

**THE DEVELOPMENT OF REGENERATION
AND TRANSFORMATION SYSTEMS
FOR *Eucalyptus* spp.**

by

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PREFACE

The experimental work described in this thesis was carried out in the Department of Biology, University of Natal, Durban, from January 1993 to December 1994, under the supervision of Drs Paula Watt and Sharmane MacRae.

These studies represent original work by the author and have not been submitted in any form to another university. Where use was made of the work of others it has been duly acknowledged in the text.



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ABSTRACT

In South Africa, *Eucalyptus* breeding programmes are aimed at the selection of fast-growing varieties, with appropriate wood characteristics and/or resistance to pests and diseases. However, the slow growth rate, long generation time and heterozygosity of trees make this a difficult task. Such problems may be overcome by the adoption of a biotechnological approach for plant propagation and modification. Towards this end, the aims of this investigation were to establish protocols for the micropropagation of *Eucalyptus grandis* and for the *Agrobacterium*-mediated transformation and subsequent plant regeneration of this important species.

The usefulness of transformed cells and/or tissues is dependent upon the availability of methods for their regeneration into plants. Consequently, methods for plant regeneration via indirect organogenesis from leaf discs and cell suspension cultures were investigated. Organogenic calli were produced from leaf explants on MS medium with 16 mg.l⁻¹ ferric citrate, 20 g.l⁻¹ sucrose, 1 mg.l⁻¹ NAA and 0.05 mg.l⁻¹ BA. Shoots were induced on MS medium containing 1 mg.l⁻¹ ZEA and 0.2 mg.l⁻¹ IAA, and subsequently rooted on medium containing MS nutrients supplemented with 1 mg.l⁻¹ IAA. Cell suspension cultures were established but not regenerated via indirect organogenesis. Additionally, various media were investigated in order to obtain somatic embryos from cell suspension cultures. The MS media supplemented with 30 g.l⁻¹ sucrose, 12 mg.l⁻¹ ABA and/or 40 g.l⁻¹ PEG were found to be most suitable, resulting in the production of embryoids; germination results are not available at this stage.

In order to establish methods for the transformation of both leaf discs and cell suspension cultures of *Eucalyptus*, a triparental mating was performed between *Escherichia coli* pJIT119 (donor), *A. tumefaciens* LBA4404 (recipient), and *E. coli* HB101::pRK2013 (helper), resulting in the transconjugant LBA4404 (pJIT119);

insertion of the pJIT119 plasmid was demonstrated using agarose gel electrophoresis. The transconjugant C58C1 (pMP90) (pJIT119) was also used. Protocols for the transformation of both leaf discs and cell suspension cultures were established, and resulted in the production of putatively transformed calli which were GUS positive and withstood selection on kanamycin ($50 \mu\text{g.ml}^{-1}$) and/or sulfadiazine ($50 \mu\text{g.ml}^{-1}$). Also, Southern blotting analysis indicated that the gene transfer process was successful. Due to difficulties in the regeneration of plants from transformed calli transgenic plants were not obtained.

Future research strategies and applications of the developed protocols to *Eucalyptus* breeding programmes are discussed.

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LIST OF ABBREVIATIONS

A	absorbance
ABA	abscisic acid
<i>als</i>	acetolactate synthase
<i>aroA</i>	gene encoding EPSP synthase
aug	augmentin
BA/BAP	6-benzylaminopurine
bp	base pair
BSA	bovine serum albumin
CaCl ₂	calcium chloride
CaMV	cauliflower mosaic virus
cef	cefotaxime
Ci	Curie
cm	centimetre
CP-TMV	coat protein gene of tobacco mosaic virus
CP-PVX	coat protein gene of potato virus X
CsCl	cesium chloride
CTAB	cetyltrimethylammonium bromide
°C	degrees Celsius
2,4-D	2,4-dichlorophenoxyacetic acid
DIC	differential interference contrast microscopy
DNA	deoxyribonucleic acid
EDTA	ethylene diaminetetracetic acid
EPSP synthase	3-phosphoshikimate-1-carboxyvinyl-transferase
f _{mass}	fresh mass
g	gravitational force
g	gram
GA ₃	giberellic acid
GC	GENECLEAN TM
<i>gus</i>	gene encoding -glucuronidase
GUS	β-glucuronidase
<i>Hm</i>	gene encoding HC-toxin reductase
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
<i>ipt</i>	isopentenyl transferase
KIN	kinetin
km/ <i>kan</i>	gene encoding resistance to kanamycin
kb	kilobases
l	litre
LA	Luria Bertani medium
M	molar (mole.l ⁻¹)
m	metre
mm	millimetre
MgCl ₂	magnesium chloride
min	minutes
mg	milligram
ml	millilitre
mM	millimolar
mmol	millimole
mol	mole
MS	Murashige and Skoog (1962) nutrient formulation
n	number of observations
NAA	1-naphthylacetic acid
NaCl	sodium chloride
<i>neo</i>	neomycin
ng	nanogram
nm	nanometre

nos	nopaline synthase
<i>nptII</i>	gene encoding neomycin phosphotransferase
OD	optical density
PEG	polyethylene glycol
pH	hydrogen ion concentration
Ph/Ch	phenol-chloroform
PPFD	photosynthetic photon flux density
PVP	polyvinylpyrrolidone
Ri	root-inducing
RGR	relative growth rate
RNA	ribonucleic acid
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
spp.	species (plural)
sul	sulfonamide
<i>sul</i>	gene encoding resistance to sulfonamides
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
T-DNA	transferred-DNA
TE	Tris EDTA
Ti	tumour-inducing
TP	transit peptide
Tween 20	polyoxyethylene sorbitan monolaurate
μ	micro
μCi	micro Curie
$\mu\text{E.m}^2.\text{s}^{-1}$	micro Einsteins per metre ² per second
μg	microgram
μl	microlitre
μm	micrometre
V	volts
v/v	volume per volume
<i>vir</i>	virulence
w/v	weight per volume
X-Gluc	5-bromo-4-chloro-3-indolyl- β -D-glucuronic acid
ZEA	zeatin
%	percent

CHAPTER 1: INTRODUCTION

1.1 Background

The growing demand for forest products throughout the world, including South Africa, makes it essential to find methods for increasing productivity, especially as land suitable for timber is becoming scarce (Van Wyk, 1985). Thus a priority exists to attempt to grow these species in marginal land that, until recently, has been considered inappropriate for their cultivation. For this reason, emphasis is being placed on the development of new techniques, as well as the application of existing ones, for the production of superior trees.

Eucalypts are regarded as one of the most productive forest crops, because of their rapid growth, useful products and wide adaptability (Turnbull and Boland, 1984). *Eucalyptus* species are grown mainly for the essential oils extracted from their leaves (Gupta and Mascarenhas, 1987), pulp wood used in the paper industry (Van Wyk, 1990; Le Roux and Van Staden, 1991b), and mining wood (Lakshmi Sita, 1986; Van Wyk, 1990; Watt *et al.*, 1991). The growth and distribution of eucalypt species worldwide, is limited mainly by climatic constraints such as temperature and water availability (Sommer and Wetzstein, 1984) and, in this country, inadequate rainfall to support good tree growth is the major factor limiting forestry expansion (Denison and Quaile, 1987). In South Africa, 11.55 % of the total land area is utilised for forest plantations (Anon, 1993) and 77 % of this area is under *Eucalyptus* plantation (Furze and Cresswell, 1985). Of this, *Eucalyptus grandis* is the most commonly grown eucalypt occupying approximately 29 % of all commercial forestry land (Graz and Von Gadow, 1990; Anon, 1993). In the 1991/1992 period 19.8 % of new afforestation in South Africa was achieved with *E. grandis* (Anon, 1993).

1.2 Propagation and improvement of *Eucalyptus* spp.

The ultimate goal of a plant breeder is to increase the frequency of desirable genes in a breeding population (Von Arnold *et al.*, 1990). In *Eucalyptus* breeding programmes, the priorities include selection for increased biomass by fast-growing trees (Gupta and Mascarenhas, 1987; Le Roux and Van Staden, 1991a) and selection of varieties which are disease and insect resistant (Grierson and Covey, 1984a; Von Arnold *et al.*, 1990), have appropriate wood characteristics and can grow in a wide variety of environmental conditions. Traditionally, propagation of *Eucalyptus* has been carried out from seed, but time to flowering is very long (5-7 years) and heterozygosity leads to great variation between trees (Lakshmi Sita, 1986). Hence, techniques of vegetative propagation, such as cuttings or graftings (Biondi and Thorpe, 1981; Libby, 1991) have been employed in clonal forestry programmes. However, by the time the tree has reached the age at which it can be successfully evaluated and selected, it has often passed the stage at which it can be propagated vegetatively (Furze and Cresswell, 1985; Gupta and Mascarenhas, 1987). Another problem is the loss of rooting capacity of cuttings of some species or clones (Biondi and Thorpe, 1981).

Clonal forestry programmes are based on the premise that land utilisation is more effective if plantations are planted with highly selected, phenotypically and genotypically uniform material. It involves the use of cuttings, which are rooted and multiplied vegetatively prior to field trials (Denison and Quaile, 1987). The clones that perform well are then used for commercial production. However, in order to commence production of *Eucalyptus* on a commercial scale, 10 000 plants are required, to be planted in a hedge (Denison and Kietzka, 1993). From a hedge of *E. grandis*, an average of 12 - 17 plants are produced per parent every 4 weeks, and the average rooting efficiency is 60 % (B. Herman, pers. comm.). Thus, it takes 3 years to produce the required number of plants to begin commercial production (B. Herman, pers. comm.).

With the advent of biotechnology, and in particular *in vitro* micropropagation, it was realised that these new techniques had the potential to improve the speed of propagation of selected clones. Below is a discussion of some of the areas of biotechnology, namely micropropagation and recombinant DNA technology, which have already proven to be, or have potential in the propagation and improvement of forest species, such as *Eucalyptus*.

1.3 Micropropagation of *Eucalyptus* spp.

Micropropagation is a process that involves the multiplication and maintenance of selected genotypes *in vitro* under sterile conditions. It has been widely used for agricultural crops, and has more recently been applied to forest species. The available protocols for the micropropagation of *E. grandis* were reviewed extensively by Le Roux and Van Staden (1991b). Subsequent reports have included the establishment of protocols for plant regeneration via indirect somatic embryogenesis from leaf explants (Watt *et al.*, 1991), the production and maintenance of cell suspension cultures of *E. grandis* (Blakeway *et al.*, 1993), as well as the recent report on the micropropagation and establishment in soil of *E. grandis* hybrids (Jones and Van Staden, 1994). When this investigation was initiated, there were no protocols available for the regeneration of *E. grandis* via indirect organogenesis. Recently, however, Lainé and David (1994) have published the methodology for plantlet regeneration of mature *E. grandis* via this route. Also, despite the availability of methods for the production and maintenance of cell suspension cultures of *E. grandis* (Blakeway *et al.*, 1993), methods for plantlet regeneration from cell suspension cultures have not yet been reported.

The *in vitro* multiplication of forest species, such as *Eucalyptus*, offers the potential to reduce the time required to introduce a clone into commercial production (Denison and Kietzka, 1993). Work at Mondi Forests (Hilton, South Africa) has shown that, with regard to *E. grandis*, the the time taken to produce the 10 000 plants required for commercial production (Section 1.2) can be reduced from 3 years, when using

cuttings, to 6 - 8 months using methods of tissue culture. Furthermore, not only is the propagation time reduced, but the performance of the plants produced *in vitro* are enhanced. Field trials have shown that, after 2 years, the tissue culture-produced plants outperform the seedlings produced from cuttings with respect to both survival and height (Hope *et al.*, 1994).

1.4 Recombinant DNA technology and its applicability to plant gene transfer systems

Chopra and Sharma (1991) have described the entire living world as one gene pool, where there are theoretically no limits to the possibility of creating desired gene assemblies. This was made possible through the development of recombinant DNA technology. Using these technologies, new techniques have been developed to circumvent the problems of conventional breeding and have made it possible to transfer genes among different species, and even different organisms (Jones and Lindsey, 1988; Von Arnold *et al.*, 1990; Chopra and Sharma, 1991; Christou, 1991; Gasser and Fraley, 1992; De Block, 1993). The unprecedented flexibility of the source of the coding region that is used for the genetic modification of plants, is the main advantage of genetic engineering over more traditional methods (Gasser and Fraley, 1992).

The advent of recombinant DNA technology has led to the possibility of plant cell transformation, which is the incorporation of specific and useful pieces of exogenous DNA into the plant genome (Bright *et al.*, 1986; Gasser and Fraley, 1992). It is now technically possible to identify, isolate, modify, transfer and obtain expression of a number of specific genes in a target crop species (Jones and Lindsey, 1988; Christou, 1991). Several methods exist whereby this DNA transfer can be achieved. These techniques all have as a common base the requirement, at least to a certain degree, of a tissue culture approach, while they differ mainly in the manner in which the DNA is delivered to the plant cell (De Block, 1993).

The approaches to the genetic modification of plant species range from *Agrobacterium*-mediated transformation (Grierson and Covey, 1984b; Mantell *et al.*, 1985a; Uchimiya *et al.*, 1989; Fillatti, 1990; Zambryski, 1992) to direct gene transfer techniques (Davey *et al.*, 1989; Fillatti, 1990; Lee *et al.*, 1991) such as electroporation (Lindsey and Jones, 1990b; Potrykus, 1991; Sawahel and Covey, 1992; Van Wert and Saunders, 1992) and microprojectile bombardment (Sanford, 1990; Christou *et al.*, 1991; Batty and Evans, 1992; Christou, 1993; Yang *et al.*, 1993). *Agrobacterium*-mediated transformation is the most commonly used technique, especially for the insertion of foreign DNA into dicotyledonous plant species (Fillatti, 1990), because it is simple, efficient, inexpensive and usually DNA insertion is a precise event (De Block, 1993). Chriqui *et al.* (1991) have successfully transformed *E. globulus* and *E. gunnii* using *Agrobacterium*, however this has not been reported for *E. grandis*, the species of interest in this study.

Although transformation technology has reached a high level of refinement, there are still many unknowns which can interfere with the generation of stable transformed plants which express the transgene in the expected way (De Block, 1993). One such example is the fact that, while few data are available, it seems that the most regenerable cell type is not necessarily the most transformable cell type (Colby *et al.*, 1991). This could be the reason why regeneration is often the limiting step in transgenic plant production (Wilde *et al.*, 1992), since individual cells are transformed, and these cells are of no practical use unless methods exist for their regeneration into plants. This problem is particularly profound in forest species, because regeneration of plants have been especially difficult to achieve with these species (Riemenschneider *et al.*, 1987).

Once systems for the efficient regeneration of plants from transformed cells and/or tissues have been established, it is important to investigate whether the acquired traits are likely to be transferred to progeny of the transgenic plant. Reported instances for

herbaceous crops indicate that most genes are stably inherited and show Mendelian monogenic segregation (Uchimiya *et al.*, 1989), but this remains to be demonstrated in woody species.

Although gene transfer can be routinely accomplished in many species (Uchimiya *et al.*, 1989) (particularly herbaceous crops), the present methods of gene delivery for higher plants offer the experimentalist no control over the gene insertion site, and little control over the number of copies of the recombinant gene (Goodman, 1990). However, by choosing various promoters, researchers can now target gene expression to specific organs and, in many cases, to specific cell types within these complex tissues (Gasser and Fraley, 1992). This has not, however, been reported in woody species.

1.5 Applications of biotechnology to forestry

The application of biotechnological tools in crop improvement programmes can be effective in different, complementary ways. These include speeding up the processes of conventional breeding via micropropagation of selected genotypes, creating variability through tissue culture and evolving novel genotypes through recombinant DNA technology (Chopra and Sharma, 1991). In addition, biotechnology can decrease the costs of what has traditionally been an energy and labour intensive forestry industry (Christou, 1991). There is a considerable potential for improvement in forestry, which, because of the long life cycle of trees, has not benefited from breeding to the same extent as annual and short-lived perennial crops (Riemenschneider *et al.*, 1987; Krugman, 1988; Hull, 1991). Thus the application of biotechnology may accelerate the genetic improvement process of economically important forest species (Riemenschneider *et al.*, 1987; Krugman, 1988). In light of the long-time scales involved in the generation of forestry products, it is important to focus on targets that are worthwhile pursuing commercially (Schuch, 1991). However, in order to successfully apply the relevant techniques of genetic engineering

to the improvement of woody species, the identification and isolation of potentially useful genes for forestry is necessary (Christou, 1991). These genes ultimately need to be recovered in mature plants which can then be used in a breeding programme. To date, there are very few examples in which a crop plant has been produced which possesses a stably modified economically, or potentially economically, useful phenotype (Jones and Lindsey, 1988). This is because applied biotechnological research has focused on simple traits in which the transfer of a single gene may lead to the desired target effect, such as herbicide resistance (Schuch, 1991). However, most traits that are economically important for the forestry industry, such as enhanced vigour or increased yield, are multigenic traits (De Block, 1993). In this regard, the genes involved in lignin biosynthesis are being investigated (Dean and Eriksson, 1992). Those authors postulate that as knowledge in this area develops, it will be possible to reconstruct trees which bear more fruit, yield better timber, and provide tailor-made fibres, which will obviously have profound effects in the forestry industry.

The greatest indirect benefit of using biotechnology may simply be the resulting general advances in many types of basic knowledge (Riemenschneider *et al.*, 1987). For example, the ability to introduce foreign genes into plants will provide new insight into the molecular basis of the regulation and function of plant genes. In this way, it will be possible to study the regulation of genes expressed during tree development, and their subsequent contribution to the development of the tree and the quality of the resulting product. This will ultimately result in the ability to modify the tree crop using biotechnology (Ahuja, 1987b).

1.6 Aims of this investigation

If biotechnology is to be of use in the forestry industry, the availability of methods for consistent plantlet regeneration, as well as the adaptation of existing methods for genetic modification, are required for the species of interest. In this study, the first objective was the development of protocols for the production of plantlets of *Eucalyptus* via indirect organogenesis from leaf discs and cell suspension cultures (Section 2.2.5), and via indirect somatic embryogenesis from cell suspension cultures (Section 2.2.6). The production of transgenic plants, using the established techniques of plant regeneration, were the subsequent aims of this investigation. Since *Agrobacterium*-mediated transformation is the most commonly used method for gene transfer in dicotyledonous plant species, it was the method of choice in this investigation. In order to establish methods for *Agrobacterium*-mediated transformation of *Eucalyptus*, the production of an appropriate vector was necessary, and was undertaken by performing a triparental mating (Section 3.2.2). Protocols for the transformation of both leaf discs and cell suspension cultures were then investigated using the *Agrobacterium* strains C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119) (Section 3.2.3). Analysis of transient expression and stable integration of the inserted genes were also undertaken.

CHAPTER 2: ESTABLISHMENT OF *In Vitro* CULTURE SYSTEMS FOR *Eucalyptus*

2.1 Introductory remarks and literature review

2.1.1 General aspects of *in vitro* cell and tissue culture

The ability of an individual cell to grow and divide in an autonomous manner relies on the phenomenon of totipotency whereby an individual cell, given the appropriate conditions, can regenerate into a whole organism (Mantell *et al.*, 1985b; Duncan and Widholm, 1986; Lindsey and Jones, 1990a; Allan, 1991). This process results in a large number of 'clones' being produced from a small explant of parent tissue. The initiation of organised development is a complex phenomenon, influenced by both extrinsic and intrinsic factors (Thorpe, 1983; Warren, 1991). Although much is known about the manipulation of extrinsic factors such as culture medium composition, knowledge about regulation at the molecular level is still lacking.

2.1.1.1 Routes of differentiation

Plant regeneration *in vitro* can occur via two routes: (1) organogenesis, the production of shoots followed by root formation or (2) somatic embryogenesis, the formation of fully formed embryos, which can be induced to germinate (Evans *et al.*, 1981; Thorpe, 1983; Ammirato, 1986). Either of these processes can occur directly from the explant or indirectly via a callus stage. Callus is a mass of undifferentiated cells (Constabel, 1984; Duncan and Widholm, 1986; Collin and Dix, 1990; Allan, 1991), which is formed by aseptically transferring a sterile explant onto a nutrient medium supplemented with plant growth regulators. It consists of a mass of tissue with a low level of organisation (Constabel, 1984; Collin and Dix, 1990; Allan, 1991), and can theoretically be maintained indefinitely by routine subculture onto fresh medium (Collin and Dix, 1990; Allan, 1991). By manipulating the medium, whole plants can

be regenerated from the cultured cells (Duncan and Widholm, 1986) by either embryogenesis or organogenesis.

2.1.1.2 Culture systems

There are numerous culture systems which are available for the *in vitro* propagation of plant tissues, but only those relevant to this study will be mentioned here.

One of the most successful methods of micropropagation of many dicotyledonous species uses the direct organogenic route via axillary bud proliferation. The steps involved are leaf multiplication, shoot elongation and plantlet rooting (Evans *et al.*, 1981; Flick *et al.*, 1983; Thorpe, 1983; Ammirato, 1986; Christianson, 1987) and, because of the resulting high yields and production of true-to-type clones (Nashar, 1989), this method is used in large-scale micropropagation of many horticultural (Jones, 1992; Yeoman, 1986), agricultural (Conger, 1981; Yeoman, 1986) and forest (Biondi and Thorpe, 1981; Sommer and Caldas, 1981; Durzan, 1988) species.

One of the most commonly used culture systems is callus. As has been mentioned in Section 2.1.1.1, callus formation occurs at a cut surface of a piece of tissue, such as a piece of leaf material, and results in the production of a mass of undifferentiated cells. Callus can be induced, in the presence of suitable concentrations of the correct plant growth regulator ratios, to form plantlets via organogenesis (Flick *et al.*, 1983; Christianson, 1987) or embryogenesis (Ammirato, 1983; Ammirato, 1987; Komamine *et al.*, 1990), or can be manipulated to form cell suspension cultures (King, 1984; Lindsey and Jones, 1990a).

Cell suspension cultures are produced when friable calli are introduced into a liquid medium and agitation causes the cells to disperse throughout the liquid (Dodds and Roberts, 1985a; Collin and Dix, 1990; Allan, 1991). Suspension cultures grow faster than callus cultures and require regular subculture for maintenance of viability

(Schröder *et al.*, 1989). Cell suspension cultures can be induced to regenerate into plantlets (Park and Son, 1988; Binh *et al.*, 1992; Levi and Sink, 1992; Qiao *et al.*, 1992; Teng *et al.*, 1992; Sharma *et al.*, 1993) or can be used to produce callus (Bergmann, 1977; Caboche, 1980; Blakeway *et al.*, 1993).

Other specific culture systems for plant regeneration include the production and culture of protoplasts (Vasil *et al.*, 1990; Sidikou-Seyni *et al.*, 1992; Scarpa *et al.*, 1993; Peeters *et al.*, 1994; Wang and Lörz, 1994), anthers (Hu and Zeng, 1984; Büter *et al.*, 1993; Pugliesi *et al.*, 1993; Maximova and Kolova, 1994; Ormerod and Caligari, 1994; Thengane *et al.*, 1994), pollen grains (Dodds and Roberts, 1985b; Yeoman, 1986), ovaries (Agrawal and Gebhardt, 1994) and inflorescences (Mohamed-Yasseen *et al.*, 1993; Balan, 1994; Kalia and Crisp, 1994; Teixeira *et al.*, 1994; Verdeil *et al.*, 1994).

2.1.2 Factors affecting *in vitro* culture systems

2.1.2.1 Choice and preparation of explant

The major requirements for effective explant tissue are a high cell division potential and morphogenetic plasticity (Warren, 1991). These criteria are usually met by meristematic or rapidly growing tissues, since mature tissues tend to be morphogenetically 'determined' (Warren, 1991) and are not very susceptible to dedifferentiation (Durzan, 1984). Callus will usually develop from any explant, although the choice of explant depends on the requirements of the research objective (Allan, 1991). Plant parts that have been used as explants include nodal segments (Cortezzi Graça and Mendes, 1989; Blomstedt *et al.*, 1991; Bhat *et al.*, 1992), root sections (Sood, 1994), leaf pieces (Bolyard *et al.*, 1991; Jäger *et al.*, 1993), inflorescences (Chen *et al.*, 1985; Vasil *et al.*, 1990; Mohamed-Yasseen *et al.*, 1993; Kalia and Crisp, 1994; Teixeira *et al.*, 1994), pollen (Dodds and Roberts, 1985b; Yeoman, 1986), cotyledons (Jang and Tainter, 1991; Griga, 1993; Pugliesi *et al.*, 1993), hypocotyls (Scarpa *et al.*, 1993; Lee *et al.*, 1994) and zygotic embryos (Cao *et*

al., 1992; Guo and Liang, 1993; Kaeppler and Phillips, 1993). Factors such as tissue source, age of organ, season in which the explant was obtained, explant size and quality of explant source also affect reproducibility of experiments and require consideration (Thorpe, 1980; Warren, 1991). After the explant has been chosen, it needs to be surface sterilised, if not already sterile, and placed onto the required medium (Thorpe, 1980; Allan, 1991). Explant orientation and contact with the medium may also be important factors to be considered (McClelland and Smith, 1990; Allan, 1991; Warren, 1991).

