was investigated.

The previous investigation (Table 3.5) indicated that bud explants had a requirement for both glutamine and casein hydrolysate while petioles did not. However, for comparative purposes, the media used here included both additives (Table 3.6). After six weeks in culture on the callus induction medium CI-2, calli with somatic embryos (at early globular but mostly globular stage of development) were transferred to embryo development media with cytokinin (2iP) and no auxin. They were maintained on the embryo development medium containing 2.5 $mg.l^{-1}$ 2iP for four weeks and then transferred to 1 $mg.l^{-1}$ 2iP for two weeks (Table 3.6). For both explant types, exposure of calli to 2.5 $mg.l^{-1}$ 2iP for four weeks led to development of globular embryos which, in petiole explants, continued to develop to the heart-shaped stage (Fig. 3.2B) after two weeks on 1 $mg.l^{-1}$ 2iP. On exposure to media with 1 $mg.l^{-1}$ 2iP, somatic embryos on calli originating from bud explants remained at the advanced globular stage with a moderate amount of embryos (Table 3.6).

Table 3.6: **Qualitative estimation of the effect of lowering the concentration of 2iP from 2.5 mg.l^{-1} to 1 mg.l^{-1} on the embryo development medium after 4 weeks in culture.** Before transfer to embryo development media calli were produced from bud and petiole explants from induction medium CI-2 (Table 2.1). The embryo development medium also contained B5 nutrients, 0.5 g.l^{-1} casein hydrolsate, 0.5 g.l^{-1} glutamine, 50 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite. Results after four weeks on medium with 2.5 mg.l^{-1} 2iP and after two weeks on 1 mg.l^{-1} 2iP. n= 10-30.

Proportion of callus and embryos:
+ small
++ moderate
+++ largest

Explant type	4 weeks on 2.5 mg.l^{-1} 2iP			2 weeks on 1 mg.l^{-1} 2iP		
	Early globular embryos	Globular embryos	Heart-shaped embryos	Early globular embryos	Globular embryos	Heart-shaped embryos
Buds	-	++	-	-	++	-
Petioles	-	++	-	-	-	+++

In all studies presented thus far, it was found that buds were difficult to handle and sometimes produced less embryogenic callus than petioles (some results not shown). Thus the frequency of embryogenic culture initiation from buds differed from treatment to treatment resulting in inconsistency. This variation could have been the result of their small size, which led to smaller amounts of calli. The larger the surface area of callus the larger the amounts of nutrients being absorbed (Wilhelm, 1999) and this would improve the callus response with regard to further development. Therefore, petioles were selected for subsequent studies.

In an attempt to improve embryo development, various concentrations of 2iP were tested on the embryo development media, *viz.* 0.5, 1, 2.5 and 4 $mg.l^{-1}$ 2iP. Explants used were petioles and these were cultured for six weeks. Of all the concentrations of 2iP tested, 1, 2.5 and 4 $mg.l^{-1}$ 2iP were equally effective in embryo development with bipolar embryos developing to the cotyledonary stage after 6 weeks (Table 3.7). Very few embryos at the torpedo stage of development were observed on the medium lacking 2iP (Table 3.7). Cotyledonary embryos with more than two cotyledons, partially or completely fused together, were sometimes present indicating abnormal embryo formation (Fig. 3.4). Although somatic embryos with an abnormal number of cotyledons may germinate into plantlets under well-adjusted culture conditions (Canhoto *et al.*, 1999), Lazerri *et al.* (1987) found that the ability of somatic embryos to germinate was positively affected by embryo normality.

Figure 3.4: **Abnormal somatic embryos of *E. grandis* with cotyledons fused together.** Bar = 1 mm.

Generally, once an auxin is removed from the culture medium, somatic embryos switch to a program of development and maturation (e.g. Emons, 1994; George, 1996). However, a few embryos developed to the torpedo shape in the medium without 2iP or auxin (Table 3.7). Nevertheless, transfer of calli to an auxin-free and cytokinin-free medium was not sufficient to induce further embryo development to the cotyledonary stage as is the case with some species (Muralidharan *et al.*, 1989; Muralidharan & Mascarenhas, 1995; Merkle, 1995; Kintzios *et al.*, 1999). The cytokinin 2iP appears essential to promote such development to the cotyledonary stage. For some species the actual amounts of hormones required in the medium depends a great deal on the relative availability of its endogenous counterparts in the plant tissue used. While somatic embryos cultured for four weeks on medium with 2.5 $mg.l^{-1}$ 2iP (Table 3.6) developed to the globular stage, leaving somatic embryos for six weeks in the embryo development medium with the same 2iP level (Table 3.7) had a marked positive effect on embryo development. Embryos reached the cotyledonary stage, with longer exposure to 2iP. However, it was noted in various studies that exposure to this medium for longer than six weeks resulted in the embryos drying out and in phenolic production. Subsequent investigations employed 2.5 $mg.l^{-1}$ 2iP (intermediate concentration) with six weeks culture time.