2.1.2.2 Maintenance of aseptic cultures

Surface sterilisation procedures with domestic disinfectants (such as sodium hypochlorite) are usually sufficient to eliminate surface contaminants. Despite these procedures, media contamination does still occur, often due to endogenous contaminants in the plant tissues (Gordon and Brown, 1988; Warren, 1991). The use of antimicrobial agents may overcome these problems, as long as they are broad-spectrum (or specific to the particular contaminant) and are non-toxic to the plant material. The effectiveness of various antibiotics has been investigated and is well-documented (see Kneifel and Leonhardt, 1992, for a review). Recently, the use of microbial culture filtrates have been implicated in the control of contaminants in plant tissue culture systems (Hussain *et al.*, 1994). Maintenance of aseptic conditions obviously requires sterile culture manipulations, such as autoclaving of media and utensils, as well as performing culture techniques in a laminar flow hood. Aseptic techniques have been reviewed by Dodds and Roberts (1985d).

2.1.2.3 Culture environment

Understanding the various factors involved in the control of plant growth *in vitro* can greatly improve the quality of micropropagated plants and prevent abnormalities that result in low survival rates *ex vitro* (Ziv, 1991a; Debergh *et al.*, 1992). There are many factors that require consideration when *in vitro* culture systems are undertaken.

and no factor operates in isolation. However, the interaction between these factors is not well understood and, therefore, in this review the factors will be considered separately.

Chemical conditions

Numerous media formulations for the culture of various plant species have been formulated and published. Plant cell culture media are based on mixtures of many components, combining all essential inorganic and organic elements, a carbon source and appropriate growth regulators. The most common media are Murashige and Skoog (MS) (Murashige and Skoog, 1962), Gamborg's B5 medium (Gamborg *et al.*, 1968), White's medium (White, 1943) and Woody Plant Medium (WPM) (Lloyd and McCown, 1980). Other commonly used media for woody plant cell culture are described by McCown and Sellmer (1987).

Many plants will grow on a wide range of media, but others require specific media formulations and additives. Most media are well-defined, but non-defined complex components such as coconut milk and casein hydrolysate are sometimes required or are beneficial (Thorpe, 1980; Ammirato, 1986; Allan, 1991). The effects of the concentrations and forms of carbohydrates and nitrogen, which are vital to any culture system, have been reviewed by Thompson and Thorpe (1987) and Kirby *et al.* (1987), respectively. Carbon is vital, since most culture systems are heterotrophic, and is usually provided in the form of sucrose, although glucose and other sugars are sometimes used to supplement the sucrose (Atanassov and Brown, 1984; David *et al.*, 1984; Spangenberg *et al.*, 1986; Sundberg and Glimelius, 1986; Watt *et al.*, 1991). Nitrogen is generally supplied in the form of nitrates or ammonia, usually in combination, while amino acid mixtures have also been used (Ammirato, 1986).

Plant growth regulators play an important role in the induction and control of morphogenesis (Ammirato, 1986). Each type of plant growth regulator has a wide

range of physiological effects in different plants and even in different plant parts, depending on, amongst other factors, the presence or absence of other plant growth regulators, genetic make-up and physiological status of the target tissue (Minocha, 1987). Plant growth regulators do not act in isolation, but critical balances must be maintained. The sequential application of different plant growth regulators may also be important (Ammirato, 1986), but the type of regeneration that is being attempted will determine the optimum plant growth regulator regime to adopt (Warren, 1991). In this regard, the importance of phytohormones or plant growth regulators such as abscisic acid (ABA), kinetin (KIN), zeatin (ZEA), indole-acetic acid (IAA), naphthylacetic acid (NAA) and benzyl aminopurine (BAP) for the initiation and regulation of morphogenesis has been reviewed extensively (Thorpe, 1983; Ammirato, 1986; Minocha, 1987; Reynolds, 1987).

The balance between auxins (IAA, NAA) and cytokinins (BAP, ZEA, KIN) determines whether unorganised growth ensues or shoots and roots develop (Ammirato, 1986). In general, high auxin concentrations suppress organised growth, while the ratio between auxin and cytokinin influences the balance between root and shoot growth, with cytokinins inhibiting rhizogenesis (root formation) (Warren, 1991).

Minocha (1987) comments that the difficulty of interpreting experimental results obtained from plant cell culture arises from the fact that the chemical composition of the medium changes significantly within hours or days of the initial transfer of cells to the medium. These changes involve, among others, the change in pH of the medium. The pH is usually set between 5.0 and 6.0 during medium preparation, but pH drifts occur during autoclaving and culture (Thorpe, 1980; Wetzstein *et al.*, 1994). Culture medium pH affects nutrient availability and uptake and has been shown to influence a number of plant developmental processes *in vitro* (Minocha, 1987). However, not much is known about the influence of actual pH values of the medium on organised

development *in vitro*, other than the fact that certain tissues have definite pH preferences.

Another problem frequently encountered in the initial culture stages is browning and eventual death due to the excessive production of polyphenols (Tulecke, 1987). This problem can sometimes be overcome by the incorporation of adsorbents into the medium such as activated charcoal and polyvinylpyrrolidone (PVP) or anti-oxidants such as ascorbic acid (Tulecke, 1987; Warren, 1991). Activated charcoal has frequently improved cultures by absorption of growth inhibitors and media components such as endogenous phytohormones and/or residual plant growth regulators and vitamins that are no longer required in the medium (Constantin *et al.*, 1977; Ammirato, 1986; Warren, 1991; Druart and De Wulf, 1993). However, because activated charcoal catalyses the hydrolysis of sucrose during autoclaving, it is necessary to autoclave activated charcoal and sucrose separately (Druart and De Wulf, 1993).

Physical conditions

An appreciation of the effect that various components of the physical environment have on morphogenesis has gradually been emerging, but less emphasis has been placed on this area than on the effects of the chemical environment (Hughes, 1981; Warren, 1991).

As may be expected, light has a strong effect on plant morphogenesis *in vitro*. Not only is the presence or absence of light important, but also the photoperiod, light intensity, light quality and wavelength (Ammirato, 1986; Thorpe, 1980; Kozai, 1991). The radiant energy requirements are different for tissue cultures than for autotrophic plants, since the former are supplied with a carbohydrate source (usually sucrose). However, the light that may be required for certain photomorphogenic events (Thorpe, 1980) of the tissue cultured material is often not considered. The spatial arrangement

of light sources may also be important since lateral illumination tends to promote better plant growth and give better plant shape due to the evenness of the light interception by the plant (Kozai, 1991).

Temperature effects have not been thoroughly evaluated and general practice has been to maintain cultures at a constant temperature of approximately 25 °C (Thorpe, 1980). Kozai (1991) has shown that there is a temperature fluctuation of about 1 °C between the light and dark periods in culture, and that the dry and fresh weights of plants are not affected by this. It must be understood that the range of suitable temperatures and the temperature optimum will vary from plant to plant (Hughes, 1981; Ammirato, 1986). For example, the temperature for optimal performance of temperate and tropical species will be expected to differ considerably, and must be taken into account.

The manner in which cultures are grown can also markedly affect morphogenesis (McClelland and Smith, 1990). This includes the nature of the gelling agent used (Gorinova *et al.*, 1993), the culture vessel (McClelland and Smith, 1990; Kozai, 1991), the relative humidity within the vessel (Debergh *et al.*, 1992) and the gaseous exchange between the vessel and the air outside (Kozai, 1991). All these factors are important for plant growth and development.

Agar and gelatine are the most commonly-used gelling agents (Gorinova *et al.*, 1993), but the use of other novel gelling agents have been investigated in order to find a reasonably low-cost gelling agent for industrial use (Bhattacharya *et al.*, 1994). However, various workers, have reported recently that the optimum gelling agent seems to depend on the plant species in question. For example, microcrystal cellulose seems to be better than other gelling agents for *Nicotiana tabacum* cultures (Gorinova *et al.*, 1993) and *Eucalyptus grandis* shoot cultures grown on Gelrite were superior to those grown on other solidifying agents (MacRae and Van Staden, 1990). For some

applications, the use of liquid cultures is advantageous, since cells are evenly exposed to nutrients and hormones. However, although this allows for precise manipulations and control of development, cultures in solid media are generally easier and cheaper to maintain (Ammirato, 1986).

The type of culture vessel and vessel closure affects the gaseous composition and the light environment and hence vitrification and plant growth *in vitro* (McClelland and Smith, 1990; Kozai, 1991). For tissue culture, closed containers are used to avoid contamination by micro-organisms. As a result, the composition of the head space in the culture vessel is different from the ambient conditions in terms of relative humidity and carbon dioxide content. The high relative humidity is the major driving force behind the phenomenon of vitrification or glassiness (Debergh *et al.*, 1992), which is a physiological disorder frequently affecting woody plants in culture. Gaspar *et al.* (1987) describe vitrified leaves as thick and wrinkled or curled and brittle, while such stems are broad, thick and translucent. The poor growth of vitrified shoots is accompanied by low rates of multiplication, rooting and survival on transfer to soil: they wilt quickly and are very susceptible to infections (Gaspar *et al.*, 1987). The occurrence of vitrification is random and thus practical strategies for its avoidance are important.

Different methods have been proposed to lower the relative humidity and thus the occurrence of vitrification, such as increasing the agar concentration. However, the gelling agent also affects the availability of water and dissolved substances (Ziv, 1991a). In this way, an increase in agar concentration can reduce vitrification, but it may also lower the propagation rate (von Arnold and Eriksson, 1984). Other methods of lowering the relative humidity include overlaying the medium with a layer of lanolin or paraffin (Wardle *et al.*, 1983) or by using loosely capped culture vessels (Dillen and Buysens, 1989).

2.1.2.4 Consequences of tissue culture - variability and instability

It is now widely accepted that plants regenerated from somatic cells *in vitro* can vary in phenotype and genotype (Bright *et al.*, 1986), due to the phenomenon of somaclonal variation proposed by Larkin and Scowcroft (1981). Somaclonal variation is variation that arises from genetic instabilities during the *in vitro* culture process, and which are thought to arise from stresses imposed on cells during culture (Cheliak and Klimaszewska, 1990). The observed somaclonal variation and thus instability seems to increase with a decrease in the level of organisation of a culture, and has been found in essentially all plant species that have been regenerated via *in vitro* culture systems (Scowcroft and Larkin, 1988; Antonetti and Pinon, 1993).

The precise cause of somaclonal variation is not known, but several factors may be involved. Changes in chromosome number and structure (Bright *et al.*, 1986), changes in ploidy (Larkin and Scowcroft, 1981) and chromosome rearrangements (Scowcroft and Larkin, 1988) are some of these factors. The extent of somaclonal variation may be affected by age of the plant, explant source, regeneration pathway, medium composition, cultural conditions and length of time in culture, or combinations of these factors (Ahuja, 1987a; Marks and Myers, 1994). Somaclonal variation is undesirable if clonal fidelity is required, but it may also be regarded as a new source of genetic variability for plant improvement (Evans and Sharp, 1986), when rapid plant screening and *in vitro* selection procedures are available (Bright *et al.*, 1986; Widholm, 1988; Collin and Dix, 1990).

2.1.3 Hardening off of regenerated plants

The clonal propagation of several plant species may be achieved through explant establishment, rapid shoot multiplication and development, and finally acclimatisation or hardening off, followed by establishment of plants in the field (Murashige, 1974). The ultimate success of micropropagation depends on the ability to transfer plants out of culture on a large scale, with high survival rates and good performance (Bhojwani

and Dhawan, 1989). Successful acclimatisation of micropropagated plants is influenced by both the conditions during the propagation stage and the conditions during the rooting and acclimatisation phases (Van Telgen *et al.*, 1992). Acclimatisation or hardening off *in vitro* involves the exposure of plants to reduced relative humidity and an environment which will allow the shoot system to acclimatise to normal growth conditions (Ziv, 1986). In the laboratory, this is usually achieved by covering the potted-out plants with plastic bags and either removing the bags for progressively longer periods or by punching an increasing number of holes in the bags (Warren, 1991). On a commercial scale, mist houses are generally used for acclimatisation.

Inevitably, *in vitro* culture conditions which promote rapid growth and maximum shoot proliferation often result in the formation of structurally and physiologically abnormal plants, which do not survive *ex vitro* (Van Telgen *et al.*, 1992). This is often due to the formation of aberrations characterised by abnormal leaf morphology, altered mesophyll structure, malfunctioning stomata and a marked reduction in cuticular waxes (Ziv, 1986; Bhojwani and Dhawan, 1989), which cause desiccation during plant transfer from culture. This emphasises the importance of hardening off or acclimatisation *in vitro* for plant survival *ex vitro*. Other factors that can improve the performance of cultures *in vitro* and may affect survival and performance after planting in soil have been discussed in Section 2.1.2.3.

2.1.4 Micropropagation in forestry

The development of new micropropagation techniques and the perfection of those already in use, is an important area of consideration in the potential application of these techniques to forestry (Yeoman, 1986). As the world demand for wood and its derivatives is increasing, there is an urgent need for the production of large numbers of improved, fast-growing trees (Biondi and Thorpe, 1981; Mascarenhas and Muralidharan, 1989). The potential benefits of micropropagation of elite genotypes

for production of clonal planting stock for afforestation or reforestation have long been recognised (Vasil, 1990; Gupta *et al.*, 1991; Denison and Kietzka, 1993). Integration of plant tissue culture methods with established breeding practices is therefore now regarded as a powerful tool for plant improvement (Smith and Drew, 1990). An area which has so far been neglected in the literature, but which is essential to the success of such an endeavour, is the replicated field testing of tissue culture-derived plants to determine the effectiveness of the micropropagation system (Gupta *et al.*, 1983; Gupta *et al.*, 1991; Hammatt, 1992; Mullin and Park, 1992; Rockwood and Warrag, 1994).

2.1.4.1 *In vitro* propagation of woody species

Most of the work on micropropagation has centred on the agronomically important crop species and the *in vitro* propagation of woody species has lagged behind them (Rao and Lee, 1986). The work that has been undertaken on woody plants has generally been on those species that are economically important, particularly those forestry species that are found in the Northern hemisphere (Table 2.1). Although the genera of these trees may be the same as those found in the Southern hemisphere, it does not mean that the established protocols will be directly applicable, since species and even varietal differences may affect the responsiveness in culture (Ammirato, 1986). Generally, organogenesis is the most reliable and therefore the most commonly used technique for the regeneration of forest species *in vitro* (Table 2.1) (Haissig, 1989). However, despite successful plantlet regeneration in some woody species (Table 2.1), only a few have been successfully transferred to soil and established in the field (Howard *et al.*, 1989; Jones and Hadlow, 1989; Mascarenhas and Muralidharan, 1989; Kristiansen, 1991; Zimmerman and Miller, 1991), and this an area of research that needs to be expanded. Some of the recent reports (1987 - 1994) on the *in vitro* propagation of woody species are outlined in Table 2.1.

Table 2.1: Recently (1987-1994) published studies on the propagation of some woody plants using *in vitro* techniques.

Species	Explant	Culture system	Results	Reference
<i>Abies fraseri</i>	cotyledon, hypocotyls	organogenesis	plantlets	Saravitz and Blazich, 1991
<i>Dalbergia latifolia</i>	nodal explants	organogenesis	plants	Raghava Swamy <i>et al.</i> , 1992
<i>Euonymus europaeus</i>	embryos	somatic embryogenesis	plants	Bonneau <i>et al.</i> , 1994
<i>Hevea brasiliensis</i>	immature seeds	somatic embryogenesis	plants	Etienne <i>et al.</i> , 1993
<i>Fraxinus excelsior</i>	cotyledonary node	organogenesis	plants	Hammatt and Ridout, 1992
<i>Morus bombycis</i>	internodal segments	organogenesis	shoots	Jain and Datta, 1992
<i>Mussaenda erythrophylla</i>	stem segments	somatic embryogenesis	plants	Das <i>et al.</i> , 1993
<i>Picea abies</i>	embryos	organogenesis	rooted shoots	von Arnold and Hakman, 1987
<i>Picea glauca</i>	embryos	somatic embryogenesis	embryogenic tissue	Park <i>et al.</i> , 1993
<i>Picea glauca</i>	suspension cultures	somatic embryogenesis	somatic embryos	Dunstan <i>et al.</i> , 1993
<i>Picea mariana</i>	embryonic shoots	organogenesis	plantlets	Ho, 1989
<i>Picea pungens</i>	embryos	somatic embryogenesis	plantlets	Afele <i>et al.</i> , 1992
<i>Pinus echinata</i>	cotyledons	organogenesis	plantlets	Jang and Tainter, 1991
<i>Pinus elliotii</i>	cotyledons	organogenesis	plants	Lesney <i>et al.</i> , 1987
<i>Pinus sylvestris</i>	axillary buds	organogenesis	rooted shoots	Zel <i>et al.</i> , 1988
<i>Pinus virginiana</i>	cotyledons	organogenesis	plantlets	Jang and Tainter, 1991
<i>Populus tremula</i>	buds	organogenesis	plants	Mandal, 1989
<i>Quercus acutissima</i>	embryos	somatic embryogenesis	plants	Kim <i>et al.</i> , 1994
<i>Quercus suber</i>	embryos	somatic embryogenesis	somatic embryos	Manzanera <i>et al.</i> , 1993
<i>Robinia pseudoacacia</i>	seeds/embryos	somatic embryogenesis	plants	Arrillaga <i>et al.</i> , 1994
<i>Tecomella undulata</i>	nodal segments	organogenesis	plants	Rathore <i>et al.</i> , 1991
<i>Tecomella undulata</i>	axillary buds	organogenesis	rooted shoots	Bhansali, 1993
<i>Ulmus americana</i>	leaves	organogenesis	plantlets	Bolyard <i>et al.</i> , 1991
<i>Ulmus parvifolia</i>	leaves	organogenesis	shoots	Bolyard <i>et al.</i> , 1991

2.1.4.2 *In vitro* propagation of *Eucalyptus* spp.

As mentioned previously, hardwood species, such as *Eucalyptus*, have a worldwide distribution, limited primarily by temperature and water restraints (Sommer and Wetzstein, 1984). In order to extend the *Eucalyptus* culture area specific traits, such as cold resistance, are being investigated through conventional breeding techniques and biotechnological approaches (Teulières and Boudet, 1991; Denison and Kietzka, 1993). As the breeding of *Eucalyptus* is a slow and difficult process because of the long generation time and the problem of carrying out controlled crosses in large numbers (Gupta and Mascarenhas, 1987), a number of attempts have been made to micropropagate *Eucalyptus* species (Table 2.2).

From the information presented in Table 2.2, micropropagation of *Eucalyptus* species have been most successful via the route of organogenesis, with very few reports on the establishment of protocols for *Eucalyptus* regeneration via embryogenesis. In a review on the field performance of micropropagated forest species, Gupta *et al.* (1991) state that micropropagated plants of various *Eucalyptus* species that had been established in the field showed more uniform growth than did plants raised from seed collected from the same trees.

Table 2.2. Recently (1987-1992) published papers on the *in vitro* culture of *Eucalyptus* species other than *Eucalyptus grandis*.

Species	Explant	Culture system	Reference
<i>E. camaldulensis</i>	nodal segments + buds	organogenesis	Gupta and Mascarenhas, 1987
	leaves	organogenesis	Muralidharan and Mascarenhas, 1987
<i>E. citriodora</i>	embryo	somatic embryogenesis	Muralidharan and Mascarenhas, 1987
	somatic embryos	somatic embryogenesis	Muralidharan <i>et al.</i> , 1989
<i>E. dunnii</i>	nodal segments	organogenesis	Cortezzi Graça and Mendes, 1989
<i>E. globulus</i>	nodal segments + buds	organogenesis	Gupta and Mascarenhas, 1987
<i>E. macarthurii</i>	nodal explants	organogenesis	Le Roux and Van Staden, 1991a
<i>E. radiata</i>	epicormic shoots	organogenesis	Donald and Newton, 1991
<i>E. regnans</i>	nodal explants	organogenesis	Blomstedt <i>et al.</i> , 1991
<i>E. saligna</i>	nodal explants	organogenesis	Le Roux and Van Staden, 1991a
<i>E. sideroxylon</i>	nodal explant	organogenesis	Burger, 1987
	axillary shoots	organogenesis	Cheng <i>et al.</i> , 1992
<i>E. smithii</i>	nodal explants	organogenesis	Le Roux and Van Staden, 1991a
<i>E. tereticornis</i>	nodal segments	organogenesis	Das and Mitra, 1990
	nodal segments + buds	organogenesis	Gupta and Mascarenhas, 1987
<i>E. torrelliana</i>	nodal segments + buds	organogenesis	Gupta and Mascarenhas, 1987
<i>E. viminalis</i>	nodal segments	organogenesis	Wiecheteck <i>et al.</i> , 1989

2.2 MATERIALS AND METHODS

2.2.1 Plant material

Seeds of *Eucalyptus grandis* S/N M6 (Mondi Forests, S.A.) were used to produce aseptic plant material for initiation of cultures, except cell suspension cultures for which shoots of *E. grandis* x *camaldulensis* were used (Blakeway *et al.*, 1993).

2.2.2 Seed germination

Seeds of *E. grandis* were separated from the husks using a dissecting microscope. They were then surface sterilised for 15 minutes in 3.5 % sodium hypochlorite containing 0.2 ml.l⁻¹ Tween 20. After three washes in sterile distilled water, they were immersed in 4 % hydrogen peroxide for 10 minutes, rinsed thoroughly in sterile distilled water and placed in 50 mg.l⁻¹ each of penicillin and streptomycin (Highveld Biological, S.A.) for 10 minutes. They were then germinated on solid germination medium containing Murashige and Skoog nutrients (MS) (Murashige and Skoog, 1962), 10 g.l⁻¹ sucrose, 1 g.l⁻¹ casein hydrolysate, 4 g.l⁻¹ Gelrite and 0.1 g.l⁻¹ Benlate, pH 5.7 by incubating in the dark at 24°C - 27°C for 6 days, and then under a 16 hour photoperiod at 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetic photon flux density (PPFD) for approximately four weeks.

2.2.3 Shoot multiplication

The seedlings obtained via the germination procedure outlined in Section 2.2.2 were immersed in a mixture of 100 $\mu\text{g}\cdot\text{ml}^{-1}$ each of penicillin and streptomycin for 20 minutes. The roots were removed and the shoots were placed in shoot multiplication medium which contained MS nutrients, 30 g.l⁻¹ sucrose, 0.01 mg.l⁻¹ NAA, 0.2 mg.l⁻¹ BAP and 4 g.l⁻¹ Gelrite, pH 5.7. The cultures were maintained at 24°C - 27°C under a 16 hour photoperiod at 200 $\mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for four to six weeks. Thereafter the multiplied shoots were transferred onto a leaf expansion medium containing half-strength MS nutrients, 30 g.l⁻¹ sucrose and 4 g.l⁻¹ Gelrite, pH 5.7. These leaves were

utilised as explants for callus production (Section 2.2.5) and leaf transformation (Section 3.2.3.2).

2.2.4 Cell suspension cultures

Cell suspension cultures were established from friable calli of *E. grandis x camaldulensis* on medium containing modified MS nutrients, 0.5 - 5 mg.l⁻¹ 2,4-D and 30 g.l⁻¹ sucrose (Blakeway *et al.*, 1993). Some of the cultures used in this investigation were prepared by Ms F. Blakeway (Biology Department, University of Natal, Durban), while the others were prepared according to the protocol described by Blakeway *et al.* (1993).

2.2.5 Plantlet regeneration via indirect organogenesis

2.2.5.1 Regeneration from cell suspension cultures

Aliquots from cell suspension cultures established on 2,4-D were removed during the exponential growth phase and placed in medium devoid of growth regulators for 4 weeks. Subsequently, they were transferred to media containing MS nutrients, 30 g.l⁻¹ sucrose, 4 g.l⁻¹ Gelrite, 0.1 - 5 mg.l⁻¹ KIN and 0 - 1 mg.l⁻¹ NAA, pH 5.7, and incubated in the dark for approximately 15 weeks, subculturing onto fresh media every 5 weeks. Calli were flooded with 1 mg.l⁻¹ citric acid and 1 mg.l⁻¹ ascorbic acid before each transfer, to prevent excess phenolic production. Thereafter they were transferred onto media containing either 1 mg.l⁻¹ or 5 mg.l⁻¹ KIN, in the presence and absence of 100 mg.l⁻¹ adenine sulphate, in the light. After approximately 16 weeks they were placed onto hormone-free medium and kept in the light. Another similar experiment was run, where KIN was replaced by BA at an initial concentration range of 0.01 - 5 mg.l⁻¹. The calli were then transferred onto media containing either 1 or 5 mg.l⁻¹ BA, with or without 100 mg.l⁻¹ adenine sulphate, and maintained in the light.

2.2.5.2 Regeneration from leaf discs

Leaf pieces were cut from the sterile shoots produced *in vitro* (Section 2.2.3) and placed on callus induction medium containing MS nutrients, 20 g.l⁻¹ sucrose, 16 mg.l⁻¹ ferric citrate, 0.5 - 5 mg.l⁻¹ NAA, 0 - 2 mg.l⁻¹ BA and 4 g.l⁻¹ Gelrite, pH 5.7, in the dark. Following callus formation (4 weeks), the calli were transferred to shoot proliferation medium (MS nutrients, 20 g.l⁻¹ sucrose, 0.2 mg.l⁻¹ IAA, 1 mg.l⁻¹ ZEA and 4 g.l⁻¹ Gelrite, pH 5.7) and kept in the dark for about a week. Thereafter they were transferred into the light (16 hour photoperiod at 200 $\mu\text{E.m}^{-2}.\text{s}^{-1}$). Shoots (50-60 mm long) were placed onto rooting medium containing MS nutrients, 1 mg.l⁻¹ IAA, 20 g.l⁻¹ sucrose and 4 g.l⁻¹ Gelrite, pH 5.7. The cultures were incubated at 24°C - 27°C in a growth room with a 16 hour photoperiod at 200 $\mu\text{E.m}^{-2}.\text{s}^{-1}$ for two to three weeks.

Alternatively, leaf pieces were placed onto the callus induction medium of Lainé and David (1994), containing MS nutrients, 30 g.l⁻¹ sucrose, 4 g.l⁻¹ Gelrite, 0.45 mg.l⁻¹ NAA and 0.45 mg.l⁻¹ BA, pH 5.7. The callus that formed (3 weeks) was placed onto shoot induction medium containing MS nutrients, 30 g.l⁻¹ sucrose, 4 g.l⁻¹ Gelrite, 1.2 mg.l⁻¹ BA and 0.1 mg.l⁻¹ NAA, pH 5.7 (Lainé and David, 1994).

2.2.6 Plantlet regeneration via indirect somatic embryogenesis

Cell suspension cultures grown in either 0 mg.l⁻¹ or 3 mg.l⁻¹ 2,4-D were used in these investigations. Cell suspensions, maintained in MS medium supplemented with 3 mg.l⁻¹ 2,4-D, were plated onto solid media containing MS nutrients, 30 g.l⁻¹ sucrose, 4 g.l⁻¹ Gelrite and 1 or 3 mg.l⁻¹ 2,4-D, pH 5.7, for 5 weeks in the dark. The calli were then transferred to MS medium comprising 1 or 3 mg.l⁻¹ 2,4-D and either 30 g.l⁻¹ sucrose or 23 g.l⁻¹ sucrose plus 7 g.l⁻¹ supplementary sugars (1 g.l⁻¹ each of ribose, xylose, arabinose, glucose, mannose, galactose and fructose). After 7 weeks in the dark, the calli were transferred to maintenance medium (MS nutrients, 30 g.l⁻¹ sucrose and 4 g.l⁻¹ Gelrite, pH 5.7) for 8 weeks in the dark. Subsequent transfers onto

maintenance medium with activated charcoal (4 g.l⁻¹) with or without casein hydrolysate (1 g.l⁻¹) were also undertaken.

Alternatively, cells (grown in 0 mg.l⁻¹ 2,4-D) were plated onto solid maintenance medium without 2,4-D. Following callus formation (6 weeks), half of the calli were kept at 25°C, while the others were treated at 10°C for 72 hours. Following the cold treatment, these calli were returned to 25°C. After 4 weeks at 25°C, the calli were transferred to medium containing 4 g.l⁻¹ activated charcoal, 1 g.l⁻¹ casein hydrolysate or a combination of both.

Cell suspension cultures grown in liquid medium containing 0 mg.l⁻¹ 2,4-D were plated on solid medium without 2,4-D (MS nutrients, 30 g.l⁻¹ sucrose, 4 g.l⁻¹ Gelrite, pH 5.7). Callus that was produced on this medium was subcultured onto fresh medium containing 12 mg.l⁻¹ ABA and/or 40 g.l⁻¹ PEG. Following embryoid production, the calli containing the embryonic structures were transferred onto various germination media and incubated in the light. The basic embryo germination medium contained MS nutrients, 30 g.l⁻¹ sucrose and 4 g.l⁻¹ Gelrite, pH 5.7, while one of the variations on this medium contained 4 g.l⁻¹ activated charcoal, and the other one was supplemented with 4 g.l⁻¹ activated charcoal, 0.01 mg.l⁻¹ NAA, 0.1 mg.l⁻¹ BA and 0.1 mg.l⁻¹ GA₃.

2.2.7 Microscopy and photography

The various stages of germination, multiplication and regeneration were recorded using a Nikon FM2 camera with a 60 mm Mikro Nikkor macro lens. Cell suspension culture-derived calli and associated structures were screened using a Wild M3 stereomicroscope and recorded using a Wild Photoautomat MPS 55 system. Individual callus cells were stained with 1 % Safranin Red stain (Harrigan and McCance, 1966), observed using an Olympus Vanox AHBS3 light microscope and recorded using an Olympus C-35AD-4 camera.

2.2.8 Data analyses

Average values were calculated from the data recorded during the different stages of plantlet regeneration via indirect organogenesis. Where appropriate, Duncan's Multiple Range Test (SAS, 1982) was used to assess differences in the recorded mean values of the variables investigated. Alphabetical values were assigned to the mean values recorded for each treatment. Mean values that did not share the same letter, were recognised as being significantly different from each other.

2.3 Results and Discussion

2.3.1 *In vitro* shoot production from axillary buds

The utilisation of seedlings that are aseptically produced *in vitro* circumvents the problems related to contamination of field-grown tissues (Durand-Cresswell *et al.*, 1982). These seedlings can then be bulked up for further use in other micropropagation systems. Another advantage of the use of seeds and seedling production for *in vitro* culture is for the micropropagation of valuable seed stock, such as the seed from a few frost-tolerant individuals (McComb and Bennett, 1986). In this study, seeds of *E. grandis* were germinated on MS nutrients, 10 g.l⁻¹ sucrose, 1 g.l⁻¹ casein hydrolysate and 0.1 g.l⁻¹ Benlate, pH 5.7, in the dark for 6 days and then in the light for about 4 weeks (Figure 2.1 A). Shoots from the germinated seedlings were then placed into multiplication medium (MS nutrients, 30 g.l⁻¹ sucrose, 0.01 mg.l⁻¹ NAA and 0.2 mg.l⁻¹ BAP) (Figure 2.1 B), in the light for 3 - 4 weeks, followed by transfer to leaf expansion medium consisting of half strength MS nutrients and 30 g.l⁻¹ sucrose. After 4 - 6 weeks on expansion medium, leaves from the multiplied shoots became large enough (approximately 1.5 x 1 cm) to be used as explants for callus production (Figure 2.1 C). These young leaves were used as explants since they are known to respond well in culture (Watt *et al.*, 1991) and were free of contaminants. The presence of contaminants is one of the major problems encountered in this laboratory with the use of mature leaves from field-grown material (unpublished). These leaves were also found to be suitable explants for transformation studies, as will be discussed in Chapter 3.

2.3.2 Plantlet regeneration from leaf discs

The process of organogenesis involves the multiplication of leaf material, elongation of shoots and rooting of plantlets (Flick *et al.*, 1983; Dixon, 1985). As previously discussed (Section 2.1.4.2), the use of direct organogenesis via axillary bud proliferation has been reported for *E. grandis* (De Fossard *et al.*, 1977; Durand-

Cresswell and Nitsch, 1977; Hartney, 1980; Franclet and Boulay, 1982; Furze and Cresswell, 1985; Sankara Rao and Venkateswara, 1985; McComb and Bennett, 1986; Warrag *et al.*, 1990) (Section 2.1.4.2). However, with the exception of the recent work by Lainé and David, 1994, no other reports of indirect organogenesis have been published. The establishment of the protocol for indirect organogenesis was deemed important in this study because of its applicability to genetic transformation. A well-established method for genetic transformation involves the use of leaf discs, as will be discussed in Chapter 3, and a prerequisite for all transformation systems, including this one, is that plantlets can be regenerated from transformed cells or tissues (Snyman *et al.*, 1992; Debeaujon and Branchard, 1993). This is often the limiting step in the development of successful protocols for transgenic plant production (Wilde *et al.*, 1992). Furthermore, a system for callus production and subsequent plant regeneration has many other potential applications, such as screening for somaclonal mutants and for mutagenesis studies.

The method documented here for the regeneration of *E. grandis* via indirect organogenesis is based on work performed previously in this laboratory (Blakeway, 1992). Leaves from young seedlings multiplied *in vitro* (Section 2.2.3) were cut in half and placed onto callus induction medium containing MS nutrients, 16 mg.l^{-1} ferric citrate, 20 g.l^{-1} sucrose and combinations of NAA ($0.5 - 5 \text{ mg.l}^{-1}$) and BA ($0 - 2 \text{ mg.l}^{-1}$) in the dark. The role of ferric citrate is unknown, but it was recommended by Lakshmi Sita *et al.* (1979) and preliminary experiments in this laboratory (unpublished) indicated that it has a positive effect on callus initiation. Similarly, the dark treatment has been found to be beneficial for callus induction (unpublished work from this laboratory), and it was also used by Lainé and David (1994). After a 28 day incubation period in the dark, the percentage of explants with callus was recorded (Figure 2.2 A,B). Optimum callus formation (90.3 - 94.2 % explants with callus) was found on the media containing $1 - 5 \text{ mg.l}^{-1}$ NAA and $0 - 0.05 \text{ mg.l}^{-1}$ BA (Figure 2.2



6a

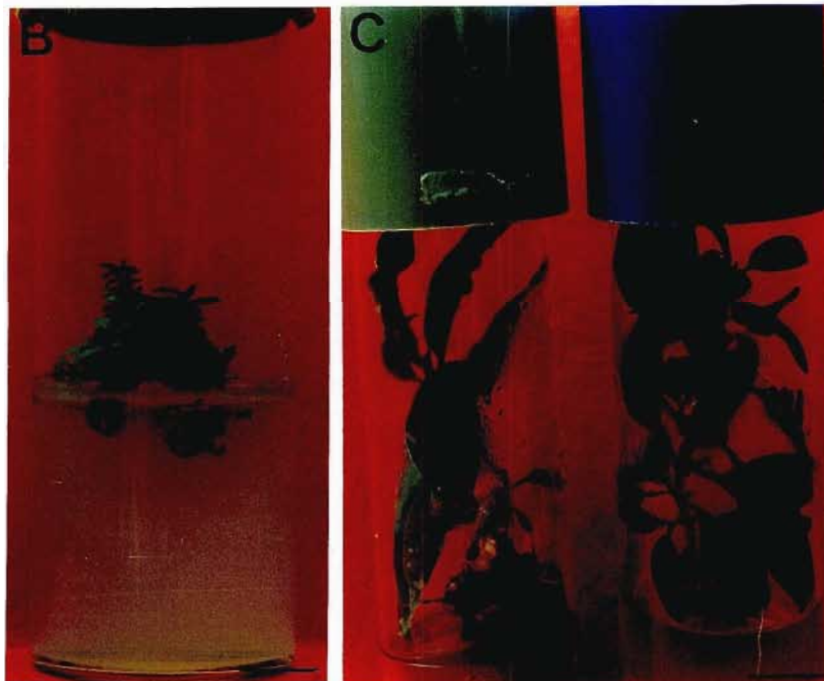


Figure 2.1: Production of sterile plantlets *in vitro*. (A) Seedlings were germinated on a medium containing MS nutrients, 10 g.l^{-1} sucrose, 1 g.l^{-1} casein hydrolysate, 4 g.l^{-1} Gelrite, in the dark for 6 days and then under a 16 hour photoperiod at $200 \mu\text{E.m}^{-2}.\text{s}^{-1}$ PPFD for four weeks (bar = 9.3 mm) (B) Shoots on multiplication medium comprising MS nutrients, 30 g.l^{-1} sucrose, 0.01 mg.l^{-1} NAA, 0.2 mg.l^{-1} BA and 4 g.l^{-1} Gelrite were incubated in the dark for 4 - 6 weeks (bar = 7.1 mm). (C) Multiplied shoots were transferred to leaf expansion medium (half-strength MS nutrients, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite) and expanded leaves were used as explants in further studies (bar = 9.3 mm).

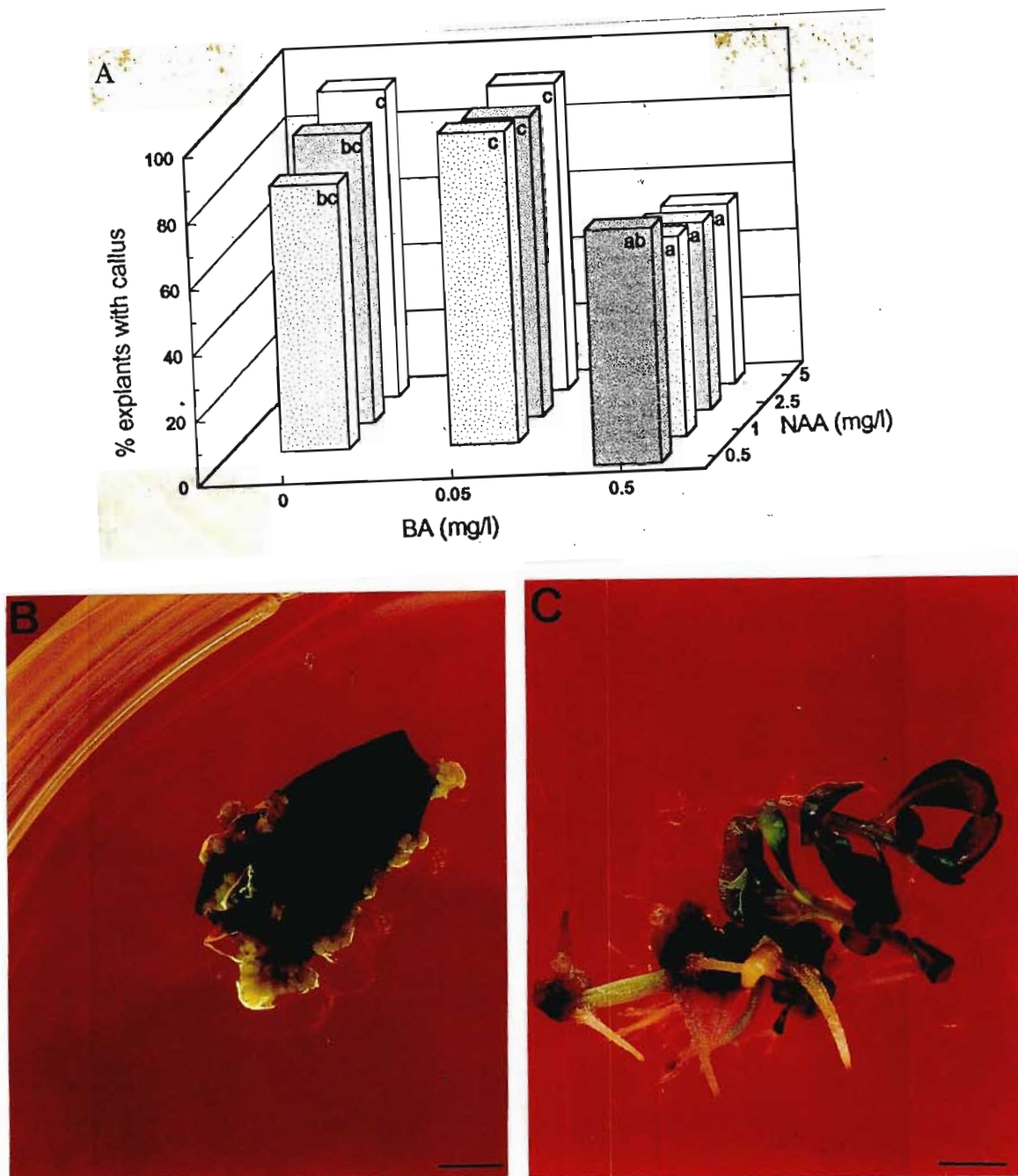


Figure 2.2: The effect of NAA and BA on callus formation from leaf explants. (A) Percent explants with callus, (B) callus produced from leaf discs (bar = 4.5 mm), and (C) shoot initiation, after 4 weeks in the dark (bar = 6 mm). Callus induction media consisted of MS nutrients, 20 g.l⁻¹ sucrose, 16 mg.l⁻¹ ferric citrate, 0.5 - 5 mg.l⁻¹ NAA, 0 - 2 mg.l⁻¹ BA and 4 g.l⁻¹ Gelrite, pH 5.7. Levels of significant difference (Duncan's Multiple Range Test) are given (n = 15 - 25).

A), although only a small amount of callus (less than 0.1 g fresh mass/explant) was formed on each explant. It was noted at this stage that shoot initiation, with a few (1 - 5) shoots per callus, had occurred in the regenerating calli (Figure 2.2 C).

Following the callus and shoot induction stage, all leaf pieces which had produced callus were placed onto shooting medium comprising MS nutrients, 20 g.l⁻¹ sucrose, 0.2 mg.l⁻¹ IAA and 1 mg.l⁻¹ ZEA. They were left in the dark for 3 days to prevent the production of phenolics (Furze and Cresswell, 1985) and then transferred to the light for a further 18 days. The results representing the number of calli with shoots (Figure 2.3) after 21 days on shooting medium were related to the NAA and BA concentrations in the initial callus induction media (Figure 2.2 A). As can be seen in Figure 2.5, the medium containing 0.05 mg.l⁻¹ BA and 1 mg.l⁻¹ NAA, which was one of the media that allowed for the best callus formation, also produced the greatest shooting percentage (63.6 % calli with shoots). On average, 4 - 6 shoots were formed per explant on these media.

Blakeway (1992) found that the most effective rooting medium for *E. grandis* shoots produced via indirect organogenesis contained 1 mg.l⁻¹ IBA. The rooting efficiencies reported by that worker were relatively high (77.8 %), and Lainé and David (1994) also reported good (85 %) rooting of elongated *E. grandis* shoots on medium containing 0.25 mg.l⁻¹ IBA. On the other hand, Lakshmi Sita *et al.* (1979) found that IAA induced high rooting so, in this study, the use of IAA was investigated. Thus, the calli with shoots were placed onto rooting medium (MS nutrients, 20 g.l⁻¹ sucrose and 1 mg.l⁻¹ IAA) in the light, and these results are presented in Figures 2.4 (A and B). Rooting seemed to be affected by the initial treatment (i.e. the callus induction medium), since explants that had initially been on one of the optimal callus induction media (e.g. 0.05 mg.l⁻¹ BA and 1 mg.l⁻¹ NAA) ultimately produced a relatively high rooting percentage when placed onto rooting medium (45.5 % of explants produced roots) (Figure 2.4 B). The results obtained indicate, therefore, that IBA was more

effective in terms of rooting efficiency (77.8 - 85 %) than IAA (45.5 %), and therefore IBA should be used in future investigations.

The developed protocol for plant regeneration via indirect organogenesis is summarised in Table 2.3. Although the number of explants which produced callus was high (90.3 - 94.2 %), the amount of callus per explant was low (less than 0.1 g fresh mass per explant), with an average of 4 - 6 shoots forming per explant. In order for this technique to be commercially viable, improvement in callus yield, and hence shoot production are required. Difficulties in this regard have also been experienced by Lainé and David (1994) who, using a different protocol, reported a 38.5 % frequency of bud production, which does not differ significantly from the average yield obtained in this study (43.5 %).

The recent work of Lainé and David (1994) was also on *E. grandis* clones, and for this reason the media formulations reported by those workers were attempted here. Preliminary results obtained in this study revealed that callus formation could be obtained after only 1 week in culture (Figure 2.5 A), while in some cases, precocious shoots formed after 2 weeks in culture (Figure 2.5 B). At the time of this report, the calli have been transferred onto the shoot initiation medium and no further results have been obtained.

Table 2.3: The best protocol and media used for the production of plantlets from leaf discs via indirect organogenesis.

Stage	Medium	Conditions	Time
callus induction	MS + 16 mg.l ⁻¹ ferric citrate + 20 g.l ⁻¹ sucrose + 0.05 mg.l ⁻¹ BA + 1 mg.l ⁻¹ NAA	dark	28 days
shooting	MS + 20 g.l ⁻¹ sucrose + 0.2 mg.l ⁻¹ IAA + 1 mg.l ⁻¹ ZEA	dark	3 days
		light	21 days
rooting	MS + 20 g.l ⁻¹ sucrose + 1 mg.l ⁻¹ IAA	light	18 weeks

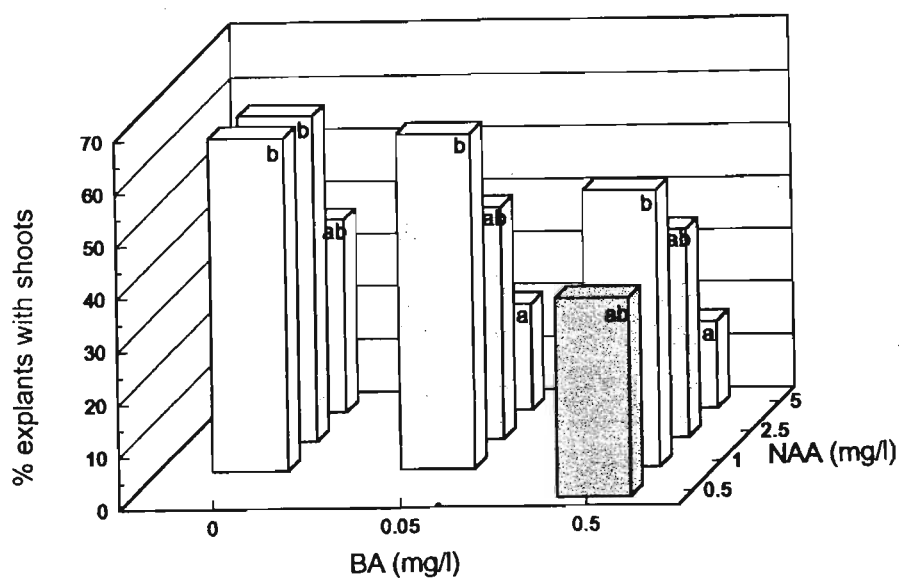


Figure 2.3: Shoot formation from leaf disc-derived callus as influenced by the initial callus induction medium. The details of the callus induction medium are as described in Figure 2.2. The shooting medium consisted of MS nutrients, 20 g.l⁻¹ sucrose, 0.2 mg.l⁻¹ IAA, 1 mg.l⁻¹ ZEA and 4 g.l⁻¹ Gelrite. Cultures were maintained in the dark for 3 days prior to transfer into the light and results were recorded after 21 days. n = 10 - 15. Levels of significant difference (Duncan's Multiple Range Test) are given.

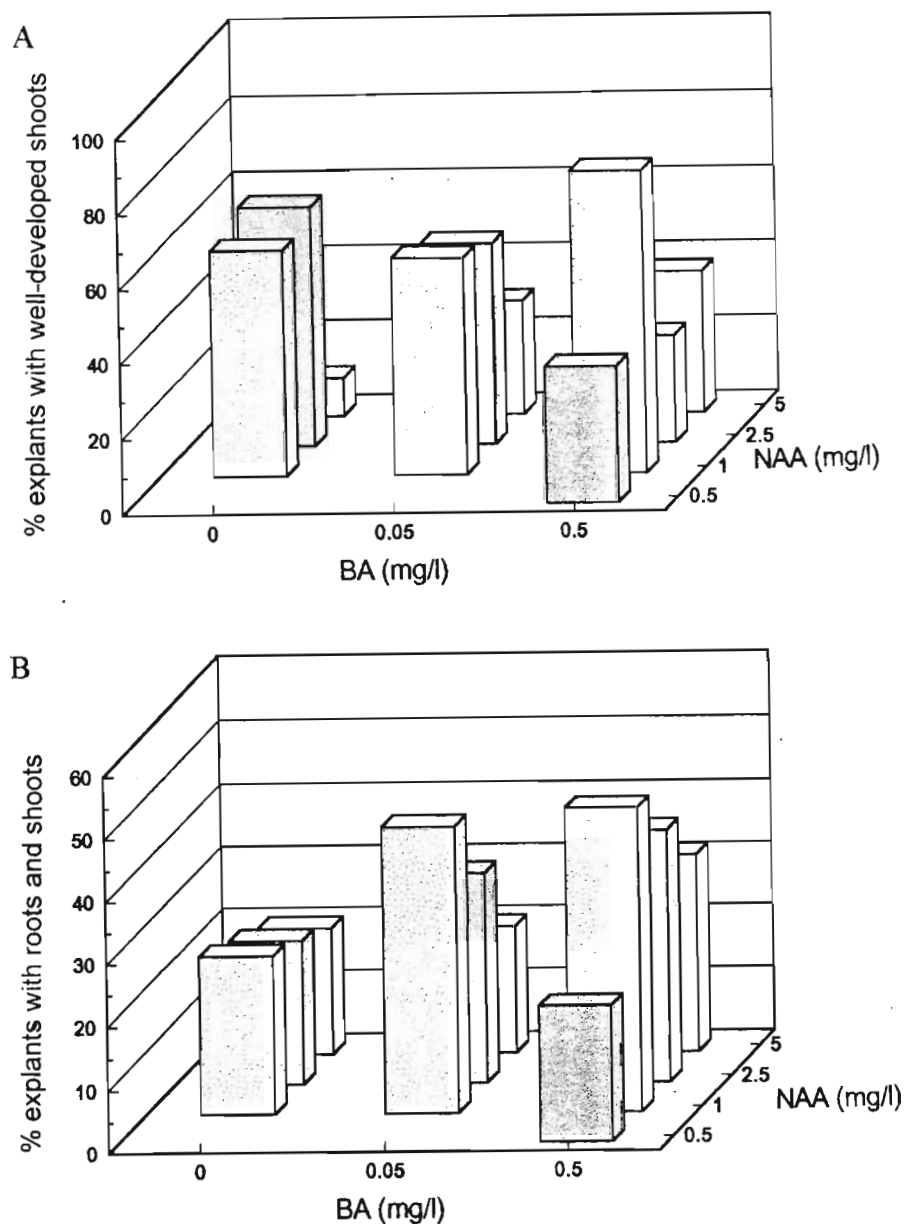


Figure 2.4: Rooting of shoots produced *in vitro* as influenced by the initial callus induction medium. Details of the callus induction are as in Figure 2.2. (A) Explants with well-developed shoots and (B) explants with roots and shoots, after being in rooting medium for 18 weeks. Rooting medium comprised MS nutrients, 20 g.l⁻¹ sucrose, 1 mg.l⁻¹ IAA and 4 g.l⁻¹ Gelrite, and cultures were maintained in the light.