Bozhkov *et al.* (1992) have shown that addition of ABA with a cytokinin increased the embryo yield on Norway spruce. The use of ABA stems from the implications of involvement of the natural ABA in maturation of zygotic embryos and also from its prior use in somatic embryos of angiosperms (Ammirato, 1973; Ackerson, 1984). At histological level ABA is known to influence the internal organization of developing somatic embryos (Salajova *et al.*, 1999). Thus, the effect of 2iP combined with various levels of ABA in the embryo development medium was investigated using the selected 2iP concentration (2.5 $mg.l^{-1}$). Calli with few early globular and lots of globular embryos from the callus induction medium CI-2 were used.

Table 3.7: **Effect of plant growth regulators on embryo development after six weeks in the embryo development medium.** Embryogenic calli produced on callus induction medium CI-2 (Table 2.1) were transferred onto B5 nutrients, 0.5 $mg.l^{-1}$ glutamine, 0.5 $mg.l^{-1}$ casein hydrolysate, 50 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite supplemented with various combinations of plant growth regulators. n= 25-50.

Proportion of callus and embryos:
+ small
++ moderate
+++ largest

PGR ($mg.l^{-1}$)	Early globular/Globular embryos	Heart-shaped embryos	Torpedo embryos	Cotyledonary embryos
–	-	-	+	-
(0.5) 2iP	-	-	-	++
(1.0) 2iP	-	-	-	+++
(2.5) 2iP	-	-	-	+++
(4.0) 2iP	-	-	-	+++
(2.5) 2iP+ (2) ABA	++	-	-	-
(2.5) 2iP+ (5) ABA	-	-	++	+++
(2.5) 2iP+ (10) ABA	-	-	+	+++
(5.0) ABA	+++	-	-	-

On the medium with 5 $mg.l^{-1}$ ABA, and the medium with 2 $mg.l^{-1}$ ABA and 2iP, somatic embryos did not develop any further than the early globular and globular stages (Table 3.7). When 2.5 $mg.l^{-1}$ 2iP was combined with 5 or 10 $mg.l^{-1}$ ABA, somatic embryos developed to the cotyledonary stage with the yield slightly higher on 2.5 $mg.l^{-1}$ 2iP combined with 10 $mg.l^{-1}$ ABA (Table 3.7). These cotyledonary embryos appeared comparatively bigger in size than on the medium with 2.5 $mg.l^{-1}$ 2iP alone. Although the actual size was not measured, such an effect of ABA on embryo size was similar to that seen by Tian & Brown (2000) on embryo development medium of soybean cultures. Those authors attributed the large embryo size to faster expansion of embryos as a consequence of faster histodifferentiation and development. Addition of a cytokinin to ABA-containing medium in experiments conducted by von Arnold & Hakman (1988) on Norway spruce reduced the embryo yield. In this study, ABA alone had no effect on embryo development, its effect was only observed when combined with 2iP, a result similar to that obtained by Bozhkov *et al.* (1992). Histological studies may elucidate the actual effect of ABA on embryo development.

c) Selection of embryo maturation medium

In an attempt to improve embryo development and maturation, and to determine whether the developmental stage at which somatic embryos are exposed to ABA had an effect on embryo maturation, calli with somatic embryos produced on CI-2 were cultured on the embryo development medium ED8 (medium described in Table 2.3). After four weeks calli were then taken from this medium (by which time embryos were torpedo-shaped) and transferred to a maturation medium containing 2.5 $mg.l^{-1}$ 2iP and 10 $mg.l^{-1}$ ABA (Table 3.8). The control medium to which embryos were also transferred had 10 $mg.l^{-1}$ ABA only and no 2iP.

On transfer of calli with torpedo embryos to medium containing 2.5 $mg.l^{-1}$ 2iP and 10 $mg.l^{-1}$ ABA, embryos developed to the cotyledonary stage (Table 3.8). Again, combined addition of ABA and 2iP led to formation of cotyledonary embryos with a bigger size than embryos cultured for 6 weeks on medium ED8 (described in Table 2.3). On medium containing 2.5 $mg.l^{-1}$ 2iP and 10 $mg.l^{-1}$ ABA, large numbers of cotyledonary embryos were observed. On

Table 3.8: **Effect of plant growth regulator combinations on embryo maturation.** Embryogenic calli produced on callus induction medium CI-2 (Table 2.1) were transferred onto medium ED8 (Table 2.3). After 4 weeks calli were transferred to two different embryo development media ED14 which was a control medium (B5 nutrients, 10 $mg.l^{-1}$ ABA, 0.5 $mg.l^{-1}$ glutamine, 0.5 $mg.l^{-1}$ casein hydrolysate, 50 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite) and ED12 whose nutrient composition was the same as ED14 but also with 2.5 $mg.l^{-1}$ 2iP. n = 30-90.

Proportion of callus and embryos:
+ small
++ moderate
+++ largest

Treatment	Early globular/ Globular embryos	Heart-shaped embryos	Torpedo embryos	Cotyledonary embryos
(2.5) 2iP for 4 weeks	+	-	+++	-
(2.5) 2iP for 4 weeks, then 2 weeks on (10) ABA	-	-	+	-
(2.5) 2iP for 4 weeks, then 2 weeks on (2.5) 2iP + (10) ABA	-	-	-	+++
(2.5) 2iP for 4 weeks, then 4 weeks on (2.5) 2iP + (10) ABA	-	-	-	+++
(2.5) 2iP for 4 weeks, then 6 weeks on (2.5) 2iP + (10) ABA	-	-	-	+++

the control media, ABA alone failed to enhance embryo development to the cotyledonary stage, and the torpedo embryos eventually turned brown and deteriorated.

The lack of response to ABA, when supplied alone, is in contrast with many reports where ABA as the only plant growth hormone in the embryo development medium, stimulated somatic embryo maturation. Application of ABA to soybean cultures at the globular stage promoted embryo growth and development, but it had no positive effect when applied at the later stages of embryo development (Tian & Brown, 2000). Such findings are supported by the view that ABA stimulates protein accumulation of soybean zygotic embryos cultured *in vitro* during the early phase of embryogenesis (Ackerson, 1984). In *E. grandis* this was definitely not the situation as application of ABA at the globular stage had no effect (Table 3.7). A positive effect of ABA was observed only when it was applied with 2iP after four weeks on the embryo development medium where embryos had developed to the torpedo shape (Fig. 3.3A). It is worth noting that the cotyledonary embryos had a normal number of cotyledons (2 cotyledons/ embryo) and embryos were easy to separate from each other. These data show that in the presence of 2iP, ABA does have an effect on embryo development and possibly maturation, the extent of which is unknown. Although, these results are not conclusive, they seem to contradict, in part, the findings of Jain & Ishii (1997) that ABA does not play a useful role in maturation of somatic embryos of *E. grandis*. Furthermore, although the embryos obtained in this study possessed a gross morphology similar to their zygotic counterparts with a well-defined root and shoot apex, embryo axis and two cotyledons per embryo, they still lacked the germination capacity on transfer to germination media.

In an attempt to induce germination, calli with cotyledonary embryos matured on ABA and 2iP containing media were subjected to desiccation using the slow drying procedure (Capuana & Debergh, 1997). This involved keeping embryos in empty Petri dishes in the laminar flow cabinet for 24 hours prior to culture on the germination media containing 1 or 4 $mg.l^{-1}$ BAP. The moisture content of the embryos after desiccation was not measured. Embryo drying, which is a natural feature of seed development and enhances seed

germination (Kermode & Bewley, 1985; Roberts, *et al.*, 1990), was tested since it is known to promote somatic embryo germination in a number of species such as grape (Gray, 1989), rapeseed (Kott & Beversdorf, 1990) and horse chestnut (Capuana & Debergh, 1997). In zygotic embryos, embryo drying leads to synthesis of proteins and enzymes characteristic of those found in germinating seedlings thereby promoting transition of embryos from the developmental state to a state in which they have the capacity to grow. When embryos produced on ED12 medium (described in Table 2.3) were sampled after two, four and six weeks for the desiccation treatment they were at the cotyledonary stage (Table 3.8). However, those sampled after six weeks had produced phenolics and started turning brown. Calli with such brown embryos produced more phenolics when transferred to rehydration media (germination media supplemented with BAP). Greening (chlorophyll production) of cotyledonary embryos on calli sampled after two and four weeks, which may indicate germination, was observed after about three weeks on germination media containing BAP. However, embryos were not dissected to observe whether they were germinating. This greening persisted for about twelve weeks but no germination occurred.