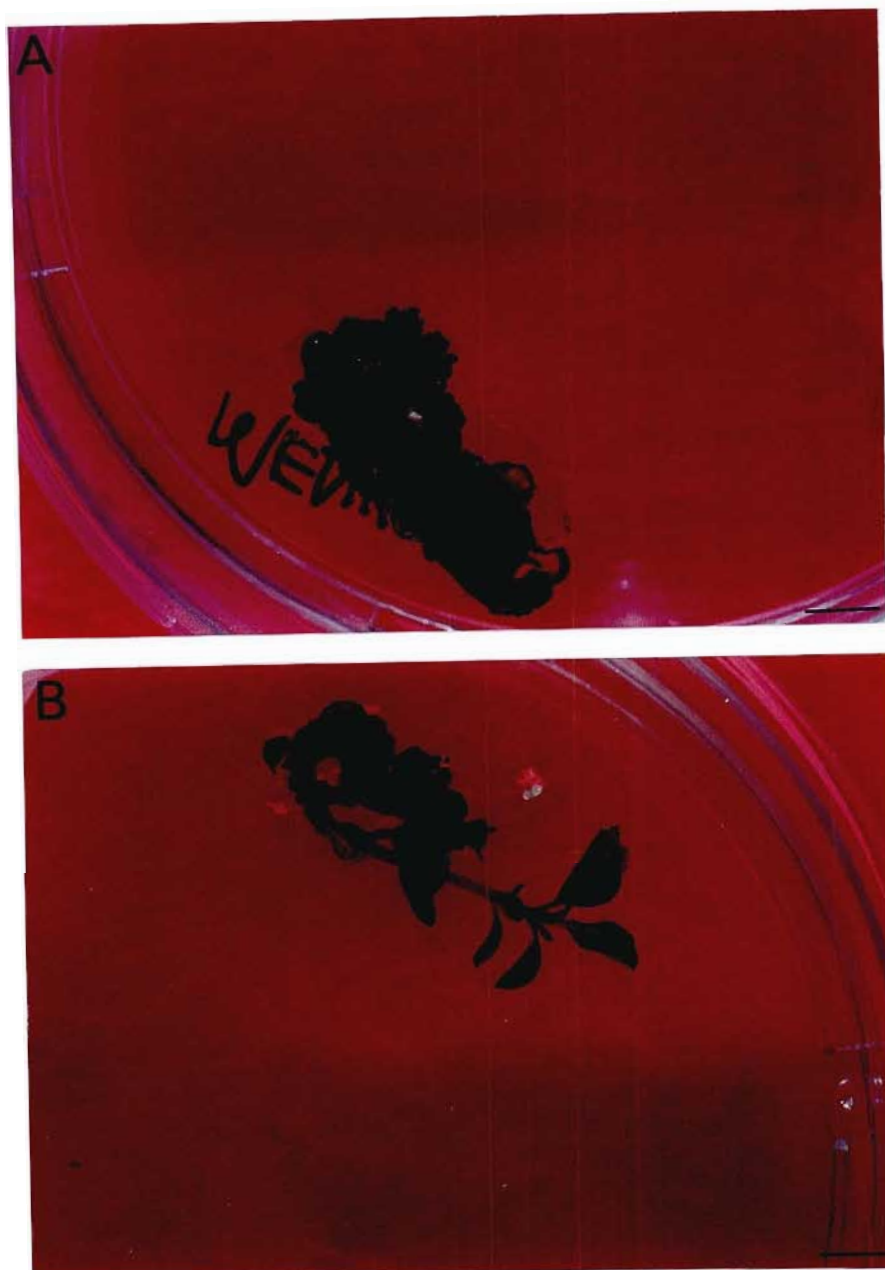


Figure 2.5: Callus and shoot formation from leaf explants. (A) Callus produced after 1 week on callus induction medium (bar = 6.3 mm), and (B) precocious shoot formation on callus induction medium after transfer into the light (1 week) (bar = 6.3 mm).

2.3.3 Production of cell suspension cultures

Cell suspension cultures (Figure 2.6) were initiated from friable calli obtained from the leaf explants (Section 2.3.2) according to the method of Blakeway *et al.* (1993). In this study, some of the cell suspension cultures were provided by Ms F. Blakeway (Biology Department; University of Natal, Durban), while others were initiated *de novo* following the technique reported by Blakeway *et al.* (1993). The fact that only a small amount of callus was produced per leaf explant (approximately 0.1 g fresh mass/explant) (Figure 2.2 B), was a problem for the initiation of large volumes of cell suspension cultures. This is because there is a critical inoculum density below which the culture will not grow (Stuart and Street, 1969; Ammirato, 1984), and which varies with species, clone and culture medium (Street, 1973). In order to overcome this problem, small cell suspension culture volumes (20 ml) (Figure 2.6 A) were initiated from the initial small amounts of callus. These liquid cultures were then plated onto solid medium to form second generation callus. As Blakeway *et al.* (1993) investigated the three types of calli (Types I - III) formed in this way, and established that Type I callus was the most suitable for cell suspension culture initiation, this was used as the explant to initiate large (100 ml) cell suspension culture volumes (Figure 2.6 B). Although the doubling time of these cultures was 9 - 12 days (Blakeway *et al.*, 1993), the cell suspension cultures were subcultured into fresh medium in the early stationary phase, approximately every 14 days. From these cultures, cells (1.5 ml) in the exponential phase were plated onto filter paper discs on solid callus induction medium comprising MS nutrients, 30 g.l⁻¹ sucrose and 3 mg.l⁻¹ 2,4-D, and allowed to proliferate to form callus (Figure 2.7). This callus was then used (1) to initiate new cell suspensions, (2) in the studies on plant regeneration from cell suspension cultures (Section 2.3.4), or (3) as the explant in transformation studies (Chapter 3).

The use of single cell systems, such as that of cell suspension cultures reported here, have many advantages. There is a rapid response time to experimental conditions

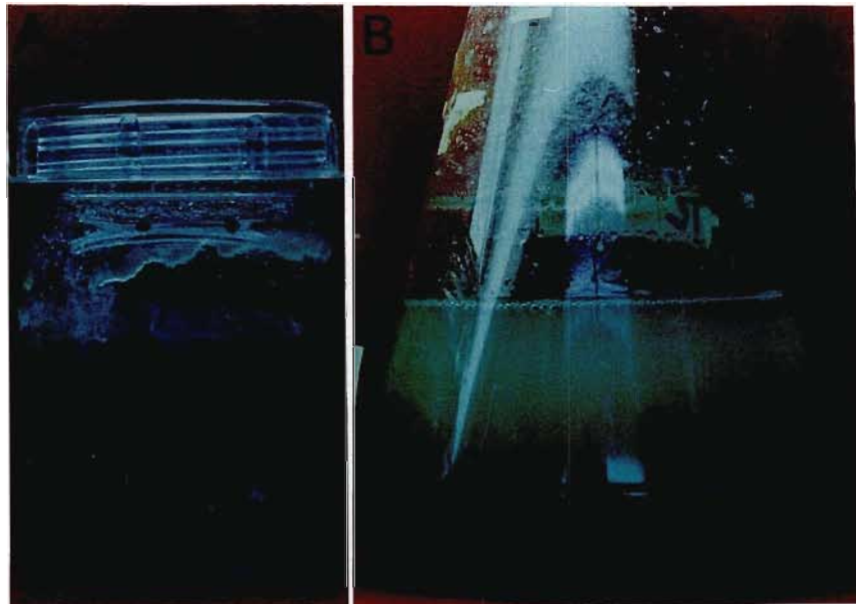


Figure 2.6: Cell suspension cultures of *Eucalyptus*. (A) Small cell suspension culture volumes were initiated from small amounts of callus derived from leaf discs (bar = 13.2 mm), and (B) large cell suspension culture volumes were produced from plated cell suspensions (bar = 13.5 mm). The liquid medium for suspension culture initiation contained MS nutrients, 30 g.l⁻¹ sucrose and 3 mg.l⁻¹ 2,4-D.



Figure 2.7: Callus produced from plated cell suspension cultures. Callus was obtained by plating 1.5 ml cells (in the exponential growth phase) onto filter paper discs on solid callus induction medium (MS nutrients, 30 g.l⁻¹ sucrose and 3 mg.l⁻¹ 2,4-D), in the dark, for 6 weeks (bar = 8 mm).

(Krogstrup, 1990), they can be used for large-scale somatic embryo production (Becwar *et al.*, 1988) and hence low cost plant multiplication, and they are ideal systems for mutant isolation and selection (van den Bulk, 1991). They can also be used as recipients for insertion and integration of foreign genes (Fraley *et al.*, 1986; Durzan, 1988), as will be addressed in Chapter 3. A disadvantage of some cell suspension culture populations is the cell cycle asynchrony that may occur. Together with the heterogeneity that may also be encountered in some cell suspension culture populations, this is a hindrance to the use of cell suspension cultures and results in only marginal success in certain physiological and biochemical studies carried out using these systems (Wang and Phillips, 1984).

2.3.4 Plantlet regeneration from cell suspension cultures

At the time of initiation of this study, the major drawback of this cell suspension culture system for *E. grandis* and its hybrids (Blakeway *et al.*, 1993) was the lack of available methods for the regeneration of plants from the cultures, as this limits the use of cell suspension cultures in transformation studies. As yet, plantlet regeneration has not been achieved from cell suspension cultures of many woody species, and there are no reports of regeneration from cell suspension cultures of *E. grandis*. As there are two routes of plant regeneration, organogenesis and embryogenesis, both approaches were investigated in this study. Organogenesis has been reported to be influenced by the addition of growth regulators such as NAA, KIN, BA and adenine sulphate (Murashige, 1974), while the removal of 2,4-D (Warren, 1991), manipulation of carbon source (Levi and Sink, 1992), addition of activated charcoal (Evans *et al.*, 1981), ABA and PEG (Dunstan *et al.*, 1994) as well as cold-treatment (Kavathekar *et al.*, 1977; Hughes, 1981) have been used for the induction of embryogenesis. In this study, all of these factors were investigated (Table 2.4).

Table 2.4: Overview and summary of the treatments used and the results obtained in an attempt to regenerate plants from cell suspension cultures - (A) an attempt to obtain plantlet regeneration via indirect organogenesis (Section 2.3.4.1); (B) - (E) an attempt to produce plants via somatic embryogenesis (Sections 2.3.4.2 - 2.3.4.5). All media are based on MS nutrients and vitamins (Murashige and Skoog, 1962). (ABA = abscisic acid; AC = activated charcoal; AdS = adenine sulphate; BA = benzylaminopurine; CH = casein hydrolysate; 2,4-D = 2,4-dichlorophenoxyacetic acid; KIN = kinetin; NAA = 1-naphthylacetic acid; PEG = polyethylene glycol; S = sucrose; suppl. sug. * = supplementary sugars (1 g.l⁻¹ each of ribose, xylose, arabinose, glucose, mannose, galactose and fructose).

	Treatment				Results
	Callus Induction		Regeneration		
	Stage 1	Stage 2	Stage 3	Stage 4	
A	0 - 1 mg.l ⁻¹ NAA + 0.1 - 5 mg.l ⁻¹ KIN	1 mg.l ⁻¹ KIN			green calli, some small red-pigmented areas
		1 mg.l ⁻¹ KIN + 100 mg.l ⁻¹ AdS			green calli, some small red-pigmented areas
		5 mg.l ⁻¹ KIN			green calli, some small red-pigmented areas
		5 mg.l ⁻¹ KIN + 100 mg.l ⁻¹ AdS			green calli, some small red-pigmented areas
	0 - 1 mg.l ⁻¹ NAA + 0.01 - 5 mg.l ⁻¹ BA	1 mg.l ⁻¹ BA			green calli, some small red-pigmented areas
		1 mg.l ⁻¹ BA + 100 mg.l ⁻¹ AdS			green calli, some small red-pigmented areas
		5 mg.l ⁻¹ BA			green calli, some small red-pigmented areas
		5 mg.l ⁻¹ BA + 100 mg.l ⁻¹ AdS			green calli, some small red-pigmented areas
B	1 mg.l ⁻¹ 2,4-D	0 mg.l ⁻¹ 2,4-D	0 mg.l ⁻¹ 2,4-D + 4 g.l ⁻¹ AC	0 mg.l ⁻¹ 2,4-D + 4 g.l ⁻¹ AC + 1 g.l ⁻¹ CH	embryogenic calli
	3 mg.l ⁻¹ 2,4-D	0 mg.l ⁻¹ 2,4-D	0 mg.l ⁻¹ 2,4-D + 4 g.l ⁻¹ AC	0 mg.l ⁻¹ 2,4-D + 4 g.l ⁻¹ AC + 1 g.l ⁻¹ CH	embryogenic calli
C	1 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S + 4 g.l ⁻¹ AC	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S + 4 g.l ⁻¹ AC + 1 g.l ⁻¹ CH	embryogenic calli
	1 mg.l ⁻¹ 2,4-D + 23 g.l ⁻¹ S + 7 g.l ⁻¹ suppl. sug.*	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S + 4 g.l ⁻¹ AC	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S + 4 g.l ⁻¹ AC + 1 g.l ⁻¹ CH	embryogenic calli
	3 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S + 4 g.l ⁻¹ AC	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S + 4 g.l ⁻¹ AC + 1 g.l ⁻¹ CH	embryogenic calli
	3 mg.l ⁻¹ 2,4-D + 23 g.l ⁻¹ S + 7 g.l ⁻¹ suppl. sug.*	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S + 4 g.l ⁻¹ AC	0 mg.l ⁻¹ 2,4-D + 30 g.l ⁻¹ S + 4 g.l ⁻¹ AC + 1 g.l ⁻¹ CH	embryogenic calli
D	0 mg.l ⁻¹ 2,4-D at 28°C	0 mg.l ⁻¹ 2,4-D at 28°C	0 mg.l ⁻¹ 2,4-D + 4 g.l ⁻¹ AC		embryogenic calli
			0 mg.l ⁻¹ 2,4-D + 1 g.l ⁻¹ CH		embryogenic calli
	0 mg.l ⁻¹ 2,4-D at 28°C	0 mg.l ⁻¹ 2,4-D at 4°C for 72 hours then back to 28°C	0 mg.l ⁻¹ 2,4-D + 4 g.l ⁻¹ AC + 1 g.l ⁻¹ CH		embryogenic calli
			0 mg.l ⁻¹ 2,4-D + 4 g.l ⁻¹ AC		embryogenic calli
E	12 mg.l ⁻¹ ABA	no supplement			embryoids
		4 g.l ⁻¹ AC			embryoids
		4 g.l ⁻¹ AC + 0.01 mg.l ⁻¹ NAA + 0.1 mg.l ⁻¹ BAP + 0.1 mg.l ⁻¹ GA ₃			embryoids
	40 g.l ⁻¹ PEG	no supplement			embryoids
		4 g.l ⁻¹ AC			embryoids
		4 g.l ⁻¹ AC + 0.01 mg.l ⁻¹ NAA + 0.1 mg.l ⁻¹ BAP + 0.1 mg.l ⁻¹ GA ₃			embryoids
12 mg.l ⁻¹ ABA + 40 g.l ⁻¹ PEG	no supplement			embryoids	
	4 g.l ⁻¹ AC			embryoids	
		4 g.l ⁻¹ AC + 0.01 mg.l ⁻¹ NAA + 0.1 mg.l ⁻¹ BAP + 0.1 mg.l ⁻¹ GA ₃		embryoids	

2.3.4.1 The effect of growth regulators and adenine sulphate

Although the precise requirements of plant growth regulators for callus production and plantlet regeneration via indirect organogenesis may differ between species, the general approach, of manipulation of exogenous auxin and cytokinin levels, formed the basis of regeneration techniques for a wide variety of species (Lindsey and Jones, 1990a). The effects of combinations of certain plant growth regulators ($0 - 1 \text{ mg.l}^{-1}$ NAA and $0.1 - 5 \text{ mg.l}^{-1}$ KIN, $0 - 1 \text{ mg.l}^{-1}$ NAA and $0.01 - 5 \text{ mg.l}^{-1}$ BA) on plant regeneration was investigated by incorporating them into solid callus induction medium (Table 2.4 A). Cultures were kept in the dark for approximately 15 weeks, subculturing every 5 weeks. Although the cells proliferated rapidly to form callus, no signs of organogenesis were visible (Figure 2.8; Table 2.4 A). Screening microscopically substantiated this observation. These cultures were then used to investigate the effects of the addition of adenine sulphate, by subculturing the calli onto callus induction medium containing either 1 or 5 mg.l^{-1} KIN or BA (depending on which cytokinin had been used previously), with or without 100 mg.l^{-1} adenine sulphate. The effects of adenine sulphate were documented by Murashige (1974), who stated that this 'substance' has been shown to promote shoot initiation and proliferation. Adenine sulphate has been used by many other workers, some of whom found that it promotes shoot initiation and proliferation (Earle and Torrey, 1965a,b; Thorpe and Murashige, 1968; Smith and Murashige, 1970; Thorpe and Murashige, 1970; Miller and Murashige, 1976; Rangan, 1976; Kitto and Young, 1981; Papachatzis *et al.*, 1981; Meyer, 1982; Srivastava and Steinhauer, 1982; Frett and Dirr, 1983; Harris and Mason, 1983; Anderson, 1984; Samartin *et al.*, 1984; Norton and Norton, 1985; Paterson and Everett, 1985), while others have found that it enhances embryo development and maturation (Mauney, 1961; Raghavan and Torrey, 1963, 1964; Mauney *et al.*, 1967; Mitra and Chaturvedi, 1972; Tisserat *et al.*, 1979).

The cultures that had been placed onto medium containing a cytokinin and adenine sulphate were maintained in the light for 16 weeks. The resultant callus that

proliferated had green and red pigments (Figure 2.9 A,B; Table 2.4 A) which, after microscopic examination could be attributed to the presence of large numbers of well-developed chloroplasts (Figure 2.9 C). These findings correlate with those of other workers (Shahin and Shepard, 1980; Paterson and Everett, 1985; Lainé and David, 1994) who found that calli had green spots when placed in the light. The green calli obtained by Paterson and Everett (1985) formed shoots in the light, while Lainé and David (1994) reported that pink and green nodules appeared in the callus before bud emergence. However, no signs of organogenesis were evident in the present study.

2.3.4.2 The effect of 2,4-D removal and the addition of activated charcoal

In most systems, embryogenesis is brought about simply by the removal of auxin, which can be aided by the addition of activated charcoal (Ammirato, 1983; Mantell *et al.*, 1985b; Ammirato, 1986; Warren, 1991). In the present study, cell suspension cultures grown in 3 mg.l^{-1} 2,4-D were plated onto media containing either 1 or 3 mg.l^{-1} 2,4-D for callus induction (5 weeks), followed by transfer to hormone-free maintenance medium for 7 - 8 weeks (Table 2.4 B). Thereafter the calli were transferred onto hormone-free maintenance medium supplemented with 4 g.l^{-1} activated charcoal for 8 weeks. As microscopic screening revealed embryogenic calli with no embryogenic structures, the calli were transferred to maintenance medium containing activated charcoal with and without 1 g.l^{-1} casein hydrolysate. Further screening showed that the embryogenic calli had not differentiated further, and only embryogenic and non-embryogenic cells were visible (Figure 2.10; Table 2.4 B).

2.3.4.3 The effect of carbon source

As mentioned previously (Section 2.3.4.2), an auxin is usually required for the induction of embryogenic cells, while removal of the auxin fosters embryo development and maturation (Ammirato, 1983; Mantell *et al.*, 1985b; Ammirato, 1986; Warren, 1991). However, other factors such as carbon source have also been shown to affect embryogenesis. In an attempt to obtain embryogenesis in the present

study, the carbon source in the medium was altered. The carbon source most commonly used in plant culture media is sucrose, however, the addition of carbohydrates other than sucrose has been suggested to enhance embryogenesis (Thompson and Thorpe, 1987; Levi and Sink, 1992). Verma and Dougall (1977) reported that embryo maturation was more rapid on media containing additional sugars than on media with sucrose as the sole carbohydrate source. Watt *et al.* (1991) found that the inclusion of additional simple sugars (arabinose, fructose, galactose, glucose, mannose, ribose and xylose) increased embryo germination from embryogenic calli produced from leaf explants of *Eucalyptus* and suggested that these sugars may provide the necessary precursors for cell wall formation. Other authors that have used a combination of sugars are Atanassov and Brown (1984) (*Medicago sativa*); Carlberg *et al.* (1984) (*Solanum tuberosum*); David *et al.* (1984) (*Pinus pinaster*); Grosser and Collins (1984) (*Trifolium rubens*); Spangenberg *et al.* (1986) (*Brassica napus*) and Sundberg and Glimelius (1986) (*Brassica napus*).

In view of the above-mentioned reports and in an attempt to obtain embryogenesis, the carbon source in the medium was altered in the present study. A comparison was made between the use of 30 g.l⁻¹ sucrose and 23 g.l⁻¹ sucrose plus 7 g.l⁻¹ supplementary sugars (1 g.l⁻¹ each of ribose, xylose, arabinose, glucose, mannose, galactose and fructose). Either 1 or 3 mg.l⁻¹ 2,4-D was included in the medium in order to stimulate cell division and thus callus formation. On closer microscopic examination, the 12 - 15 week-old callus contained both embryogenic and non-embryogenic cells (Figure 2.10) but no well-developed embryogenic structures were found (Table 2.4 C).

Activated charcoal has been found to enhance somatic embryo development (Ammirato, 1983; Evans *et al.*, 1981; Ammirato, 1987) since it absorbs plant growth regulators, such as auxins, which have been shown to inhibit embryo maturation, thereby delaying embryo germination and thus plantlet production (Ammirato, 1983.



Figure 2.8: Large amounts of cell suspension culture-derived callus formed after 15 weeks. The calli were produced on media containing combinations of NAA and KIN or NAA and BA (bar = 8 mm). Morphologically the calli were not distinct from one another, and a representative example is presented above.

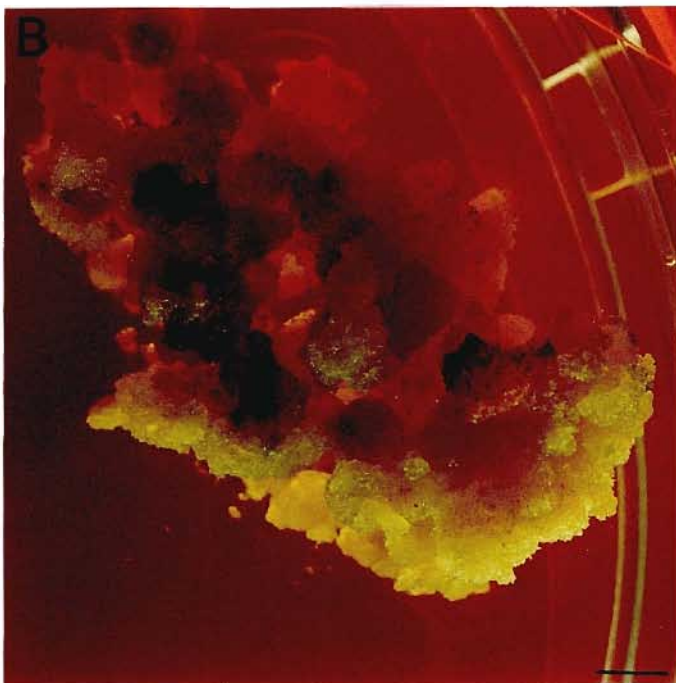
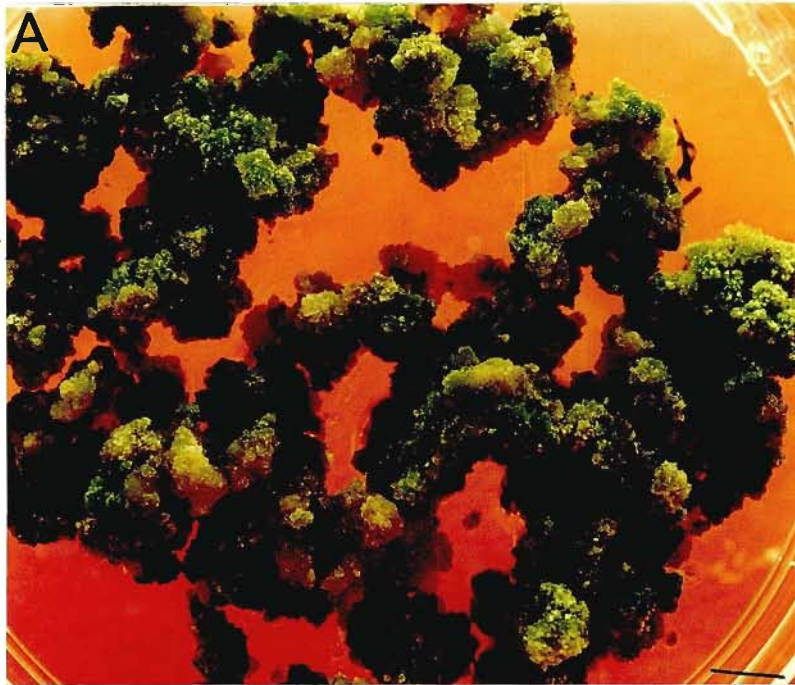


Figure 2.9: Examples of calli formed on media containing either KIN or BA, with or without adenine sulphate. The calli that were maintained, in the light, on media containing different plant growth regulator combinations could not be distinguished on the basis of morphology, and representative examples are illustrated. (A,B) Callus cells contained red and green pigments (A: bar = 14.8 mm; B: bar = 25 mm); (C) microscopic detail of individual callus cells revealed numerous chloroplasts (bar = 25 μm).