In conclusion, embryos pretreated with ABA seemed to have survived desiccation but still lacked the germination capability. Therefore, it cannot be concluded that ABA-treated embryos were physiologically mature. Abscisic acid alone was detrimental to embryogenesis and proliferation of embryogenic cultures regardless of the stage at which it is supplied. In contrast, combination of ABA with 2iP had a marked improvement on embryo development and maturation whether these growth regulators were added to globular or torpedo shaped embryos. No embryo germination was obtained after desiccation treatments.

3.2.3 Selection of somatic embryo germination medium and conditions

Manipulation of culture conditions may lead to increased frequencies of somatic embryogenesis as this may affect expression of one or more genes which are important in triggering embryo formation (Dodeman *et al.*, 1997). To achieve complete embryo conversion into plantlets, several plant growth regulators were tested for their ability to

provide optimum nutritive and environmental conditions. All germination studies were undertaken using calli produced from CI-2 (described in Table 2.1) which were then transferred to an embryo development medium ED8 (described in Table 2.3) where cotyledonary embryos developed (Fig. 3.3B). Calli with these cotyledonary embryos were then placed on a number of different germination media (Tables 2.4, 2.5 and 2.6), including a hormone-free medium, and subjected to different physical and chemical treatments. The choice of the various additives, plant growth regulator combinations and treatments tested for plantlet production was based on media formulations that were successful with other tree species. Results for these studies are only discussed in the text (i.e. no Table of data is given) as plantlet regeneration was only obtained with two of the media tested.

Light is known to promote initiation and growth of shoots (Ammirato, 1986; Hussey, 1978), and in *Eucalyptus* species germination has been achieved under light conditions (Muralidharan *et al.*, 1989; Watt *et al.*, 1991). When this was tested in this study by comparing the influence of light against dark conditions on embryo germination, it was found that neither treatment promoted embryo germination (results not shown). Further investigations on embryo germination were carried out under light conditions.

Unlike the results of Muralidharan & Masarenhas (1995) on *E. citriodora,* the hormone-free medium EG6 (described in Table 2.6) failed to promote embryo germination in *E. grandis*.

Addition of glutamine and/or casein hydrolysate on embryo germination media presented in Table 2.5 also had no beneficial effect on embryo germination (results not shown). Germination media with increased concentrations (1, 2.5, 4 and 5 $g.l^{-1}$) of casein hydrolysate also failed to promote embryo germination (results not shown). Amino acids in the culture medium have been found to improve or even be essential for embryo germination (Radojevie *et al.*, 1988; George, 1996).

Activated charcoal was tested for its effect on embryo germination as it is well known to enhance *in vitro* growth via somatic embryogenesis as well as in organogenesis (Fridborg *et al.*, 1978; Watt *et al.*, 1991; Druart & De Wulf, 1993). In red fescue, pretreatment of

embryogenic callus with activated charcoal increased the level of precocious germination possibly due to adsorption of 2,4-D and other inhibitors (Zaghmout & Torello, 1988). In this study, addition of activated charcoal (4 or 10 $g.l^{-1}$) on germination media containing cotyledonary embryos from petiole explants did not result in embryo germination (results not shown) and calli just became brown and black due to phenol production. Activated charcoal is known to decrease the time taken by embryos to germinate while also absorbing substances such as auxins and other toxins that would inhibit embryo germination (Pan & van Staden, 1998). However, in the present case, activated charcoal played little, if any, role in phenol adsorption. Activated charcoal also adsorbs plant growth regulators and other organic compounds which are beneficial to growth in culture (Fridborg *et al.*, 1978; Pan & Van Staden, 1998). It is possible, therefore, that this non-selective adsorption effect may have had negative effects on the *E. grandis* calli.

With nearly all tested concentrations and combinations of plant growth regulators in germination media, no germination occurred. The exception was on MS nutrients, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite and 1 or 4 $mg.l^{-1}$ BAP. The germination response in these media was 3.3% after approximately ten weeks. According to Ammirato (1986) cytokinins may be necessary for the conversion of embryos into plantlets and one such cytokinin is BAP which is known to stimulate shoot formation (Huang & Murashige, 1977; Laparra *et al.*, 1997). The regenerated plantlets were used to observe the gross morphology and for photography (Fig. 3.5) and could therefore not be transferred to the greenhouse for hardening-off.

In a medium similar to the one that promoted embryo germination (albeit at very low percentage) with 1 or 4 $mg.l^{-1}$ BAP in which MS nutrients were replaced by B5 nutrients, embryos failed to germinate. Likewise, MS was shown to be better than B5 medium with regard to plant regeneration in sorghum (Gamborg *et al.*, 1977). These results confirm the findings of Ammirato (1986) which showed that the concentration and the form of the nitrogen source, as well as the sequence of their application, can affect normal embryo morphology which in turn affects embryo germination into plantlets. The MS basal medium has higher amounts of ammonium nitrate than the B5 medium and in eucalypts the MS medium is often used when morphogenesis is desirable (Muralidharan & Mascarenhas,

1995). The results obtained suggest that the cotyledonary embryos of *E. grandis* benefited from the additional ammonium nitrate present in the MS nutrients.

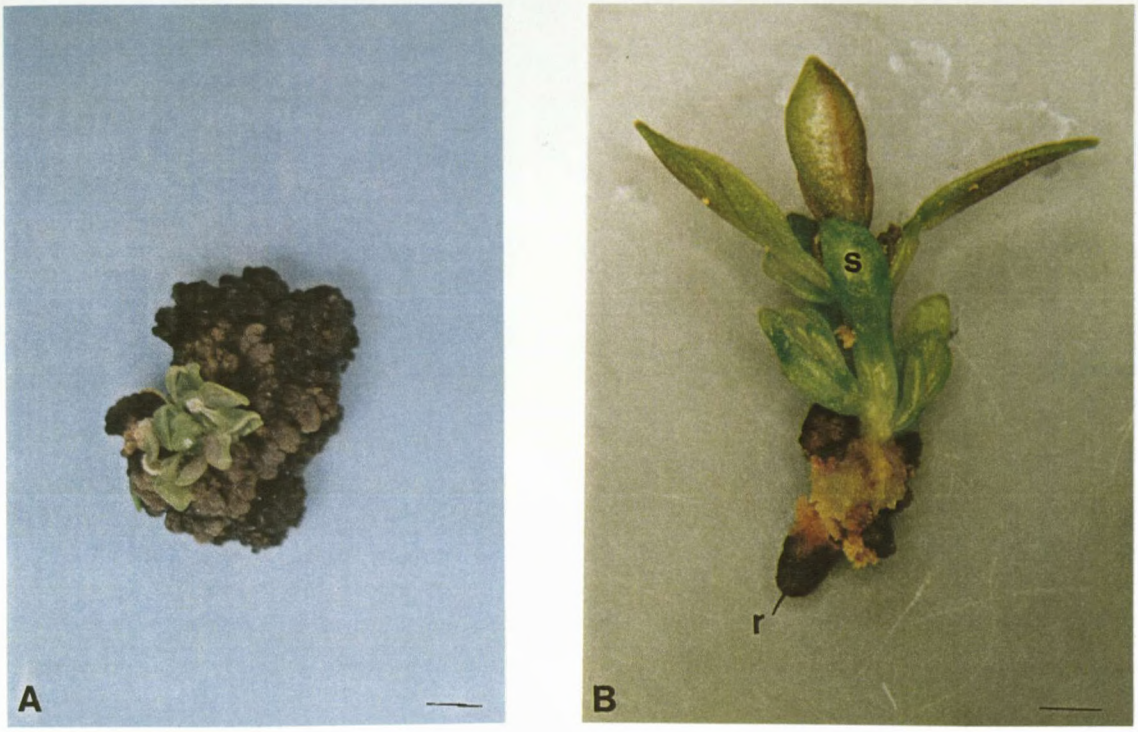

Figure 3.5: **(A) Callus mass with a cluster of plantlets protruding from the somatic embryos of *E. grandis*.** Bar= 1.5 mm. **(B) Germinating plantlet from a cotyledonary embryo with simultaneous development of shoot (s) and root (r)**. Germination took place in medium containing MS nutrients, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite and 1 or 4 $mg.l^{-1}$ BAP. Bar = 1mm.

Cold treatment is normally required by plant tissues to break dormancy allowing embryo conversion to take place (Zimmerman, 1982). Chilling of calli on different embryo germination media at 8-9°C for one, two and four weeks did not induce germination of *E. grandis* embryos (results not shown).