1986). Activated charcoal has also been found to absorb inhibitors such as 5-hydroxymethylfurfural formed in the medium by sucrose degradation during autoclaving (Weatherhead *et al.*, 1978). Watt *et al.* (1991) found that, for *Eucalyptus*, the removal of such substances promoted embryogenesis from leaf disc explants and increased plantlet regeneration. This approach was therefore tried by placing the calli that had been induced on sucrose and supplementary sugars onto media containing activated charcoal for 3 weeks, followed by subculture onto a combination of activated charcoal and casein hydrolysate. The calli on media containing either activated charcoal or activated charcoal and casein hydrolysate were screened regularly using a microscope, but no difference was found after 8 - 10 weeks on these media - embryogenic calli containing both embryogenic and non-embryogenic cells were found, but no embryogenic structures had differentiated (Figure 2.10; Table 2.4 C).

2.3.4.4 The effect of cold treatment

Another attempted approach towards the regeneration of plantlets by somatic embryogenesis, was using a cold treatment (Kavathekar *et al.*, 1977). Cells in the exponential phase were plated onto solid callus induction medium (Section 2.3.3) without 2,4-D. Following callus formation, half of the calli were maintained at 25°C, while the rest were placed at 10°C for 72 hours. Thereafter they were returned to 25°C. After 4 weeks, the calli were screened for the presence of embryogenic structures before transfer onto medium containing activated charcoal, casein hydrolysate or a combination of both. In all cases, before transfer to, and after 6 - 8 weeks on these media, the cells in the calli contained both embryogenic and non-embryogenic cells (Figure 2.10), but no differentiated structures (Table 2.4 D).

2.3.4.5 The effect of ABA and/or PEG

ABA has been shown to aid in the maturation process of immature somatic embryos of spruce (*Picea glauca x engelmannii*) (Dunstan *et al.*, 1994). The same workers also reported that PEG promoted embryo maturation in the absence of ABA, i.e. PEG

could be used as an alternative to ABA, probably because PEG exposure resulted in an increase in the endogenous levels of ABA. Consequently the effects of these two factors, singly and in combination were investigated (Table 2.4 E). Callus was initiated on solid callus induction medium (Section 2.3.3). This callus was then subcultured onto solid medium (Section 2.3.3) supplemented with 12 mg.l⁻¹ ABA and/or 40 g.l⁻¹ PEG. Following a 4 to 6 week incubation on this medium, microscopic screening of the callus indicated the presence of embryoids, ranging from globular- to torpedo-shaped structures (Figure 2.11). The number of embryoids per gram fresh mass (Table 2.5) indicate that the medium containing PEG appears to be the most efficient for the induction of somatic embryogenesis. The embryoids that were formed on these media were then placed onto different embryo germination media (Section 2.2.6), but results are not yet available.

Table 2.5: Effect of different treatments on the average number of embryos formed per gram fresh mass. (n = 3)

Treatment	Number.g⁻¹fmass
ABA	0.57
PEG	19.52
PEG + ABA	13.52



Figure 2.10: Embryogenic (e) and non-embryogenic (ne) cells from callus derived from cell suspension cultures (bar = 30 μm).

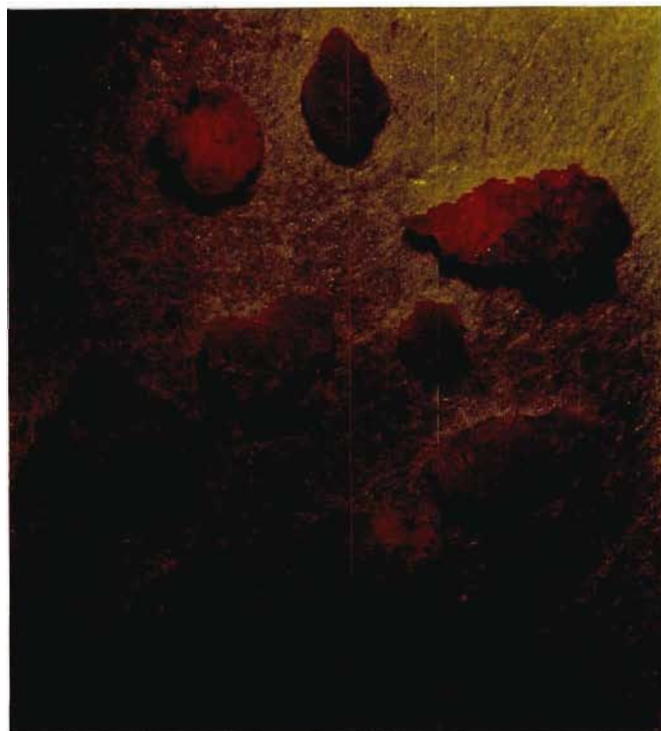


Figure 2.11: Embryoids produced on solid medium (Section 2.3.3) supplemented with 12 mg.l^{-1} ABA and/or 40 g.l^{-1} PEG (bar = 0.4 mm).

2.4 Conclusions

The *in vitro* production of plantlets from leaf discs of *E. grandis* has been achieved via indirect organogenesis. However, as mentioned previously, although the frequency of callus production was high, with 90.3 - 94.2 % of explants producing callus, the amount of callus produced per explant, and the subsequent shoot production was low. On average, 4 - 6 shoots were produced per explant, of which only approximately 43.5 % were successfully rooted. In order for the indirect organogenic technique to be commercially viable, improvements in yield are required.

The establishment and maintenance of cell suspension cultures for *E. grandis* and its hybrids has been achieved. However, at this stage, callus production from cell suspension cultures has, as yet, not led to plantlet regeneration, although many factors were manipulated in an attempt to obtain plantlet regeneration via organogenesis and embryogenesis. Recently, however, promising results have been obtained with the production of embryoids from cell suspension culture-derived callus. Therefore, it appears that the production of somatic embryos from cell suspension cultures of *Eucalyptus* could soon be accomplished, which would have great impact on the application of the cell suspension culture route for large-scale micropropagation, for the production of secondary metabolites, and for the production of transgenic plants.

CHAPTER 3: DEVELOPMENT OF GENETIC TRANSFORMATION PROTOCOLS FOR *Eucalyptus*

3.1 Literature Review

3.1.1 Factors influencing successful gene transfer into plant cells

There are three prerequisites for successful genetic transformation of a cell or tissue: introduction of the DNA into the cell, its integration into the host genome and the controlled expression of the introduced DNA (Lindsey and Jones, 1990a).

Introduction of DNA into a cell or tissue can be achieved in many ways, the most commonly used method being based on the natural gene transfer system of the soil bacterium *Agrobacterium tumefaciens* (Section 3.1.3.1). The host range of *A. tumefaciens* was initially thought to include only dicotyledonous species, however, T-DNA insertion has subsequently been demonstrated in some monocotyledonous plant species (Chan *et al.*, 1992; Hooykaas and Schilperoort, 1992; Chan *et al.*, 1993; Delbreil *et al.*, 1993; Ritchie *et al.*, 1993). Despite the fact that some monocotyledonous species are amenable to transformation by *Agrobacterium tumefaciens*, many species remain recalcitrant to this method, however, they can generally be genetically modified by other means, such as direct gene transfer (Section 3.1.3.2).

Agrobacteria also have the ability to insert a particular segment of foreign DNA (Section 3.1.3.1) into the plant genome, under the control of genetic elements within the bacterium, such as the promoter. The promoter is a regulatory element in the immediate vicinity of the transcription start site (Beilmann *et al.*, 1992), and is the DNA region which binds RNA polymerase and directs the enzyme to the correct transcriptional site so that RNA synthesis can begin (Oliver and Ward, 1985). Unless placed under the control of suitable promoter elements, bacterial genes are not transcribed after integration into the plant genome. *Agrobacterium* T-DNA is the

exception, since it contains its own promoter elements (Fraley *et al.*, 1983). Other genetic transformation methods rely on random insertion of DNA into the plant genome (Potrykus *et al.*, 1985). Successful integration is therefore influenced by factors such as DNA conformation, concentration and the type of vector used.

Different promoters have different efficiencies and thus transcriptional levels vary. The nopaline synthase (*nos*) and the 35S transcript promoters from the cauliflower mosaic virus (CaMV) are commonly used promoters (Cocking and Davey, 1987; Hensgens *et al.*, 1992). Chimeric genes that function in plants may thus be produced, and these are often marked with bacterial antibiotic-resistance genes.

An important limitation in the use of gene transfer technology is the fact that there is considerable inter-transformant variability in expression levels of introduced genes (Dunsmuir *et al.*, 1988; Hooykaas and Schilperoort, 1992; Ottaviani *et al.*, 1993). The factors governing expression of foreign genes and causing this variability in plants remain an enigma, although factors such as DNA methylation (Hooykaas and Schilperoort, 1992; Ottaviani *et al.*, 1993), DNA copy number and position in the host genome have been suggested to affect expression of the introduced gene (Gao *et al.*, 1991), and these factors may also function over long distances, such as chromatin folding (Dunsmuir *et al.*, 1988).

The correlation between copy number and gene expression in transformants has been reported to be positive (Gendloff *et al.*, 1990), indeterminate (Jones *et al.*, 1987; Dean *et al.*, 1988; Shirsat *et al.*, 1989) or negative (Hobbs *et al.*, 1990). This is further complicated by the fact that the introduction of additional copies of naturally occurring genes may have a repressive effect on gene expression (Hobbs *et al.*, 1993). The finding that transgenes can influence each other's expression as well as the expression of resident genes in transgenic plants (referred to as co-suppression) indicates that there is still much to be learnt about the nuclear processes involved in

gene regulation and genome maintenance (Kooter and Mol, 1993). One of the advances that have been made is in the chemical regulation of transgene expression in plants (Ward *et al.*, 1993), which allows for the manipulation of levels of gene expression in order to gain an understanding of the functions of individual genes. Although the constitutive expression of inserted genes is adequate at present, in the future, other inserted genes may be useful only if placed under exogenous control or regulation (Ward *et al.*, 1993).

3.1.2 Vectors for gene cloning in plants

An important part of recombinant DNA technology is the selection of a suitable vector (carrier) into which DNA sequences can be inserted (Sambrook *et al.*, 1989). In the context of plant genetic engineering, a vector may be defined as an agent which will facilitate one or more steps in the overall process of placing foreign genetic material into plants or their constituent parts (Mantell *et al.*, 1985a; Grierson and Covey, 1988). The term 'plant gene vector' applies to potential carriers for the transfer of genetic information both between plants and from other organisms, such as bacteria, to plants (Mantell *et al.*, 1985a). Likely candidates for vectors are those biological systems where entry of the nucleic acid usually occurs pathogenically (Grierson and Covey, 1984a), such as the T-DNA (transferred DNA) of the *Agrobacterium* Ti plasmid, which will be discussed in Section 3.1.3.1.

3.1.3 Commonly used strategies for gene transfer to plants

Advances in gene transfer technologies have enabled the production of both monocotyledonous and dicotyledonous transgenic plants (Jain *et al.*, 1992). Investigations have particularly concentrated on plant species which have a certain value or importance, either for economic or nutritional reasons (Sawahel and Covey, 1992). Because of the inherent limitations in all available gene transfer methods, it may be unrealistic to hope that a generally applicable method will be possible at all; a wide variety of methods may be the solution for future gene transfer problems

(Potrykus, 1991). In this regard, *Agrobacterium tumefaciens* has been found to be a very effective transfer system for genetic transformation in dicotyledonous plant species (Fillatti *et al.*, 1987) however, as mentioned most monocotyledonous plant species do not respond well to *Agrobacterium*-mediated transformation (Uchimiya *et al.*, 1989), and thus alternative direct DNA transfer methods have been developed for these species (Davey *et al.*, 1989; Sawahel and Cove, 1992; Aragao *et al.*, 1993). Transgenic plants, broadly speaking, refer to those plants in whose genomes functional foreign genes have been inserted (Uchimiya *et al.*, 1989).

3.1.3.1 *Agrobacterium*-mediated gene transfer

As mentioned, the best and most widely-used system for plant transformation is that based on the *Agrobacterium* tumour-inducing (Ti) plasmid (Grierson and Covey, 1984b; Uchimiya *et al.*, 1989; Zambryski, 1992). At present, this approach is limited by the host range of *Agrobacterium* (Nester, 1987; Binns, 1990), although an increased understanding of factors influencing host range may eventually extend these boundaries (Meredith, 1990). Because of its natural ability to genetically transform plant cells during infection, the Ti plasmid of the plant pathogen *A. tumefaciens* was identified as a potential gene vector for higher plants (Drummond, 1979; Owens and Galun, 1983). The potential of the *Agrobacterium* Ti plasmid as a vector arises from the ability of the bacterium to transfer, and stably integrate, a piece of plasmid DNA into the plant nuclear genome (Mantell *et al.*, 1985a; Uchimiya *et al.*, 1989; Fillatti, 1990).

Infection by the bacterium occurs at wound sites on the plant (Mantell *et al.*, 1985a; Grierson and Covey, 1984b; Fillatti, 1990) and results in crown gall disease. This disease is characterised by the formation of tumorous outgrowths (Grierson and Covey, 1984b; Cresswell, 1991) which produce metabolites that are utilised by the bacteria as their sole nitrogen and carbon source (Mantell *et al.*, 1985a; Ream, 1989). The ability to produce tumours on plants was found to be associated with the

possession by the bacterium of a large (more than 200 kb) Ti (tumour-inducing) plasmid (Mantell *et al.*, 1985a; Hooykaas and Schilperoort, 1992; Zambryski, 1992), while avirulent bacteria (unable to elicit tumours) do not carry the Ti plasmid (Mantell *et al.*, 1985a). In early experiments, expression was limited to the formation of tumour tissues, but subsequent findings indicated that the T-DNA could be 'disarmed' by deleting the oncogenic hormone biosynthetic genes, without interfering with its ability to integrate into plant chromosomes (Grierson and Covey, 1988). Thus the natural gene transfer system was modified in order to deliver desired genes into plant cells, with the elimination of the tumorous growth form (Mantell *et al.*, 1985a; Mozo and Hooykaas, 1992b).

The Ti plasmid contained in the bacterial cell carries most of the functions for DNA transfer and consists of two important genetic components, the T-DNA and the virulence (*vir*) region (Zambryski, 1992), with the latter being induced by compounds produced by the plant during the wound response (Stachel *et al.*, 1985; Zambryski *et al.*, 1989; Hooykaas and Schilperoort, 1992).

The T-DNA is flanked by 24 bp direct repeats, which are the only elements required *in cis* for the transfer process (Mozo and Hooykaas, 1992b). Thus, the genes that are to be transferred to plant cells are cloned between the border repeats. The second essential element, the *vir* gene complex, is the main cluster of genes controlling infection by *A. tumefaciens* (Stachel and Nester, 1986). The virulence (*vir*) region is localised on the Ti plasmid, but is not within the T-DNA (Fillatti, 1990), and it retains the ability to direct the insertion of foreign DNA into plant cells when it is located on a separate plasmid molecule (Grierson and Covey, 1988). During tumour formation, the T-DNA is transferred to the plant cell and integrated into the plant nuclear genome (Armitage *et al.*, 1988; Grierson and Covey, 1984b). The mechanisms of T-DNA transfer into plant cells are not completely understood, but they have been likened to bacterial conjugation (Zambryski *et al.*, 1989; Beijersbergen *et al.*, 1992;

Zambryski, 1992). The T-DNA is stable within the plant genome and no major rearrangements of the sequence take place (Armitage *et al.*, 1988; Hooykaas and Schilperoort, 1992). It was discovered, however, that none of the genes in the T-DNA are essential for the transfer and integration of T-DNA into the host genome (Armitage *et al.*, 1988), thus genes from unrelated plant, animal or bacterial sources, when inserted into the T-DNA, are transferred into the plant nuclear genome (Mantell *et al.*, 1985a). Hence, a vector system can be constructed where the *trans*-acting *vir* region is on a separate plasmid from that carrying the T-DNA of interest (Hoekema *et al.*, 1983; Jones *et al.*, 1992). This has allowed the development of the binary vector system (An *et al.*, 1988), in which the T-DNA is cloned into a broad-host-range plasmid, while the *vir* functions are supplied by a Ti plasmid from which the T-DNA has been deleted, i.e. a disarmed helper plasmid (De Framond *et al.*, 1983; Bevan, 1984; Mozo and Hooykaas, 1992b).

Transformation of plants through *Agrobacterium* results in the integration of at least one copy of the T-DNA into the genome of target cells (Jones *et al.*, 1987; Derolles and Gardner, 1988), however, the site of integration of the T-DNA into the plant is apparently random (Armitage *et al.*, 1988; Gao *et al.*, 1991). Due to the randomness of integration, it is possible that aberrations may result. For this reason, it is important that transformants are analysed to ensure the integrity of the inserted gene (Dunsmuir *et al.*, 1988), which may then be then transferred through Mendelian inheritance to the progeny (Goto *et al.*, 1993). There is, however, little information concerning the stability or instability of introduced genes in consecutive sexual generations (Sawahel and Cove, 1992).

A number of different explants have been used for *A. tumefaciens*-mediated transformation, such as shoot segments (Li *et al.*, 1992; Ritchie *et al.*, 1993), embryos (Chan *et al.*, 1993; Delbreil *et al.*, 1993), root segments (Li *et al.*, 1992; Warkentin and McHughen, 1992), stems (Ying *et al.*, 1992), cotyledons (Miljuš-Djukic *et al.*,

1992; Pawlicki *et al.*, 1992; Ying *et al.*, 1992; Warkentin and McHughen, 1992; Jacq *et al.*, 1993; Mansur *et al.*, 1993) and hypocotyls (Dong and McHughen, 1993; Jacq *et al.*, 1993), but the most commonly used explants are leaf discs (Horsch *et al.*, 1988; Cardi *et al.*, 1992; Chan *et al.*, 1992; Mozo and Hooykaas, 1992a; Ying *et al.*, 1992; Atkinson and Gardner, 1993; Grevelding *et al.*, 1993; Mansur *et al.*, 1993; Palmgren *et al.*, 1993). It is, however, vital that the methods exist for regeneration from the explant following transformation. The successful transformation of plants using *A. tumefaciens*-based vectors and the subsequent regeneration of transgenic plants has been achieved in a wide range of species, such as *Arabidopsis thaliana* (Sangwan *et al.*, 1992) *Oryza sativa* (Chan *et al.*, 1992; Li *et al.*, 1992; Chan *et al.*, 1993), *Daucus carota* (Pawlicki *et al.*, 1992), *Nicotiana glauca* (Mozo and Hooykaas, 1992a), *Asparagus officinalis* (Delbreil *et al.*, 1993), *Cyphomandra betacea* (Atkinson and Gardner, 1993), *Linum usitatissimum* (Dong and McHughen, 1993), *Nicotiana tabacum* (Palmgren *et al.*, 1993), *Solanum tuberosum* (Ottaviani *et al.*, 1993), *Zea mays* (Ritchie *et al.*, 1993) *Dendranthema grandiflora* (Pavingerova *et al.*, 1994), *Cucumis melo* (Vallés and Lasa, 1994) and *Glycine max* (Luo *et al.*, 1994).

Another tumorigenic plasmid, the Ri plasmid (responsible for hairy root disease) of *Agrobacterium rhizogenes*, is also being used as a vector for plant genetic manipulation (Grierson and Covey, 1988). An advantage of this system is that regeneration of whole plants from *A. rhizogenes*-transformed hairy roots is relatively simple compared with *A. tumefaciens*-transformed tissues (Grierson and Covey, 1984a). Further discussion on this plasmid is, however, beyond the scope of this report, but has been reviewed by White *et al.* (1985), Cardarelli *et al.* (1987) and Zambryski *et al.* (1989).

3.1.3.2 Direct gene transfer

Several techniques for direct DNA delivery are available and have been employed successfully in the production of transgenic plants. They range from uptake of DNA

into isolated protoplasts mediated by chemical procedures or electroporation, to injection and the use of high-velocity particles to introduce DNA into intact tissues (Davey *et al.*, 1989) (Table 3.1). The primary advantage of direct gene transfer systems is that they are not subject to host range restrictions (Fillatti, 1990; Lee *et al.*, 1991). Although the frequency of stable transformation is low, direct DNA uptake is applicable to those plants not amenable to *Agrobacterium*-mediated transformation, particularly monocotyledons (Davey *et al.*, 1989; Meredith, 1990). Using direct DNA transfer methods, foreign DNA is incorporated into the plant chromosome, although the mechanism whereby integration occurs is still unclear (Fillatti, 1990). For the purposes of this discussion, some of the more common direct gene transfer systems will be discussed.

As mentioned for *Agrobacterium*-mediated transformation (Section 3.1.3.1), different explants can be used for studies using the various direct gene transfer methods. Electroporation and PEG-mediated direct gene transfer methods generally make use of protoplasts (Lindsey and Jones, 1990b; Lyznik *et al.*, 1991; Gallie, 1993; Zaghmout and Trolinder, 1993; Zhou *et al.*, 1993; Mukhopadhyay and Desjardins, 1994; Sagi *et al.*, 1994), while the explants used for microprojectile bombardment include calli (Vasil *et al.*, 1992; Li *et al.*, 1993), meristems (Aragao *et al.*, 1993; Bilanz *et al.*, 1993), leaf pieces (Prakash and Varadarajan, 1992; Aragao *et al.*, 1993; Gallo-Meagher and Irvine, 1993; Godon *et al.*, 1993), petiole pieces (Prakash and Varadarajan, 1992), cell suspension cultures (Wang *et al.*, 1988; Cao *et al.*, 1992; Vain *et al.*, 1993), embryos (Aragao *et al.*, 1993; Li *et al.*, 1993) and cotyledons (Aragao *et al.*, 1993). The use of explants other than protoplasts circumvents the regeneration problem associated with this type of explant (Sawahel and Cove, 1992). Therefore the most commonly used method of direct gene transfer appears to be microprojectile bombardment (Table 3.1).

Table 3.1: Examples of some transgenic plants obtained using direct DNA transfer methods. .

Species	Transformation Method	Reference
<i>Asparagus officinalis</i>	electroporation	Mukhopadhyay and Desjardins, 1994
<i>Daucus carota</i>	PEG	Gallie, 1993
<i>Glycine max</i>	microprojectile bombardment	Wang <i>et al.</i> , 1988
<i>Ipomoea batatas</i>	microprojectile bombardment	Prakash and Varadarajan, 1992
<i>Musa</i> spp.	electroporation	Sagi <i>et al.</i> , 1994
<i>Nicotiana tabacum</i>	microprojectile bombardment	Godon <i>et al.</i> , 1993
<i>Oryza sativa</i>	electroporation	Xu and Li, 1994
<i>Oryza sativa</i>	microprojectile bombardment	Wang <i>et al.</i> , 1988
<i>Oryza sativa</i>	microprojectile bombardment	Li <i>et al.</i> , 1993
<i>Oryza sativa</i>	microprojectile bombardment	Cao <i>et al.</i> , 1992
<i>Phaseolus vulgaris</i>	microprojectile bombardment	Aragao <i>et al.</i> , 1993
<i>Saccharum</i> spp.	microprojectile bombardment	Gallo-Meagher and Irvine, 1993
<i>Triticum aestivum</i>	electroporation	Zhou <i>et al.</i> , 1993
<i>Triticum aestivum</i>	electroporation	Zaghmout and Trolinder, 1993
<i>Triticum aestivum</i>	microprojectile bombardment	Bilang <i>et al.</i> , 1993
<i>Triticum aestivum</i>	microprojectile bombardment	Vasil <i>et al.</i> , 1992
<i>Triticum monococcum</i>	microprojectile bombardment	Wang <i>et al.</i> , 1988
<i>Zea mays</i>	PEG	Lyznik <i>et al.</i> , 1991
<i>Zea mays</i>	electroporation	Fromm <i>et al.</i> , 1986
<i>Zea mays</i>	microprojectile bombardment	Vain <i>et al.</i> , 1993

PEG

The first direct DNA uptake method to be used with plant cells, involved treating the plant protoplasts with chemicals, such as polyethylene glycol (PEG) (Krens *et al.*, 1982; Paszkowski *et al.*, 1984; Fillatti, 1990). These chemicals increase membrane permeability by creating reversible channels in the plasma membrane, through which the DNA passively diffuses into the cytoplasm (Sawahel and Cove, 1992). A portion of the DNA then enters the nucleus and integrates into the plant genome. The disadvantage of the use of protoplasts is their recalcitrance to regeneration (Joersbo and Brunstedt, 1991). Some examples are presented in Table 3.1. More recently Lee *et al.* (1991) reported the transformation of small cell groups of rice by PEG-mediated DNA delivery, without removal of cell walls. This approach circumvents the need for protoplast isolation and the difficult and lengthy step of protoplast regeneration.

Electroporation

A variation of the gene transfer method using PEG, entails applying an electrical impulse to plant protoplasts to create reversible pores in the plasma membrane through which DNA can move, presumably by diffusion (Fromm *et al.*, 1986; Lindsey and Jones, 1990b; Potrykus, 1991; Sawahel and Cove, 1992). This electric field-mediated membrane permeabilisation technique (Van Wert and Saunders, 1992), referred to as electroporation, improved the efficiency and consistency of transformation in some species (Fillatti, 1990). However the applicability of this technique is limited (examples in Table 3.1), since it also requires regeneration of plants from protoplasts (Joersbo and Brunstedt, 1991; Sawahel and Cove, 1992).

Microinjection

The microinjection method of gene transfer has been used for many years to introduce foreign DNA into animal cells (Capecchi, 1980). This technique, applied to plant protoplasts, makes use of finely drawn out capillaries, which are used to deliver DNA directly into the cytoplasm or nucleus of a cell (Aly and Owens, 1987; Fillatti, 1990;

Neuhaus and Spangenberg, 1990; Sawahel and Cove, 1992). However, this procedure is time-consuming and laborious and is unlikely to become a routine gene transfer method (Fillatti, 1990).