In conclusion, although callus formation with large quantities of somatic embryos was induced under various media formulations, it was difficult to obtain plantlet regeneration from these somatic embryos. This was achieved only with MS nutrients, 50 $g.l^{-1}$ sucrose, 4

$g.l^{-1}$ Gelrite and 1 or 4 $mg.l^{-1}$ BAP at only 3.3% plantlet regeneration. Manipulation of culture conditions failed to improve embryo germination. In all three stages of somatic embryogenesis the period of time in culture appeared to be a very critical factor for succesful somatic embryogenesis. At present, the failure of somatic embryos to germinate into plantlets on the various media and treatments cannot be explained and, therefore, further research is required. This would involve testing other physical treatments that promote embryo germination such as combined embryo drying and desiccation. Other cytokinins and auxins that have been successful in promoting embryo germination of other dicotyledounous tree species should also be tested singly and in combinations. Transferring calli with cotyledonary embryos to a germination medium containing BAP or other cytokinins should be considered.

3.3 Testing the developed protocol on various genotypes

Having identified the developmental stages of somatic embryos and developed a protocol to be used to induce somatic embryos up to the stage where cotyledonary embryos develop, a further objective was to assess its applicability to other *Eucalyptus* genotypes. This was done by subjecting petiole explants of eight different *Eucalyptus* hybrid genotypes to appropriate sterilization protocols (see paragraph below) followed by callus induction on CI-2 medium containing B5 nutrients, 1 $mg.l^{-1}$ 2,4-D, 0.5 $g.l^{-1}$ casein hydrolysate, 0.5 $g.l^{-1}$ glutamine, 30 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Calli produced after six weeks were then transferred to the embryo development medium (B5 nutrients, 2.5 $mg.l^{-1}$ 2iP, 0.5 $g.l^{-1}$ casein hydrolysate, 0.5 $g.l^{-1}$ glutamine, 50 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite). After six weeks in this medium calli which had developed cotyledonary embryos were cultured on germination medium, composed of MS nutrients, 50 $g.l^{-1}$ sucrose, 4 $g.l^{-1}$ Gelrite and 1, 2 or 4 $mg.l^{-1}$ BAP.

In our laboratory the hybrid clones used in this study were employed for axillary bud proliferation studies by another researcher (Naidoo *et al.*, 2000). Those studies revealed that there existed differences in susceptibility to contamination among the clones, which required application of sterilization procedures suitable for each hybrid clone. This was attributed to the use of mercuric chloride, a sterilant used during the sterilization procedure of pure *E. grandis*, which contributed to high levels of necrosis in a number of hybrid clones used by

this researcher. The duration of treatment and concentration of the hypochlorite solution vary among species (Zimmerman, 1982) hence the use of different sterilization procedures for the various genotypes tested.

The callus induction medium CI-2 induced embryogenic calli from the petioles of all genotypes tested but the response varied from one genotype to another (Table 3.9). Callus production ranged from 48% (GU21, GU151) to 96% (GU297) (Table 3.9). Except for genotype NH0, the estimated amount of callus produced by explants of the various hybrids was comparable to that of *E. grandis* (Table 3.9). However, the morphology of the calli differed from that of the control (pure *E. grandis*). Callus produced was soft, yellow or brown and white with more non-embryogenic than embryogenic callus (Fig. 3.7) and in *E. grandis* calli were nodular, yellow or brown and yellow (Fig. 3.1). Production of brown exudates (phenolics) by callus cells contributed to browning in some of the calli. These results are consistent with those of other researchers on woody species who found somatic embryogenesis to be dependent on the genotype (Steward *et al.*, 1975; Lu *et al.*, 1982; Vieitez *et al.*, 1992; Vlašínová & Havel, 1999). The genotypic variations of embryogenic potential can be attributed to genotypic differences in accumulation and degradation of hormones among clones (El Hadrami *et al.*, 1991).

Table 3.9: **The effect of the established callus induction medium CI-2 (B5 nutrients, 1 mg.l^{-1} 2,4-D, 0.5 g.l^{-1} casein hydrolysate, 0.5 g.l^{-1} glutamine, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite) on embryogenic callus production and developmental stage of proembryos or embryo structures from petiole explants of different *Eucalyptus* hybrids.** Results after six weeks in culture. n= 50-65.

Proportion of callus and embryos:
+ small
++ moderate
+++ largest

Genotype	Callus formation (%)	Amount of callus	Early globular embryos	Globular embryos	Heart-shaped embryos	Torpedo embryos
E. grandis	91.25±6.39 [a]	+++	+	+++	-	-
GN1	78.00±4.67 [ab]	+++	-	-	++	+
GN107	50.00±16.67 [b]	+++	-	+	+	-
GN121	82.86±4.12 [ab]	+++	-	+	+	+
NH0	64.00±14.56 [ab]	+++	-	-	-	+
NH69	60.00±13.66 [ab]	++	-	-	+	-
GU21	48.00±16.11 [b]	+++	+	-	+	+
GU151	48.00±16.11 [b]	+++	-	-	+	-
GU297	96.00±4.00 [a]	+++	-	+	+	+

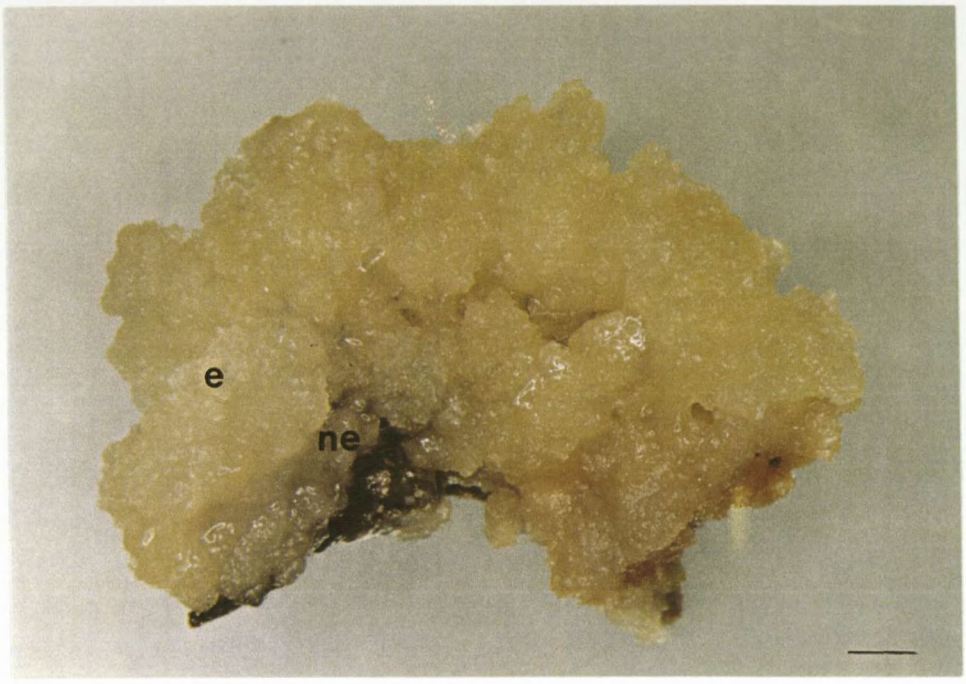

Figure 3.6: **Callus obtained from petiole explants of NH0 with soft, yellow and brown, mucilagenous, non-embryogenic regions (ne) and yellow and white embryogenic regions (e).** Bar = 1.4 mm.

Variations in the total number and developmental stages of somatic embryos among the cultured clones were observed both on the callus induction media and on the embryo development media. Although all genotypes produced callus after six weeks on the callus induction medium the number of embryos was very low compared to that of the *E. grandis* (Table 3.9). Asynchronous development of somatic embryos from calli of the same age was more pronounced in all the hybrid clones tested than in *E. grandis* (Table 3.9). Further, unlike calli from *E. grandis,* which had only the early globular and globular stages after six weeks in the induction medium, embryos from GN1, NH0, NH69 and GU151 developed into heart and torpedo stages. Only calli from explants of GU21 had early globular embryos while calli from GN107, GN121 and GU 297 had globular embryos (Table 3.9).

Six weeks after transfer of calli to the embryo development medium established for the *E. grandis,* NH0, NH69, GU21, GU151 and GU 297 had cotyledonary embryos (Table 3.10).

However, very few embryos had developed to the cotyledonary stages in these clones and such embryos did not germinate on media with BAP. Furthermore, with time, in NH0, GN1, GN107 and GN121 early globular, globular, heart and torpedo shaped embryos were found indicating secondary embryogenesis (Table 3.10). In addition, GN1, GN107 and GN121 lost the capacity for further development and produced lots of phenols which are detrimental to development of the plant (Durand-Cresswell & Nitsch, 1977) and the brown and white callus from all the clones became much softer to the touch as the embryogenic capacity declined.