Microprojectile bombardment

The cell wall is the main limitation to direct gene transfer into intact plant cells, and thus in all the previously-mentioned direct gene transfer methods protoplasts have to be used (Yang *et al.*, 1993). However, the more recently-developed technique of microprojectile bombardment ('biolistics') facilitates the transfer of exogenous DNA into intact plant cells *in situ* (Christou *et al.*, 1991; Batty and Evans, 1992; Christou, 1993; Yang *et al.*, 1993; Brown *et al.*, 1994). This technique makes use of high velocity microprojectiles, which are small enough so that penetration is not lethal (Klein *et al.*, 1990), and are used to carry DNA or other substances past cell walls and membranes (Sanford, 1990). The microprojectiles are accelerated into target cells using various devices (Rasmussen *et al.*, 1994), such as those powered by pressurised helium (Sautter *et al.*, 1991; Seki *et al.*, 1991; Brown *et al.*, 1994) or a gunpowder charge (Klein *et al.*, 1987). Bombardment of plant tissues by microprojectiles has also recently been shown to be an effective method of wounding to promote *Agrobacterium*-mediated transformation (Bidney *et al.*, 1992).

An advantage of the use of the biolistic process over other plant transformation techniques is that it appears to be effective regardless of species or tissue type (Sanford, 1990; Sawahel and Cove, 1992), and can thus be used for species recalcitrant to other transformation methods, such as monocotyledons (Sawahel and Cove, 1992) (Table 3.1). There are, however, limits to the efficiency of gene transfer to living cells, which are factors such as target area and particle size (Klein *et al.*, 1988; Sautter *et al.*, 1991). The process also requires adaptation of existing protocols to specific species or tissue types of interest (Sawahel and Cove, 1992).

Other novel strategies (silicon carbide fibres, sonication)

Kaepler *et al.* (1990) reported a novel transformation method for DNA delivery to plant cells. These workers used suspension culture cells of *Zea mays* and *Nicotiana tabacum*, which were vortexed with silicon carbide fibres and plasmid DNA containing a selectable marker gene (*gus*) (Section 3.1.4) to enable detection of inserted DNA. The silicon carbide fibres apparently act as tiny microinjection needles and facilitate DNA delivery into intact plant cells. This method seems to be simple, rapid and less expensive than other methods, as well as being applicable to all plant species for which protocols of regeneration from cell suspension cultures are available (Kaepler *et al.*, 1990). Recently, Wilson *et al.* (1994) reported the production of fertile transgenic maize plants using silicon carbide fibre-mediated transformation.

Another approach that has been reported for plant cell transformation is one utilising mild sonication. Joersbo and Brunstedt (1990) developed a unique and efficient method for the transfer of plasmid DNA into plant protoplasts of *Beta vulgaris* and *Nicotiana tabacum*. This involved a brief exposure of protoplasts to ultrasonic waves, which facilitated the uptake of plasmid DNA, without affecting plant cell metabolism. This method is also simple and inexpensive, but is limited by the lack of protocols for plant regeneration from protoplasts.

3.1.4 Genetic markers for plant transformation

One of the most important advances in gene transfer to plant cells, was the development of genetic markers, which can be used to show that foreign DNA is integrated into the plant genome and is being expressed (Reynaerts *et al.*, 1988; Walden, 1988). Genetic markers can be separated into reporter genes and selectable marker genes. Reporter genes can be used to study transient gene expression, which is easily detected (Herrera-Estrella *et al.*, 1988), occurs cytoplasmically and does not require chromosomal integration, such as the gene encoding β -glucuronidase (*gus*) (Jefferson *et al.*, 1987; Jefferson and Wilson, 1991; Hodal *et al.*, 1992; Vitha *et al.*,

1993). On the other hand, selectable marker genes allow the direct selection of transgenic cells by their ability to grow and proliferate under selective conditions (Walden, 1988; Lindsey and Jones, 1990a), such as the gene encoding neomycin phosphotransferase (*nptII*), which confers resistance to the antibiotic kanamycin (Bevan *et al.*, 1983; Herrera-Estrella *et al.*, 1988; Carrer *et al.*, 1993) and the *sulI* gene conferring resistance to sulfonamides (Guerineau *et al.*, 1989; Guerineau *et al.*, 1990). Less commonly used selectable marker genes include those conferring resistance to various herbicides (Mullineaux, 1992), such as dalapon (Buchanan-Wollaston *et al.*, 1992), the gene encoding for bacterial aspartate kinase (Perl *et al.*, 1993) and the gene encoding a bacterial dihydrodipicolinate synthase (Perl *et al.*, 1993).

3.1.5 Engineering plants to contain useful agronomic traits

As the techniques of gene transfer have developed, most attention has been focused on their potential application in crop improvement, with the aim of engineering specific traits into a wide variety of plants (Walden, 1988). Currently, the greatest constraint of genetic engineering on plant improvement is the availability of only a limited number of cloned genes for use in plant improvement (Jain *et al.*, 1992). As researchers characterise new genes that can add further value to crop plants (Jones *et al.*, 1992), transgenic plants will additionally have a greater applied significance.

Many desirable characteristics, such as enhanced vigour or increased yield, are likely to be controlled by a number of interacting processes, which have to be determined before attempting to isolate the genes of interest (Walden, 1988). Because of this, attention has been focused largely on characters which may be determined by single genes (Burr and Burr, 1985; Walden, 1988; Pimentel *et al.*, 1989; Schulz *et al.*, 1990; Mullineaux, 1992), such as resistance to herbicides (Comai *et al.*, 1985; Chaleff, 1988; Hathaway, 1989a,b,c; Mazur and Falco, 1989; Schulz *et al.*, 1990; Smith and Chaleff, 1990; Mullineaux, 1992) and diseases or pests (Cuozzo *et al.*, 1988; Raffa, 1989; van den Elzen *et al.*, 1989; Grumet, 1990; van den Bulk, 1991; Bejarano and Lichtenstein,

1992; Harms, 1992; Broglie and Broglie, 1993; Smigocki *et al.*, 1993; Baulcombe, 1994).

Resistances to herbicides are controlled by genes such as *als*, encoding acetolactate synthase (Chaleff, 1988; Haughn *et al.*, 1988) and *aroA*, encoding EPSP synthase (3-phosphoshikimate-1-carboxyvinyl-transferase) (Comai *et al.*, 1985), while resistances to viruses are regulated by genes such as CP-TMV, the coat protein gene of tobacco mosaic virus (Cuozzo *et al.*, 1988) and CP-PVX, the coat protein gene of potato virus X (van den Elzen *et al.*, 1989). Disease resistance, on the other hand, is brought about by the genes such as the *Hm* gene, encoding HC-toxin reductase (Broglie and Broglie, 1993) and the *ipt* gene, encoding bacterial isopentenyl transferase, involved in cytokinin biosynthesis (Smigocki *et al.*, 1993).

3.1.6 Genetic modification of woody species

Although traditional hybridisation methods have been utilised effectively in many plant species for the transfer of specific genes, these procedures take too long, and are impractical in long-lived forest species (Riemenschneider *et al.*, 1987; Ahuja, 1987b, 1988). The use of gene transfer techniques, such as *Agrobacterium*-mediated transformation (Section 3.1.3.1) or direct gene transfer (Section 3.1.3.2) have led to the production of transgenic plants of a number of woody species (Tables 3.2 and 3.3, respectively).

Regardless of the gene transfer method used, regeneration of plants is required after genetic modification, yet regeneration has been difficult to achieve with forest trees (Riemenschneider *et al.*, 1987), and this has limited the success obtained. Also, woody plants are, in general, difficult to transform with *Agrobacterium tumefaciens*, which may be due to the host range of *A. tumefaciens* (Ahuja, 1988). For both these reasons, the list of transgenic woody plants obtained via *Agrobacterium*-mediated

gene transfer (Table 3.2) is not extensive, when compared with that of non-woody species (Uchimiya *et al.*, 1989).

Table 3.2: Examples of transgenic woody plants obtained by *Agrobacterium*-mediated gene transfer.

Host Plant	Foreign Genes	Reference
<i>Carya illinoensis</i>	<i>gus, aph(3')II</i>	McGranahan <i>et al.</i> , 1993
<i>Eucalyptus globulus</i>	<i>gus</i>	Chriqui <i>et al.</i> , 1991
<i>Eucalyptus gunnii</i>	<i>gus</i>	Chriqui <i>et al.</i> , 1991
<i>Malus pumila</i>	<i>gus</i>	Martin <i>et al.</i> , 1990
<i>Malus x domestica</i>	<i>neo, gus, kn</i>	Sriskandarajah <i>et al.</i> , 1994
<i>Populus alba x grandidentata</i>	<i>cat, nptII</i>	Klopfenstein <i>et al.</i> , 1993
<i>Populus alba x P. tremula</i>	<i>neo, bar</i>	De Block, 1990
<i>Populus nigra</i>	<i>gus, nptII</i>	Confalonieri <i>et al.</i> , 1994
<i>Populus trichocarpa x P. deltoides</i>	<i>neo, bar</i>	De Block, 1990
<i>Prunus dulcis</i>	<i>gus, kn</i>	Miguel <i>et al.</i> , 1994
<i>Prunus persica</i>	<i>gus</i>	Martin <i>et al.</i> , 1990
<i>Robinia pseudoacacia</i>	<i>nptII</i>	Han <i>et al.</i> , 1993
<i>Rubus clones</i>	<i>cat, gus, nptII</i>	Hassan <i>et al.</i> , 1993
<i>Vitis vinifera</i>	<i>gus, nptII</i>	Baribault <i>et al.</i> , 1990

Abbreviations: *aph(3')II* = aminoglycoside phosphotransferase II; *bar* = encodes for enzyme phosphinotricin

acetyltransferase; *cat* = chloramphenicol acetyltransferase; *gus* = β -glucuronidase; *kn* = kanamycin resistance;

neo/npt = neomycin phosphotransferase.

Table 3.3: Examples of transgenic woody plants obtained by direct gene transfer methods.

Host Plants	Foreign Genes	Transfer Methods	Reference
<i>Actinidia deliciosa</i>	<i>gus, nptII</i>	PEG	Oliveira <i>et al.</i> , 1994
<i>Actinidia deliciosa</i>	<i>gus, nptII</i>	electroporation	Oliveira <i>et al.</i> , 1994
<i>Citrus sinensis</i>	<i>aph(3')II</i>	PEG	Kobayashi and Uchimiya, 1989
<i>Hevea brasiliensis</i>	<i>gus, nptII, cat</i>	microprojectile bombardment	Arokiaraj <i>et al.</i> , 1994
<i>Larix</i> spp.	<i>gus, nptII</i>	microprojectile bombardment	Duchesne <i>et al.</i> , 1993
<i>Liriodendron tulipifera</i>	<i>gus, nptII</i>	microprojectile bombardment	Wilde <i>et al.</i> , 1992
<i>Picea abies</i>	<i>gus, neo</i>	microprojectile bombardment	Robertson <i>et al.</i> , 1992
<i>Picea glauca</i>	<i>gus, cat</i>	PEG	Wilson <i>et al.</i> , 1989
<i>Picea glauca</i>	<i>gus, nptII</i>	microprojectile bombardment	Bommineni <i>et al.</i> , 1993
<i>Picea glauca</i>	<i>gus, nptII</i>	microprojectile bombardment	Charest <i>et al.</i> , 1993
<i>Picea glauca</i>	<i>gus, nptII</i>	microprojectile bombardment	Ellis <i>et al.</i> , 1993
<i>Picea mariana</i>	<i>gus, nptII</i>	microprojectile bombardment	Bommineni <i>et al.</i> , 1993
<i>Pinus radiata</i>	<i>gus, luc</i>	microprojectile bombardment	Campbell <i>et al.</i> , 1992
<i>Pinus radiata</i>	<i>gus, luc</i>	electroporation	Campbell <i>et al.</i> , 1992
<i>Populus tremula</i> x <i>P. alba</i>	<i>nptII, als, pat</i>	electroporation	Chupeau <i>et al.</i> , 1994
<i>Pseudotsuga menziesii</i>	<i>gus</i>	microprojectile bombardment	Goldfarb <i>et al.</i> , 1991

Abbreviations: *als* = acetolactate synthase; *aph(3')II* = aminoglycoside phosphotransferase II; *cat* = chloramphenicol acetyltransferase; *gus* = β -glucuronidase; *luc* = luciferase; *npt/neo* = neomycin phosphotransferase; *pat* = phosphinotricin acetyltransferase.

As mentioned previously (Section 3.1.3.2), certain methods of direct gene transfer, such as PEG-mediated gene transfer and electroporation (Section 3.1.3.2), require totipotent protoplasts, and thus methods for regeneration from protoplasts in order to

recover transgenic plants. It is not surprising therefore, that the most commonly used technique for direct gene transfer into woody species has been microprojectile bombardment (Table 3.3).

3.1.7 Release of engineered plants in the field

Despite the significant advances made in crop improvement, the commercialisation of genetically modified plants has been hampered by concerns about the impact of these plants on the environment and on human health (Nap *et al.*, 1992; Sawahel, 1994). The genes inserted into plants to date include those that confer resistance to diseases, pests and herbicides (Dale, 1993), and there is concern that the modified organisms may become pests, or will produce pests as a result of mating with other organisms in the environment (Beringer *et al.*, 1992; Dale, 1993). The need for a science-based approach to establish the safety of genetically modified plants and plant products was identified by workers in the field (Krugman, 1988; Pimentel *et al.*, 1989; Fuchs and Perlak, 1992), who decided that an assessment of the risks of introducing such plants into the environment should be based on the nature of the organism and the environment into which it is to be introduced, and not on the method by which it was produced (Dale, 1993; Sawahel, 1994). Gasser and Fraley (1992) propose that crops modified by molecular and cellular methods should pose risks no different from those modified by classical methods for the same traits. At present, 'release committees' exist in a number of countries (Beringer *et al.*, 1992), but there does not seem to be an internationally accepted framework for dealing with this issue. The concept of biological containment of transgenic plants was formulated to prevent the escape and reproduction of genetically engineered plants until adequate field testing has been conducted. For forest species, this does not seem to be a major constraint for adequate field testing (Krugman, 1988). It seems that regulation and guidance in this area are part of an evolutionary process aimed at achieving the correct balance between risks and benefits (Hull, 1991).

3.2 Materials and Methods

3.2.1 Bacterial strains and plasmid

The various *Escherichia coli* and *A. tumefaciens* strains used in this study to produce the transconjugant *A. tumefaciens* LBA4404 (pJIT119), and the antibiotics to which they are resistant, are shown in Table 3.4. The plasmid (pJIT119) was obtained from Dr Mullineaux of the John Innes Institute (Norwich, U.K.), and its configuration is shown in Figure 3.1. It carries sequences for kanamycin resistance (*km*, *neo*), GUS activity (*gus*) and sulfonamide resistance (*sul*) that may be used in the selection of transformants. The *km* sequence confers resistance to the bacterial strains during colony selection, while the *neo* sequence confers resistance to the plant cells following transformation. In addition, *A. tumefaciens* C58C1 (pMP90) (pJIT119), was obtained from Dr S. MacRae (Forestek, CSIR, Durban).

Table 3.4: The various bacterial strains used in this study, indicating their functions, the antibiotics and the antibiotic concentrations to which they are resistant.

Bacterial strain	Function	Antibiotics
<i>E. coli</i> pJIT119	donor	50 $\mu\text{g.ml}^{-1}$ kanamycin
<i>E. coli</i> HB101::pRK2013	helper	100 $\mu\text{g.ml}^{-1}$ kanamycin
<i>A. tumefaciens</i> LBA4404	recipient	100 $\mu\text{g.ml}^{-1}$ rifampicin
<i>A. tumefaciens</i> LBA4404 (pJIT119)	transconjugant	50 $\mu\text{g.ml}^{-1}$ kanamycin + 50 $\mu\text{g.ml}^{-1}$ rifampicin
<i>A. tumefaciens</i> C58C1 (pMP90) (pJIT119)	transconjugant	50 $\mu\text{g.ml}^{-1}$ kanamycin + 50 $\mu\text{g.ml}^{-1}$ rifampicin + 40 $\mu\text{g.ml}^{-1}$ gentamycin

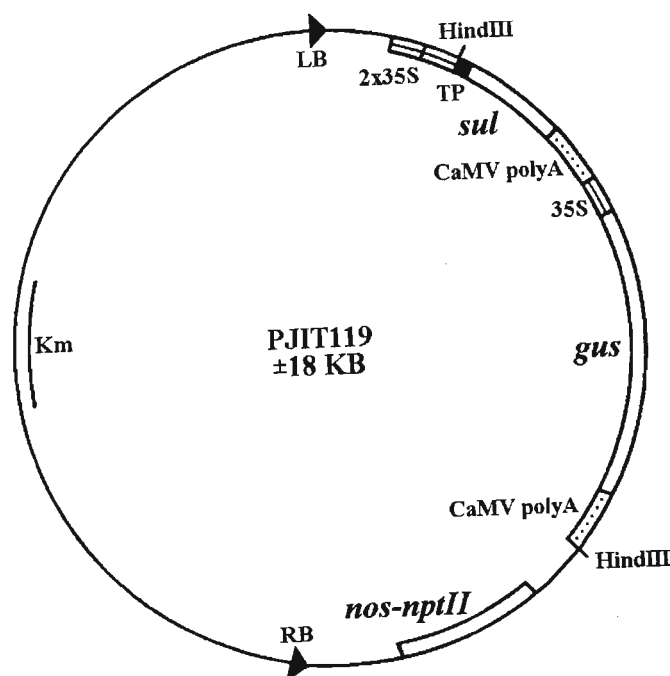


Figure 3.1: Organisation of plasmid pJIT119. The *sulI* coding sequence from pJIT92 (Guerineau *et al.*, 1990) was fused to a sequence coding for the RUBP-carboxylase small subunit (RUBISCO) transit peptide (TP). The TP-*sulI* coding sequence was placed between the CaMV 35S promoter and the CaMV gene VI polyadenylation sequences. The chimeric *sulI* gene was inserted between the left and right T-DNA borders of the binary vector pBIN19 (Bevan, 1984), which also carried the β -glucuronidase (*gus*) gene (Jefferson *et al.*, 1987) placed under the action of the 35S promoter. The plasmid (approximately 18 kb) confers resistance to both kanamycin (*km*, *neo*) and sulfonamides (*sul*) (Guerineau *et al.*, 1989).

3.2.1.1 Growth and maintenance

The *E. coli* strains were maintained and grown when necessary on Luria Bertani medium (LA) containing 10 g.l⁻¹ Bactotryptone, 5 g.l⁻¹ yeast extract, 0.5 g.l⁻¹ NaCl and 2 g.l⁻¹ glucose, pH 7.0, agar was added to 1.5 % (w/v). *A. tumefaciens* strains were maintained and grown in GT medium containing 3 g.l⁻¹ yeast extract, 10 g.l⁻¹ tryptone, 2 g.l⁻¹ glucose, 10 g.l⁻¹ sodium glycerophosphate, 1 g.l⁻¹ Tris and 0.04 g.l⁻¹ CaCl₂, pH 7.4, agar was added as for LA. When liquid media were required, the agar was omitted from the formulations (LB and liquid GT, respectively).

Cultures of all the strains, including the transconjugants, were stored in three ways: (1) they were maintained on plates of appropriate medium and antibiotics, in the fridge at 4°C for 3 - 4 weeks; (2) prepared stab cultures were kept in the dark, at room temperature, for up to 6 months; and (3) samples from each strain were stored, in 40 % glycerol, at -80°C (Draper *et al.*, 1988). When actively growing cultures were required, these stock cultures were used to inoculate liquid media, which were incubated in a shaking incubator (G24 Environmental Incubator Shaker, New Brunswick Scientific Co., U.S.A.) at the appropriate temperatures: *E. coli* cultures were prepared in LB at 37°C while *A. tumefaciens* cultures were prepared in GT at 28°C for 48 hours (time found to be required to obtain cultures in the exponential growth phase under these growth conditions).

3.2.1.2 Cell number

The relationship between optical density (OD) (at 660 nm) and cell number for each *A. tumefaciens* strain [(LBA4404 (pJIT119) and C58C1 (pMP90) (pJIT119)] was determined by serial dilutions (Figure 3.2). This was calculated as 0.6 x 10⁸ cells.ml⁻¹ corresponding to an OD of 1.2 for LBA4404 (pJIT119) and 0.2 x 10⁸ cells.ml⁻¹ for a culture with an OD of 0.8 for C58C1 (pMP90) (pJIT119).

3.2.2 Production of transconjugant *A. tumefaciens* LBA4404 (pJIT119)

3.2.2.1 Triparental mating

The protocol used was modified from that of Armitage *et al.* (1988). Single colonies of the *E. coli* donor strain pJIT119, the *E. coli* helper strain HB101::pRK2013 and the recipient *A. tumefaciens* LBA4404 were each grown for 48 hours, as described previously (Section 3.2.1).

The optical density of each culture was measured at 660 nm and when necessary, the cultures were diluted with liquid GT. Then, 0.5 ml of each strain were mixed and microfuged (36 g) for 30 seconds, and resulting pellet was resuspended in 1 ml liquid medium. From this, 200 μ l was plated onto solid GT; this triparental mating or conjugation was performed in triplicate. The plates were incubated at 28°C for 48 hours in order for the mating to proceed. Some of the colonies (potential transconjugants) were scraped off the plates and resuspended in 1 ml liquid GT. These cells were then streaked onto solid GT with 50 μ g.ml⁻¹ kanamycin (Sigma, U.S.A.) and 50 μ g.ml⁻¹ rifampicin (Sigma, U.S.A.), and allowed to incubate for two to three days at 28°C. The resultant colonies were restreaked onto fresh antibiotic plates and incubated for three days before being stored under the conditions described in Section 3.2.1.

3.2.2.2 DNA extraction

Plasmid DNA was extracted using the mini-preparation procedure of Kado and Liu (1981), which results in the selective isolation of plasmid DNA, which can be used in electrophoretic analysis. Overnight cultures of the various bacterial strains were microfuged for 30 seconds (36 g), and the pellets were resuspended in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). Lysis was accomplished by the addition of 3 % SDS (w/v) (in 50 mM Tris-hydroxide, pH 12.6). The samples were

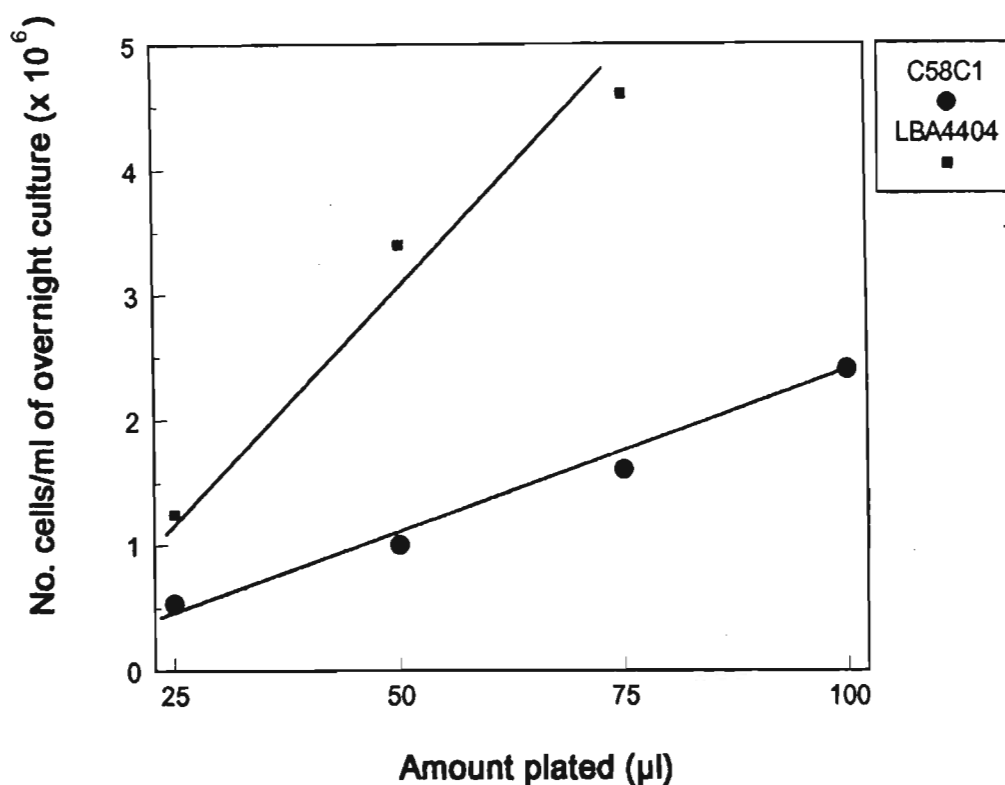


Figure 3.2: The relationship between volume plated and cell number for *A. tumefaciens* strains C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119). From overnight cultures of the two *Agrobacterium* strains, 1 ml of culture was used to measure the optical density (OD) at 660 nm, which was found to be 1.2 for LBA4404 (pJIT119) and 0.8 for C58C1 (pMP90) (pJIT119). From these cultures, dilutions were made and different volumes of bacterial culture were plated onto solid GT plates containing the appropriate antibiotics. Resulting colonies were counted, and the cell number per ml of overnight culture was calculated. For C58C1 (pMP90) (pJIT119), an overnight culture with an OD of 0.8 was calculated to contain 0.2×10^8 cells.ml⁻¹, while for LBA4404 (pJIT119), an OD of 1.2 corresponded to 0.6×10^8 cells.ml⁻¹ of overnight culture.

heat-treated in hot water (100°C for 1 minute for *Agrobacterium*; 65°C for 5 minutes for *E. coli*) and then placed immediately on ice for 5 minutes. Extraction with buffered phenol-chloroform was followed by microfugation (36 g for 20 min). The non-viscous, top layer was removed and stored at 4°C.

3.2.2.3 Agarose gel electrophoresis

A DNA grade agarose (Biorad, U.S.A.) gels (0.8 - 1.0 % w/v) was run in TBE buffer, and pBR322 (Boehringer Mannheim, Germany) was used as a standard in the gel. The loading mixtures of the plasmid DNA samples were as follows: (1) DNA samples: 16 µl sample DNA, 4 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and 5 µl loading buffer (50 % sucrose (w/v), 4 M urea, 0.1 % bromophenol blue, 10 mM EDTA), and (2) pBR322 standard: 2 µl standard, 4 µl TE buffer and 3 µl loading buffer. A total of 20 µl was loaded for each DNA sample and 8 µl was loaded for the pBR322 standard. A voltage of 60 V was applied across the 7 cm by 10 cm Hoefer (HE 33) horizontal gel apparatus for about two hours. The gel was then stained for 15 minutes with 0.5 µg.ml⁻¹ ethidium bromide (Sigma, U.S.A.), destained (15 minutes) in tap water, and then viewed under ultraviolet light using a transilluminator.

3.2.3 Plant cell transformation

3.2.3.1 Curing of *A. tumefaciens*

The effectiveness of cefotaxime (Claforan - Roussel Laboratories, S.A.) and augmentin (Beecham Pharmaceuticals (Pty) Ltd, S.A.) as bacteriostatic agents were investigated by exposing bacterial strains to increasing concentrations of the antibiotics. Samples from actively growing liquid cultures of the two *A. tumefaciens* strains, C58C1 (pMP90) (pJIT119) (0.2×10^8 cells.ml⁻¹) and LBA4404 (pJIT119) (0.6×10^8 cells.ml⁻¹), were spread onto plates consisting of solid GT supplemented with the appropriate selective agents (Section 3.2.1). Wells of diameter 1.9 cm were made in the solid media using an autoclaved cork-borer. Solutions (2 ml) of the various concentrations of the two curing antibiotics, cefotaxime and augmentin, were

placed in the wells, and the diameter of the zones of inhibition were recorded after 24 hours at 28°C (Figure 3.3, A, B).

3.2.3.2 Plant transformation protocol

Leaf discs

The basic transformation protocol was based on the classical approach reported for many other species (see protocol for tobacco in Draper *et al.*, 1988) and was performed with *A. tumefaciens* C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119). Leaf pieces were cut from sterile shoots produced *in vitro* (Sections 2.2.2 and 2.2.3), and they were placed upside down in petri dishes (9 cm in diameter) containing 20 ml liquid MS medium (MS nutrients, 30 g.l⁻¹ sucrose and 4 g.l⁻¹ Gelrite, pH 5.7), to which 6.25 x 10⁶ bacterial cells [either C58C1 (pMP90) (pJIT119) or LBA4404 (pJIT119)] had been added. Ten leaf pieces were placed in each petri dish. The petri dishes were incubated at low light intensity (50 µE.m⁻².s⁻¹ for 6, 16 or 24 hours). The leaf pieces were then placed for 1 hour in liquid MS medium containing 750 µg.ml⁻¹ of the appropriate curing antibiotic [cefotaxime for *A. tumefaciens* C58C1 (pMP90) (pJIT119) or augmentin for *A. tumefaciens* LBA4404 (pJIT119)] (Section 3.2.3.1, Figure 3.3). Control leaf pieces were incubated in liquid MS medium containing no bacterial cells or curing antibiotics. Then the leaf pieces were blotted dry on sterile absorbent paper and placed onto callus induction medium (Section 2.2.5) supplemented with 750 µg.ml⁻¹ of the appropriate curing antibiotic, with or without 50 µg.ml⁻¹ kanamycin. The explants were subcultured onto fresh medium regularly (every 2 - 3 days), and after curing was complete (2 - 3 weeks), the curing antibiotic was omitted from the medium.

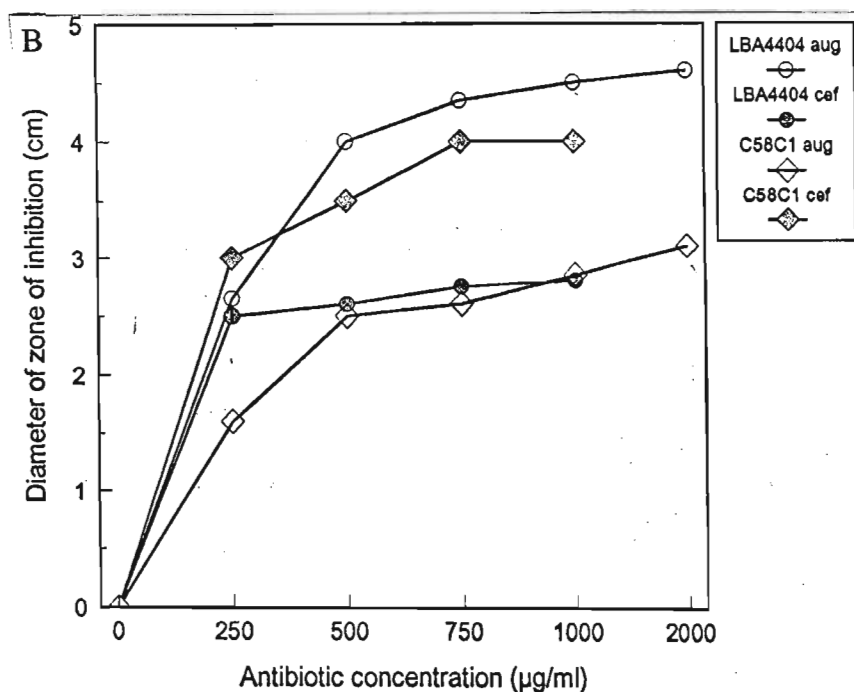
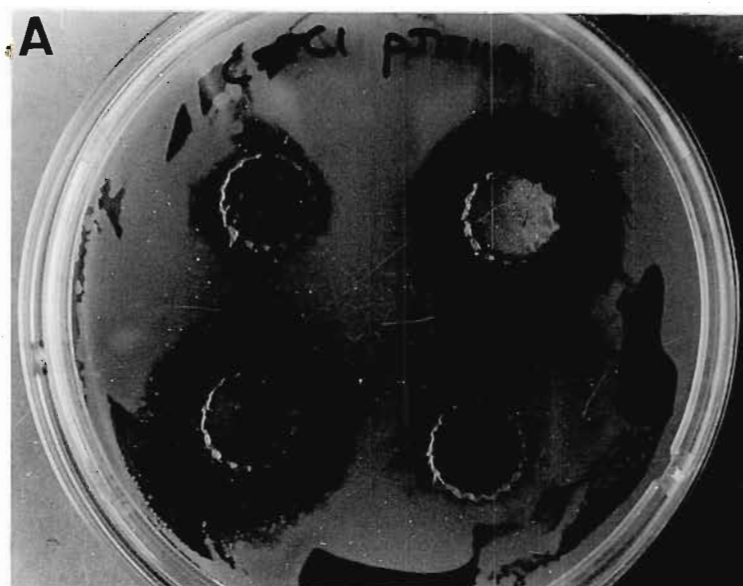


Figure 3.3: Effectiveness of various concentrations of cefotaxime and augmentin as bacteriostatic agents for *A. tumefaciens* strains, C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119). Samples from liquid cultures of the two strains were spread onto plates consisting of solid GT and the appropriate selective agents (Section 3.2.1). (A) Wells of diameter 1.9 cm were made in the media with an autoclaved cork-borer. (A, B) Solutions (2 ml) of the various concentrations of augmentin (aug) and cefotaxime (cef) were placed in the wells and the diameters of the zones of inhibition were recorded after 24 hours at 28°C.

Cell suspension cultures

The developed protocol is a modification of that reported for embryogenic carrot cell suspension cultures (Draper *et al.*, 1988). Feeder plates, consisting of 2 ml cell suspension culture (1.3×10^3 cells.ml⁻¹) and 15 ml molten nutrient medium (comprising MS nutrients, 30 g.l⁻¹ sucrose, 3 mg.l⁻¹ 2,4-D and 4 g.l⁻¹ Gelrite, pH 5.7), were prepared. A filter paper guard disc (8.5 cm in diameter) was placed on the surface of the feeder plate and a smaller filter paper transfer disc (5.5 cm in diameter) was placed over the centre of the guard disc. The plates were incubated in the dark at 25°C for 4 days. Thereafter the feeder plates were seeded by plating 0.5 ml of cell suspension (1.3×10^3 cells.ml⁻¹) onto the transfer disc. Following 7 days of incubation in the dark at 25°C, the plant cells were inoculated with 300 µl of various dilutions (1:1, 1:100, 1:250) of an overnight culture (0.2×10^8 cells.ml⁻¹) of the bacterial strain C58C1 (pMP90) (pJIT119). Transformation was allowed to proceed for 4 days before curing repeatedly with 750 µg.ml⁻¹ cefotaxime. Selection of transformed cells was undertaken in media containing 50 µg.ml⁻¹ kanamycin and/or 50 µg.ml⁻¹ sulfadiazine (Sigma, U.S.A.).

3.2.4 Selection of transgenic callus/plantlets

3.2.4.1 GUS activity

Transient expression of β-glucuronidase was investigated by means of a histochemical assay (Jefferson *et al.*, 1987). This assay was performed during co-cultivation of cell suspension cultures and bacterial cells (2 days after inoculation), after repeated curing (10 days after inoculation) and six weeks after transformation (callus stage). The GUS assay solution (100 ml; pH 7.0) consisted of 0.1 M sodium phosphate buffer (pH 7.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 0.2 % Triton X-100, and 1 mg.ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc)(Sigma, U.S.A.) (F. Botha, pers. comm.). Callus tissues were flooded with this assay solution and incubation was allowed to proceed overnight at 37°C. Cells expressing GUS were detected microscopically by the distinct blue colour which results from enzymatic

cleavage of X-Gluc. Similarly, following callus formation (6 weeks), callus pieces were incubated with X-Gluc (72 hours at 37°C) and cells expressing GUS were viewed microscopically.

3.2.4.2 Growth on selective agents

Calli from the cell suspension culture transformations were initially selected on 50 $\mu\text{g.ml}^{-1}$ kanamycin, while some of the calli from the leaf disc transformation were also selected on kanamycin (50 $\mu\text{g.ml}^{-1}$).

Following selection on kanamycin, calli were further selected on sulfadiazine. To investigate the effect of various concentrations of sulfadiazine (Sigma, U.S.A.) on control callus derived from cell suspension cultures, weighed segments of callus were placed on fresh callus medium (MS nutrients, 3 mg.l^{-1} 2,4-D, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite, pH 5.7) containing 0 to 500 $\mu\text{g.ml}^{-1}$ sulfadiazine. The relative growth rate (increase in fresh mass over initial mass of explant) was determined after 21 days.

The effects of the various concentrations (0 to 500 $\mu\text{g.ml}^{-1}$) of sulfadiazine on shoot and root growth of control plantlets was also determined by placing shoots of the same size (2 cm long) onto rooting medium (Section 2.2.5) containing the selective agent. The percentage of rooted shoots was calculated.

3.2.5 Analysis of gene integration

3.2.5.1 Probe preparation

Source of probe

The probe used in these investigations was the HindIII restriction fragment (5.9 kb) of the pJIT119 plasmid (Figure 3.1).

Plasmid generation, isolation and purification

The plasmid pJIT119 was isolated from *E. coli* cells by a modification of the protocol for large-scale plasmid preparation (Armitage *et al.*, 1988). The modifications were: (1) amplification of the plasmid in rich medium (Maniatis *et al.*, 1989) preceding the isolation protocol; (2) the *E. coli* strain was grown overnight in LB medium containing 50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin; (3) CsCl centrifugation at 100 000 rpm and 18°C for 4 hours, and (4) dialysis of the resulting plasmid solution for approximately 24 hours against six changes of TE buffer. The concentration of purified plasmid DNA (determined spectrophotometrically by absorption at 260 nm) was 50 $\mu\text{g}\cdot\text{ml}^{-1}$, and the ratio of A_{260}/A_{280} was 1.82.

Restriction enzyme digestion

Purified plasmid DNA (prepared as described above) was digested with the restriction enzyme HindIII for 3 hours at 37°C in order to yield the DNA insert of interest. Digestion mixture was as follows: 1.5 μg DNA, 2 μl HindIII (Boehringer Mannheim, Germany) and 3 μl B buffer (Boehringer Mannheim, Germany), in a final volume of 35 μl .

Agarose gel electrophoresis

Electrophoresis conditions were the same as those described in Section 3.2.2, except that a 1% low melting point agarose was used (Amersham, U.K.) and the gel was run in Tris-acetate (TAE) buffer (1 x working solution: 0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0). Loading mixtures were as follows: (1) undigested plasmid DNA sample: 10 μl DNA and 5 μl loading buffer (Section 3.2.2), (2) digested plasmid DNA: 30 μl DNA and 5 μl loading buffer, and (3) pBR322 standards: 2 μl DNA, 4 μl TE buffer and 3 μl loading buffer. Loading volumes were 35 μl for DNA samples and 8 μl for the pBR322 standards.

Probe labeling

The small band containing the DNA fragment of interest was cut out of the gel under ultraviolet light using a sterile scalpel blade. The gel slice containing the DNA was stored at 4°C and used in the labeling reaction without removal of agarose.

The oligolabelling technique of Feinberg and Vogelstein (1984) was used to incorporate [α - 32 P] dCTP into the HindIII-restricted and denatured pJIT119 fragment. The reaction mixture contained 100 ng DNA, 4 μ l each of dATP, dTTP and dGTP, 8 μ l hexanucleotide mixture (0.5 mol.l⁻¹ Tris-HCl, 0.1 mol.l⁻¹ MgCl₂, 1 mmol.l⁻¹ DTE, 2 mg.ml⁻¹ BSA) (Boehringer Mannheim, Germany), 20 μ l [α - 32 P] dCTP (10 μ Ci. μ l⁻¹) (Amersham, U.K.) and 4 μ l Klenow enzyme (Boehringer Mannheim), and the reaction was allowed to proceed for 30 minutes at 37°C. Thereafter it was terminated by the addition of 85 μ l of 2 mM EDTA.

3.2.5.2 Southern Analysis

Extraction of plant genomic DNA

a) isolation

Callus material (1 - 5 g) was frozen in liquid nitrogen and ground using a mortar and pestle. The resulting powder was incubated in 7.5 - 15 ml cetyltrimethylammonium bromide (CTAB) (Sigma, U.S.A.) isolation buffer (2 % (w/v) CTAB, 1.4 M NaCl, 0.2 % (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, with or without 1 % (w/v) PVP-40), for 30 minutes at 60°C, with occasional swirling. The sample was extracted once with chloroform-isoamyl alcohol (24:1) and centrifuged (1600 g for 10 min at 25°C) to concentrate the phases. Following transfer of the aqueous phase to a clean centrifuge tube, 2 - 3 volumes of cold isopropanol were added to precipitate the DNA. This was pelleted by centrifugation (500 g for 1 - 2 min at 25°C) and the pellet was washed for a minimum of 20 min in 10 ml wash buffer (76% (v/v) ethanol, 10 mM ammonium acetate). After centrifugation (1600 g for 10 min at 25°C), the supernatant was discarded and the DNA pellet was air-dried before

resuspension in 1 ml TE buffer (Section 3.2.2). RNA was removed by addition of RNase (final concentration $10 \mu\text{g}\cdot\text{ml}^{-1}$) and incubation at 37°C for 30 min. Then, ammonium acetate (final concentration 2.5 M) and 2.5 volumes cold ethanol were added and this solution was mixed gently to precipitate the DNA. The final DNA pellet was obtained by centrifugation (10000 g for 10 min at 4°C), allowed to dry, and solubilized in $200 \mu\text{l}$ TE buffer. This volume was increased to $500 \mu\text{l}$ for the samples that were to be subjected to a phenol-chloroform (1:2) extraction. This extraction was followed by additional ammonium acetate and ethanol precipitations, as described above.

b) purification

Two protocols for DNA purification were compared: spun-column chromatography (Sambrook *et al.*, 1989) followed by mini-dialysis against TE buffer (S. MacRae, pers. comm.) and the GENE CLEAN™ kit (Bio 101 Inc., U.S.A.) procedure. Mini-dialysis involved loading $50 \mu\text{l}$ aliquots of DNA solution onto $0.22 \mu\text{m}$ cellulose acetate filters (Millipore, U.S.A.), which were floated in petri dishes containing 20 ml TE buffer for approximately 2 hours. The protocol for GENE CLEAN™ was as recommended by the manufacturers. DNA concentration was determined spectrophotometrically by absorption at 260 nm.

Restriction enzyme digestion

Plant genomic DNA samples were digested with BamHI (Boehringer Mannheim, Germany) for 16 hours at 37°C . The digestion mixtures consisted of $25 \mu\text{l}$ DNA, $2 \mu\text{l}$ BamHI and $3 \mu\text{l}$ B buffer (Boehringer Mannheim, Germany). Lambda DNA (Boehringer Mannheim, Germany) samples were digested with a combination of HindIII (Boehringer Mannheim, Germany) and EcoRI (Boehringer Mannheim, Germany) or BamHI for 3 hours at 37°C . The digestion mixtures were as follows: (1) HindIII/EcoRI digest - $22 \mu\text{l}$ DNA, $2 \mu\text{l}$ EcoRI, $3 \mu\text{l}$ HindIII, $3 \mu\text{l}$ B buffer, and (2) BamHI digest - $22 \mu\text{l}$ DNA, $2 \mu\text{l}$ BamHI, $3 \mu\text{l}$ B buffer, $3 \mu\text{l}$ water.

Gel electrophoresis

Agarose gels (0.8 %) were run in TBE buffer. In the first gel DNA samples from control calli were loaded as follows: (1) DNA samples - 20 μ l DNA, 5 μ l loading buffer (Section 3.2.2), and (2) Lambda markers -5 μ l DNA, 6 μ l TE buffer, 6 μ l loading buffer. Loading volumes were 25 μ l for DNA samples and 15 μ l of Lambda DNA. In a subsequent gel DNA samples from transformed and control calli were made up of: (1) unrestricted DNA samples - 30 μ l DNA, 5 μ l loading buffer, (2) restricted DNA samples - 30 μ l DNA, 5 μ l loading buffer, and (3) Lambda DNA -30 μ l Lambda DNA digest, 5 μ l loading buffer. Loading volumes were 35 μ l for all lanes. Apparatus for electrophoresis was as described in Section 3.2.2.

Southern blotting

Following electrophoresis, DNA fragments were blotted onto Hybond-C extra supported nitrocellulose membranes (Amersham, U.K.) according to the method described by Scott (1988), based on the original protocol reported by Southern (1975). The filter was fixed by baking at 80°C, according to the instructions for nitrocellulose filters (Amersham, U.K.).

Hybridisation

The hybridisation protocol was as described by Scott (1988). The air-dried Hyperfilm β -max X-ray film (Amersham, U.K.) was wrapped in Saran wrap and exposed at -80°C in an X-ray film cassette for 24 - 96 hours before developing. [2 min in Ilford Phenisol developer (Ciba Geigy (Pty) Ltd, S.A.), followed by fixation in Ilford Hypam rapid fixer (Ciba Geigy (Pty) Ltd, S.A.)].

3.2.6 Microscopy and photography

Approximately six hours after inoculation of cell suspension culture cells with the *A. tumefaciens* C58C1 (pMP90) (pJIT119), bacterial attachment was investigated using

differential interference contrast light microscopy (Olympus Vanox AHBS3) and recorded using an Olympus C-35AD-4 camera. Samples were also prepared for and viewed using scanning electron microscopy (Hitachi S-520), according to conventional procedures (Vasil and Vasil, 1984). Calli were photographed using a Nikon FM2 camera with a 60 mm Mikro Nikkor macro lens. GUS activity was visualised using a Wild M3 stereomicroscope and recorded using a Wild Photoautomat MPS 55. Gels were photographed on a transilluminator, using a red filter.

3.3 Results and Discussion

3.3.1 Triparental mating and selection of transconjugants

Co-cultivation of donor [*E. coli* (pJIT119)], recipient [*A. tumefaciens* LBA4404] and helper [*E. coli* HB101::pRK2013] bacterial strains for 48 hours at 28°C, resulted in the production of putative transconjugants, which were incubated on the appropriate antibiotics (50 µg.ml⁻¹ kanamycin and 50 µg.ml⁻¹ rifampicin) (Table 3.4) in order to select for the transconjugants [*A. tumefaciens* LBA4404 (pJIT119)] (Figure 3.4). Another transconjugant [*A. tumefaciens* C58C1 (pMP90) (pJIT119)] was also used in this investigation, and it was obtained from Ms S. MacRae (Forestek, CSIR, Durban).

DNA was extracted from the various bacterial strains using a mini-preparation procedure (Kado and Liu, 1981) and an agarose gel was run to ascertain whether the plasmid from the *E. coli* donor strain (Figure 3.5, lane 5) had been transferred to the *A. tumefaciens* recipient strain (Figure 3.5, lane 2). From the DNA banding pattern seen on the gel (Figure 3.5), it was evident that a band of the correct size (6 kb), which had not previously been in the recipient bacterium, was present following the triparental mating (Figure 3.5, lane 3). This was indicative of successful conjugation. This band was also found to be present in the other transconjugant strain, C58C1 (pMP90) (pJIT119) (Figure 3.5, lane 6).

3.3.2 Leaf disc transformation

3.3.2.1 Co-cultivation of leaf pieces with *Agrobacterium*

Leaf pieces, that had been cut from sterile shoots produced *in vitro* (Section 2.2.3), were co-cultivated with the *A. tumefaciens* strains C58C1 (pMP90) (pJIT119) or LBA4404 (pJIT119) for 6 or 24 hours. Then, the leaf pieces were placed onto callus induction medium (Section 2.2.5) in the dark, and callus production (Figure 3.6) was assessed after 6 weeks in culture (Table 3.5) as a means of selecting the optimal co-cultivation time. From the data in Table 3.5 it can be seen that for both



Figure 3.4: Colonies of the transconjugant LBA4404 (pJIT119) formed on medium containing the selective antibiotics kanamycin ($50 \mu\text{g}\cdot\text{ml}^{-1}$) and rifampicin ($50 \mu\text{g}\cdot\text{ml}^{-1}$) (bar = 11.25 mm).



Figure 3.5: Agarose gel electrophoresis of plasmid DNA extracted from the various bacterial strains (arrowhead = plasmid band of interest).

Lanes: 1 - pBR322 standard

2 - *A. tumefaciens* LBA4404

3 - *A. tumefaciens* LBA4404 (pJIT119)

4 - *E. coli* HB101::pRK2013

5 - *E. coli* pJIT119

6 - *A. tumefaciens* C58C1 (pMP90) (pJIT119)

7 - pBR322 standard

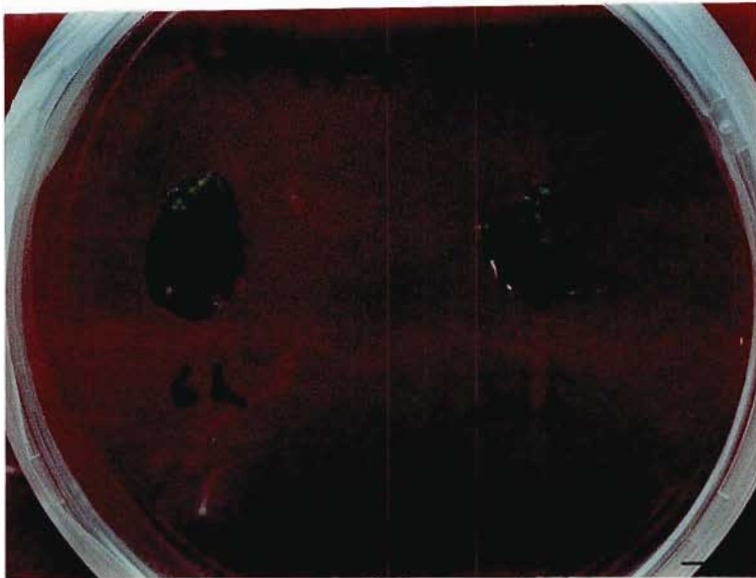


Figure 3.6: Comparison of callus formation obtained, on selective callus induction medium, from leaf explants that had been co-cultivated with bacterial cells for 6 hours or 24 hours. The medium contained MS nutrients, 16 mg.l⁻¹ ferric citrate, 20 g.l⁻¹ sucrose, 0.05 mg.l⁻¹ BA, 1 mg.l⁻¹ NAA and 50 µg.ml⁻¹ kanamycin (bar = 8.6 mm).

Agrobacterium strains, 6 hours was the more favourable length of time to use, both in terms of percent callus production and prevention of necrosis, but for practical reasons, an overnight incubation (16 hours) was used in subsequent manipulations.

Table 3.5; Effect of length of co-cultivation period of bacterial and plant cells on callus formation (% explants with callus) and explant necrosis after 6 weeks on callus induction medium. Callus production of control leaf pieces was assessed after 3 - 4 weeks on callus induction medium (n = 17 - 30).