The period of exposure to the callus induction medium containing an auxin may be the cause of the failure of embryos to continue developing beyond the preglobular stage and instead go through repetitive cycles of new early/ preglobular embryo formation. These results agree with the observation made by Jain & Ishii (1997) that induction of secondary embryogenesis together with the maintenance of embryogenic potential in woody plants depends on the species and genotype. Culture conditions need to be optimised for successful somatic embryogenesis from the genotypes tested.

In conclusion, the *E. grandis* hybrid clones studied here were recalcitrant to somatic embryogenesis possibly because of the choice of plant growth regulators and the nutrient salts on the medium. The results from clones NH0, NH69, GU21, GU151 and GU 297 (Table 3.10) for which cotyledonary embryos were obtained under the tested conditions are promising. Further, the results obtained are in agreement with the view of Nehra *et al.* (1989; 1990) and many other workers (Lu *et al.*, 1982; Chen & Marowitch, 1987) that no two genotypes can give similar responses under a given set of culture conditions, which indicates that protocol optimization may be required for each genotype.

It must be noted that these studies were carried out in autumn and according to Kärkönen *et al.* (1999) has reported that the hormonal balance of woody species in autumn greatly favours dormancy over growth. This needs to be taken into consideration when working with tissue cultures as it may result in inconsistent results.

Table 3.10: **Stage of embryos on embryo development medium after six weeks on ED8 containing B5 nutrients, 2.5 mg.l^{-1} 2iP, 0.5 mg.l^{-1} glutamine, 0.5 m g.l^{-1} casein hydrolysate, 50 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite.** n=50-65.

Proportion of callus and embryos:
+ small
++ moderate
+++ largest

Genotype	Early globular embryos	Globular embryos	Heart/torpedo-shaped embryos	Cotyledonary embryos
E. grandis	-	-	+	+++
GN1	-	+++	++	-
GN107	-	++	+++	-
GN121	-	++	+++	-
NH0	++	+	+	+
NH69	-	-	++	+
GU21	-	-	+++	+
GU151	+	-	++	+
GU297	-	+	+++	+

CONCLUSIONS AND RECOMMENDATIONS

In order to develop an efficient protocol for *in vitro* plant regeneration of *E. grandis*, the explants and optimum culture conditions for somatic embryogenesis were studied. Somatic embryogenesis was induced regardless of callus induction medium and explant type employed, but the response varied among the explants and the culture media used. A somatic embryo development medium containing 2.5 $mg.l^{-1}$ 2iP was found appropriate for embryo development to the cotyledonary stage. The data presented in this study demonstrated that simultaneous application of 2iP and ABA had a positive effect on growth, development and normal cotyledonary embryos. Further studies concerning the effect of these two growth regulators on embryo development and maturation need to be conducted.

Only a low frequency of plantlet regeneration (3.3%) was obtained, so plantlet regeneration via somatic embryogenesis from mature *E. grandis* still remains problematic. To achieve plantlet regeneration via somatic embryogenesis it is important to understand and control somatic embryo development, and to know which factors are important in subsequent embryo and plant performance. This study indicates that such a protocol for mature *E. grandis* trees may be possible in the near future.

This study also showed that somatic embryogenesis in *Eucalyptus* is genotype-dependent. Basic research aimed at increasing the number of responsive genotypes is therefore essential. To improve the protocol for these genotypes, culture conditions will also need to be optimised.

Such proposed future research is of importance as somatic embryogenesis can play a major role in tree breeding programmes. Furthermore, development of an efficient and reproducible protocol for *E. grandis* trees and its hybrids would have an impact in the South African forestry industry.

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3.5 STANDARDS, CRITERIA AND MEASUREMENT TOOLS

Standards relating to structure, process and outcome need to be identified to provide a yardstick against which to measure the quality of care. Standards indicate how well aspects should be met. In many cases they are not specific enough to be used for measurement, and therefore, criteria need to be identified to reduce ambiguity. This can be done by using the tools as measurements. Standards only define structure, process and outcome. They are not evaluating the programme. There are different kinds of tools and data collection methods. For example, observation method, which entails checklist and audit, self-report, which may be interview or questionnaire and record reviews can be used to measure the quality of care.

3.6 EVALUATION, INTERPRETATION AND ACTION

'Interpreting the findings of a quality care evaluation is an essential component of the process. It allows for the identification of discrepancies between the quality care standards of the agency and the actual practice of the nurse or ether health providers' (Stanhope and Lancaster, 2000). The thrust is to understand how well a service is moving towards its objectives so that remedial action may be taken when things seem to be going well (Section 4.1). Regular intervals for evaluation should be established within the agency, and periodic reports should be written