	Bacterial Strain				
	Control	C58C1 (pMP90) (pJIT119)		LBA4404 (pJIT119)	
Time	-	6 hours	24 hours	6 hours	24 hours
% callus formation	100	37	33	85	48
% leaf necrosis	0	7	21	0	12

3.3.2.2 Growth and maintenance of transformed calli on selective medium

The most commonly used method for the transformation of plant cells with *A. tumefaciens* involves the use of leaf discs as explants, after which transgenic plants are regenerated via indirect organogenesis (Horsch *et al.*, 1988; Cardi *et al.*, 1992; Chan *et al.*, 1992; Mozo and Hooykaas, 1992a; Ying *et al.*, 1992; Atkinson and Gardner, 1993; Grevelding *et al.*, 1993; Mansur *et al.*, 1993; Palmgren *et al.*, 1993). In this way, transformed cells are induced to multiply, resulting in the formation of 'transformed' callus. The callus cells can then be stimulated to produce transgenic plants. Therefore, a single explant cell that is transformed, can theoretically, be used to produce numerous transgenic plants.

Consequently, the indirect organogenic approach was used: after co-cultivation (16 hours) with bacterial cells from either *A. tumefaciens* strain C58C1 (pMP90) (pJIT119) or LBA4404 (pJIT119), leaf pieces were placed onto callus induction

medium (Section 2.2.5). As discussed previously (Section 3.2.1), the gene for kanamycin resistance (*neo*) is on the plasmid that was used for the transformation (Figure 3.1) so, resistance to this selective antibiotic ($50 \mu\text{g.ml}^{-1}$) (Section 3.2.3.2), was used initially as an indication of successful transformation. After 6 weeks in culture, callus proliferation from some areas of the leaf pieces was obtained on this selective medium, giving a positive indication that some areas of callus had been successfully transformed with the two *Agrobacterium* strains (Figure 3.6).

In addition to the gene conferring kanamycin resistance, the plasmid used for the transformation carries the *gus* gene (Figure 3.1), responsible for β -glucuronidase (GUS) expression (Jefferson *et al.*, 1987), commonly used by numerous workers as an initial indication of successful transformation (Baribault *et al.*, 1990; Martin *et al.*, 1990; Li *et al.*, 1992; Mozo and Hooykaas, 1992a; Pawlicki *et al.*, 1992; Warkentin and McHughen, 1992; Ying *et al.*, 1992; Chan *et al.*, 1993; Delbreil *et al.*, 1993; Dong and McHughen, 1993; Jacq *et al.*, 1993; Ottaviani *et al.*, 1993; Ritchie *et al.*, 1993; Confalonieri *et al.*, 1994; Pavingerová *et al.*, 1994; Vallés and Lasa, 1994). Consequently, following callus formation (6 weeks), pieces of leaf disc-derived callus that had been transformed with both strains of *Agrobacterium* were incubated for 4 days at 37°C in X-Gluc, the substrate used to investigate transient GUS expression. Cells expressing GUS were visualised microscopically by the blue colour which formed from the enzymatic cleavage of X-Gluc (Figure 3.7), indicating that they were successfully transformed.

Despite the positive results obtained, both with selection on kanamycin and with GUS expression, the callus production from transformed leaf pieces on medium containing kanamycin was slower (6 weeks from initiation) than that of the control leaf pieces (3-4 weeks from initiation) (Section 2.2.5), and the calli that formed directly on the kanamycin selective media were recalcitrant to regeneration. As recently reported,

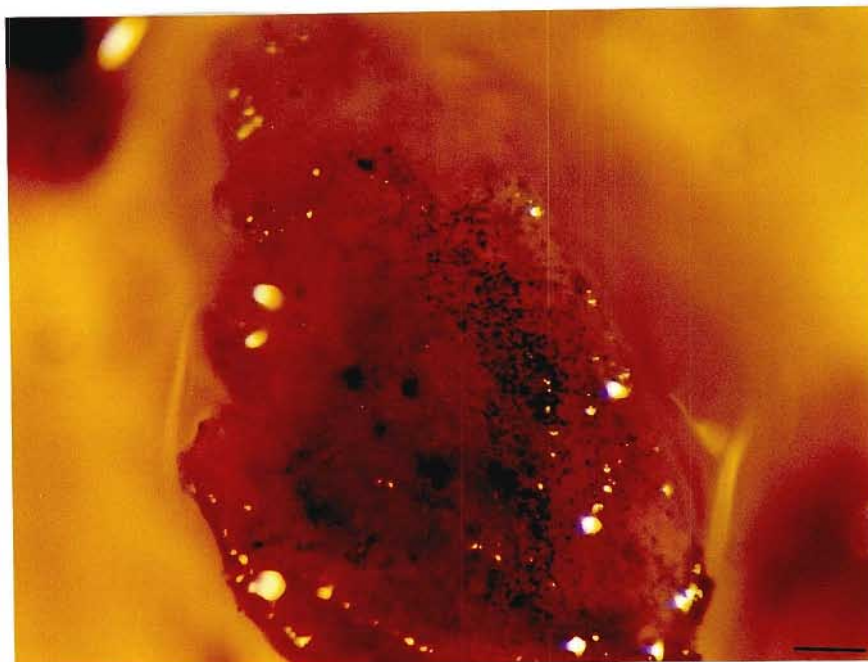


Figure 3.7: Histochemical localisation of GUS activity, obtained in the callus derived from leaf explants following transformation. Transient GUS expression is indicated by the blue cells formed as a result of the enzymatic cleavage of X-Gluc (bar = 70 μm).

the recalcitrance to regeneration could be attributed to the inhibitory effects of kanamycin on shoot emergence (Dong and McHughen, 1993; Sriskandarajah *et al.*, 1994) and regeneration (Ying *et al.*, 1992). Despite these few reports, the problem of recalcitrance of transformed cells to regeneration is not frequently mentioned in the literature, although it appears to be a common occurrence (A. Tibok, pers. comm.; B. Hockett, pers. comm.). Following the advice of Tibok, the transformation protocol, using both strains of *Agrobacterium*, was repeated with kanamycin being omitted from the callus induction medium, in an attempt to increase the rate of callus formation and produce plantlets from the transformed callus. In this instance, the omission of kanamycin from the callus induction medium resulted in the rate of callus production from transformed leaf pieces (4 weeks) increasing to that reported for the control leaf pieces (Section 2.2.5).

The callus produced on non-selective callus induction medium has recently been placed onto shoot proliferation medium (Section 2.2.5). Following shoot formation, the next stage of selection is rooting. It is commonly accepted that rooting on selective agents can be used as a means of separating transformed from non-transformed plantlets, because, as mentioned by Draper *et al.* (1988) and Lipp João and Brown (1993), this stage of development is particularly sensitive to inhibition by antibiotics. Consequently, in this study, kanamycin ($50 \mu\text{g.ml}^{-1}$) and sulfadiazine ($50 \mu\text{g.ml}^{-1}$) (Section 3.3.2.3, below) would be the selective agents utilised to select for transformed plants. Should shoots root on medium containing the selective agents, they would be likely to contain copies of the DNA of interest, however, final proof of integration of the genes of interest into the plant DNA requires that the DNA of the putative transformed tissue hybridise with the bacterial gene under appropriate conditions (Nester, 1987) using Southern blotting. Time constraints have, however, prevented the completion of this work.

3.3.2.3 Establishment of parameters to be used for selection of transformed plants on sulfadiazine

In addition to the genes conferring kanamycin resistance (*kan*) and GUS expression (*gus*), the plasmid utilised throughout this investigation (Figure 3.1) also contains the gene responsible for resistance to the antibiotic sulfadiazine (*sul*). In contrast to GUS activity which is transient (Section 3.3.2.2), the exhibition of resistance to sulfadiazine is an indication of successful gene integration, since incorporation of the DNA insert into the host chromosome is necessary for the expression of resistance (Guerineau *et al.*, 1989; Guerineau *et al.*, 1990; Hull, 1991).

It follows that, in order to select transformed plantlets on medium containing sulfadiazine, the optimal conditions for selection need to be determined, since the response may vary for different species or experimental systems. In this regard, the effect of various concentrations of sulfadiazine on the rooting of control shoots produced *in vitro* was investigated (Table 3.6; Figure 3.8). The results of this assessment (Table 3.6; Figure 3.8) indicate that a sulfadiazine concentration of 50 $\mu\text{g.ml}^{-1}$ is sufficient to enable the distinction between transformed and non-transformed shoots, and therefore is the recommended concentration for selection of transformed shoots.

Table 3.6: The effect of different concentrations of sulfadiazine on the rooting of control shoots produced *in vitro*. The percentage of rooted shoots were determined after 4 weeks on rooting medium (MS nutrients, 20 g.l^{-1} sucrose, 1 mg.l^{-1} IAA and 0 - 500 $\mu\text{g.ml}^{-1}$ sulfadiazine).

Sulfadiazine ($\mu\text{g.ml}^{-1}$)	% rooting
0	80
50	0
150	0
300	0
500	0

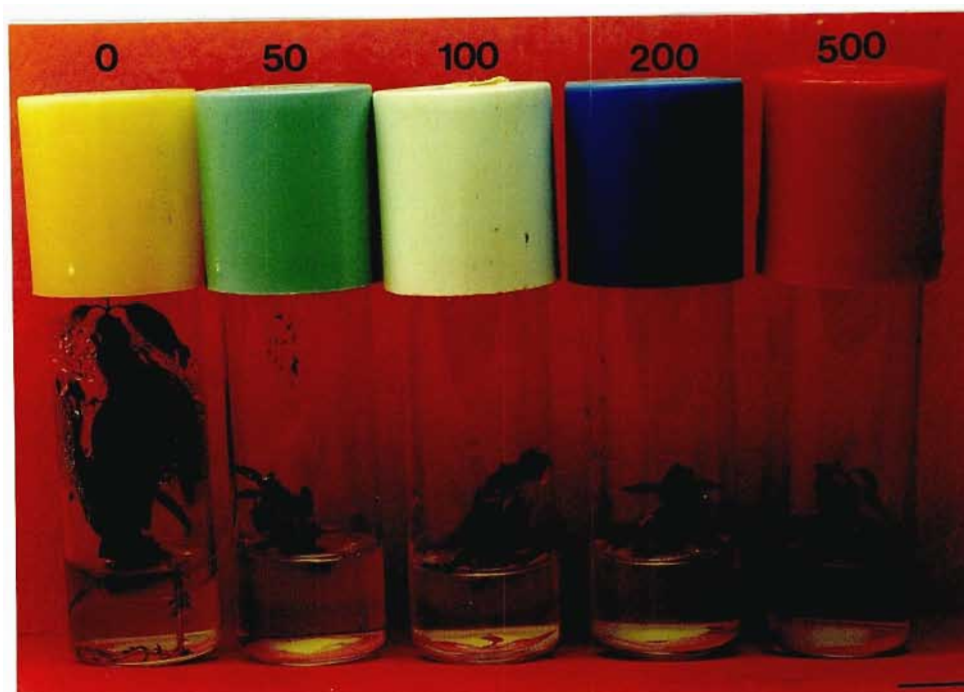


Figure 3.8: Effects of various sulfadiazine concentrations (0 - 500 $\mu\text{g.ml}^{-1}$) on the rooting of control shoots produced *in vitro*. The rooting medium consisted of MS nutrients, 20 g.l^{-1} sucrose, 1 mg.l^{-1} IAA and 0 - 500 $\mu\text{g.ml}^{-1}$ sulfadiazine, and results were recorded after 4 weeks on the rooting medium (bar = 12.5 mm).

3.3.3 Cell-suspension culture transformation

3.3.3.1 Optimisation of protocol

Cell suspension cultures of *Eucalyptus* were prepared and maintained as described in Section 2.2.4. The availability of this tissue culture system provided an opportunity for an investigation into the use of this method as an alternative means of obtaining transgenic plants. Although cell suspension cultures have been used as explants for transformation studies using microprojectile bombardment (Wang *et al.*, 1988; Cao *et al.*, 1992; Vain *et al.*, 1993), there are few reports on the use of cell suspension cultures for *Agrobacterium*-mediated transformation. An exception is the simple and efficient method for the *Agrobacterium*-mediated transformation of embryogenic cell suspension cultures of carrot which has been reported by Draper *et al.* (1988). Hence, this was the basic protocol which was adapted to be used for *Eucalyptus*.

A number of experiments were carried out in order to establish the optimal conditions for transformation. Briefly, the modified protocol for *Eucalyptus* (Section 3.2.3.2) differs from that reported by Draper *et al.* (1988) in that feeder plates contained 2.6×10^3 cell suspension culture cells and 15 ml of molten medium containing MS nutrients, 3 mg.l^{-1} 2,4-D, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite (Section 2.2.5). Filter paper guard and transfer discs (8.5 and 5.5 cm in diameter, respectively) were placed onto the surface of the feeder plates, which were incubated for 4 days in the dark. This system of utilising filter paper discs recommended by Draper *et al.* (1988) was found to be very efficient, since it facilitated the simple transfer of plated cell suspension cultures and developing calli from one medium to the next. Thereafter, the transfer discs were seeded with 0.65×10^3 cells (rather than the 250 plating units used by Draper *et al.* (1988)), and incubated for 7 days in the dark, prior to inoculation with the bacterial cells. In this regard, Draper *et al.* (1988) used a 1:1000 dilution of the bacterial culture, while various dilutions (1:1, 1:100, 1:250) of a bacterial culture of *A. tumefaciens* strain C58C1 (pMP90) (pJIT119) were considered in this study. Differential Interference Contrast Light Microscopy (DIC) (Figure 3.9 A) and

Scanning Electron Microscopy (SEM) (Figure 3.8 B) were used to visualise bacterial attachment to the suspension culture cells (Douglas *et al.*, 1985) after 24 hours of co-cultivation (Figure 3.9 A,B). It was found that very few bacteria were attached to the plant cells when 1:250 and 1:100 dilutions were used (results not shown), and thus a dilution of 1:1 was employed in further investigations. Cefotaxime, at a concentration of 750 $\mu\text{g.ml}^{-1}$, was the curing agent utilised repeatedly in this investigation (Figure 3.3), as the concentration of 250 $\mu\text{g.ml}^{-1}$ recommended Draper *et al.* (1988) was found to be insufficient for the curing of the *A. tumefaciens* strain C58C1 (pMP90) (pJIT119) (Figure 3.3).

3.3.3.2 Growth and maintenance of transformed calli on selective medium

As mentioned for transformed leaf discs (Section 3.3.2.2), the transient expression of β -glucuronidase (GUS) is used as an initial indication of successful transformation (Jefferson *et al.*, 1987). In the cell suspension culture system utilised in this study, the GUS assay was performed during co-cultivation with *A. tumefaciens* C58C1 (pMP90) (pJIT119) (2 days after inoculation with the bacteria) (Figure 3.10 A,B), after repeated curing (10 days after transformation) (Figure 3.10 C) and following callus formation (6 weeks after transformation). Positive GUS activity was detected at 2 and 10 days after transformation (Figure 3.10 A,B,C), but not at 6 weeks after transformation. This indicates that transient expression of this inserted gene (*gus*) can be detected up to 10 days after transformation, enabling the initial screening of plated cells to be undertaken prior to callus formation.

Selection of transformed cells on 50 $\mu\text{g.ml}^{-1}$ kanamycin was undertaken in conjunction with cefotaxime, immediately after co-cultivation with the bacterial cells. After curing was complete, cefotaxime was eliminated from the medium, and selection of transformed cells on kanamycin continued. As callus proliferated on the medium containing kanamycin (6 weeks), it may be presumed that the transformation was successful. However, there is no assurance that all cells were transformed,

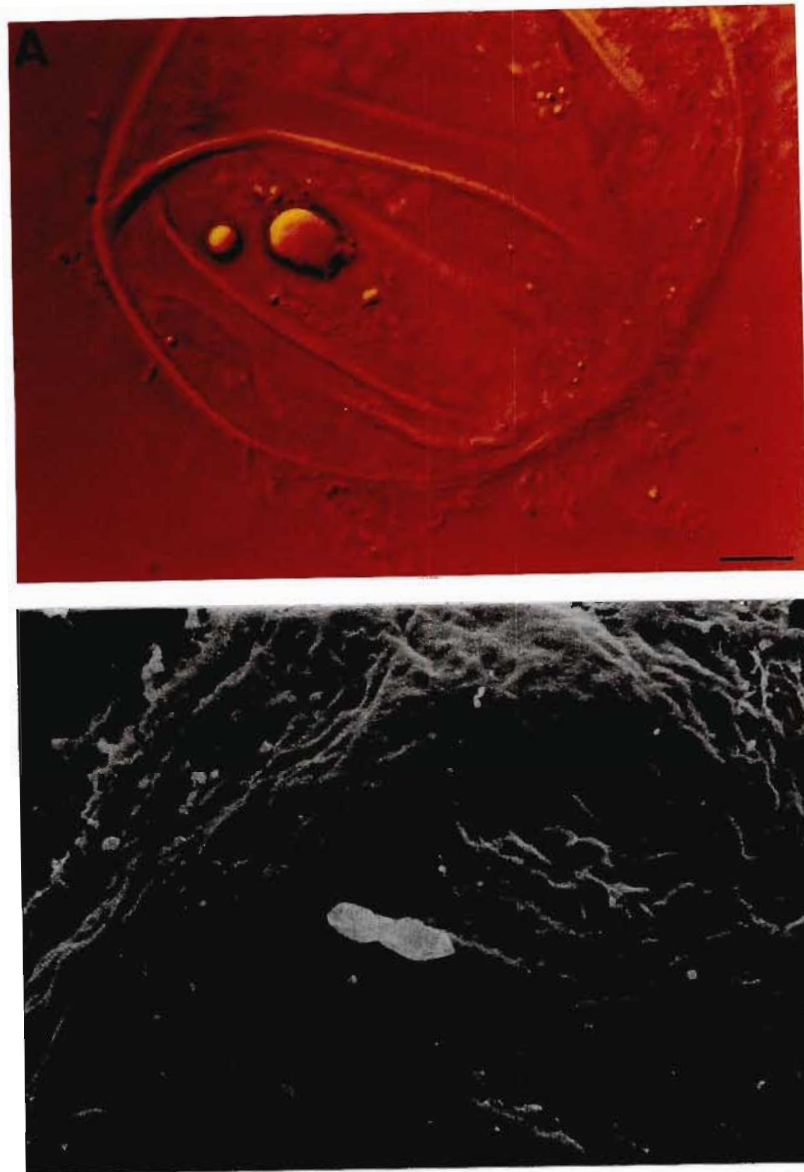


Figure 3.9: Attachment of *A. tumefaciens* C58C1 (pMP90) (pJIT119) cells to suspension culture cells. The attachment was visualised using (A) DIC (bar = 7 μm) and (B) SEM (bar = 1.8 μm).

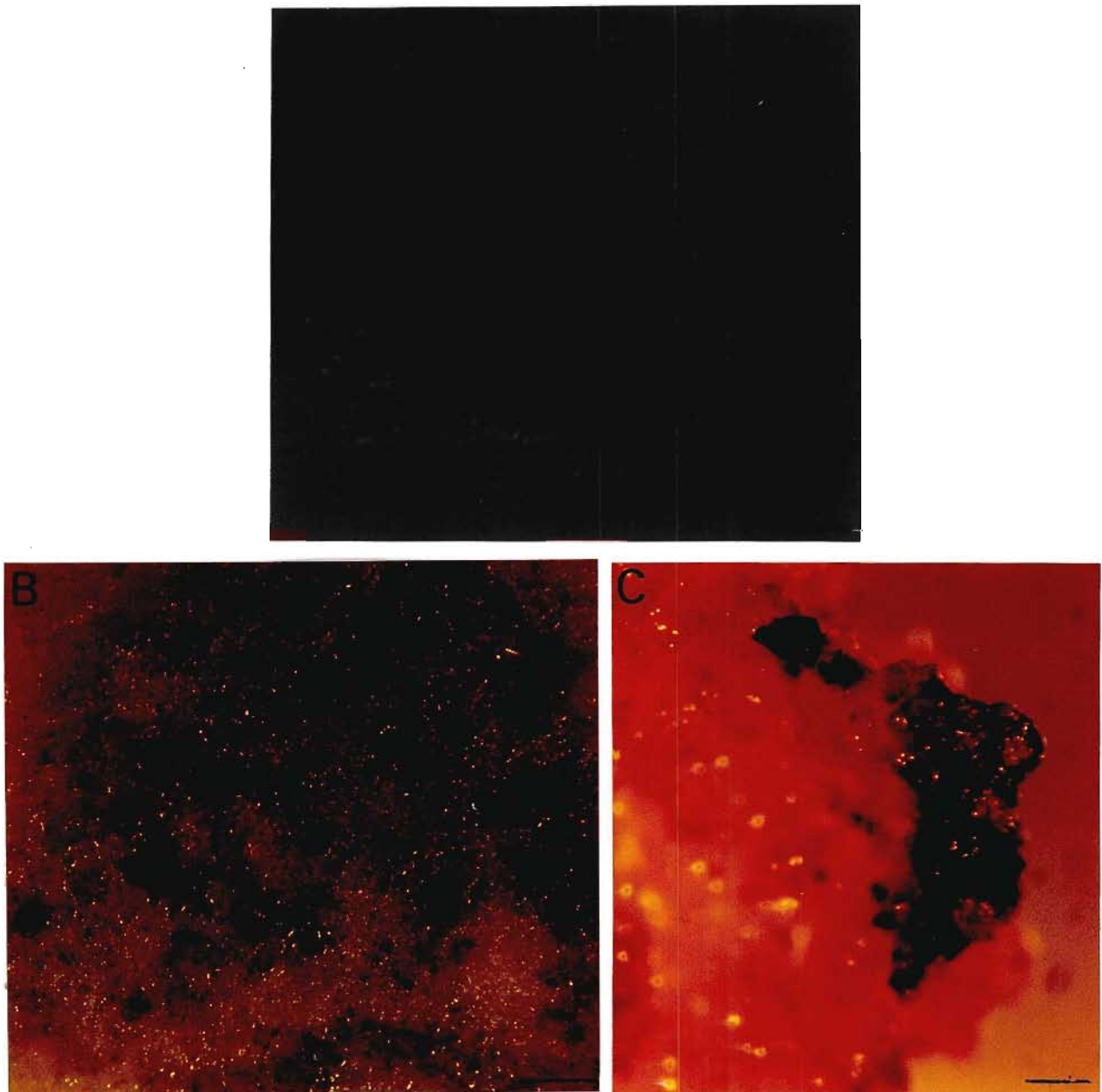


Figure 3.10: Histochemical localisation of GUS expression obtained in suspension culture cells transformed with *A. tumefaciens* C58C1 (pMP90) (pJIT119). (A), (B) Cells exhibiting transient GUS expression (blue colour) during co-cultivation with bacteria (A: bar = 13 μm ; B: bar = 0.8 mm); (C) GUS-positive cells detected after curing with 750 $\mu\text{g}\cdot\text{ml}^{-1}$ cefotaxime (bar = 0.2 mm).

since there may be a large percentage of cells which 'escape' selection on kanamycin. The 'escape' rate has recently been reported to be as high as 80 %, where non-transformed cells are not selected out of the population (Dong and McHughen, 1993; Vallés and Lasa, 1994).

As previously mentioned, the vector pJIT119 (Figure 3.1) possesses the *sul* gene for the selection of transformants on sulfadiazine. However, as for shoot cultures, it was necessary to establish the conditions for selection of transformed callus on sulfadiazine. In this regard, the effect of various concentrations of this selective agent on the growth of control callus derived from cell suspension cultures was investigated (Table 3.7). The relative growth rates of control calli were determined after 21 days on various concentrations of sulfadiazine (0 to 500 $\mu\text{g}\cdot\text{ml}^{-1}$), and from these results, the sulfadiazine concentration at which 50 % of callus growth was inhibited (I_{50}), was calculated. As this was found to be 50 $\mu\text{g}\cdot\text{ml}^{-1}$, the transformed calli were placed onto this concentration of sulfadiazine, and the resultant proliferation (Figure 3.11) suggested that successful transformation and possibly integration of the *sul* gene had occurred. However, it must be noted that rooting on sulfadiazine (Section 3.3.2.3) is obviously a more efficient indication of positive selection (a positive or negative situation), while with callus there is still some survival of control callus, even up to 500 $\mu\text{g}\cdot\text{ml}^{-1}$. For this reason, rooting on selective agents is regarded as the final positive test of selection.

The success of a transformation protocol is, however, dependent on a method for regeneration from the transformed cells. This is often the limiting step in the development of successful protocols for transgenic plant production (Wilde *et al.*, 1992), as was discovered in this study (Sections 2.2.5, 2.2.6 and 3.3.2.2). As discussed previously (Sections 2.2.5 and 2.2.6), and although this was investigated extensively, the production of plantlets from transformed callus has not yet been

achieved due to the recalcitrance of cell suspension culture-derived callus to regeneration. On the other hand, as mentioned in Section 2.3.4.5, recent results obtained in this study have indicated that the production of somatic embryos from cell suspension culture-derived callus may soon be possible. The regeneration of transgenic plants of *Eucalyptus* from transformed calli would thus be possible, using the methods established in this study.

Table 3.7: The effect of different concentrations of sulfadiazine on the relative growth rate (RGR) of control callus derived from cell suspension cultures after 3 weeks on media containing the various sulfadiazine concentrations. (* = standard deviations) Calli were cultured on solid callus medium (MS nutrients, 30 g.l⁻¹ sucrose, 3 mg.l⁻¹ 2,4-D and 4 g.l⁻¹ Gelrite) supplemented with 0 - 500 µg.ml⁻¹ sulfadiazine.

Sulfadiazine (µg.ml ⁻¹)	RGR
0	3.46 ± 0.83*
20	2.49 ± 0.39
50	2.19 ± 0.51
100	1.99 ± 0.39
200	1.45 ± 0.41
500	1.39 ± 0.25

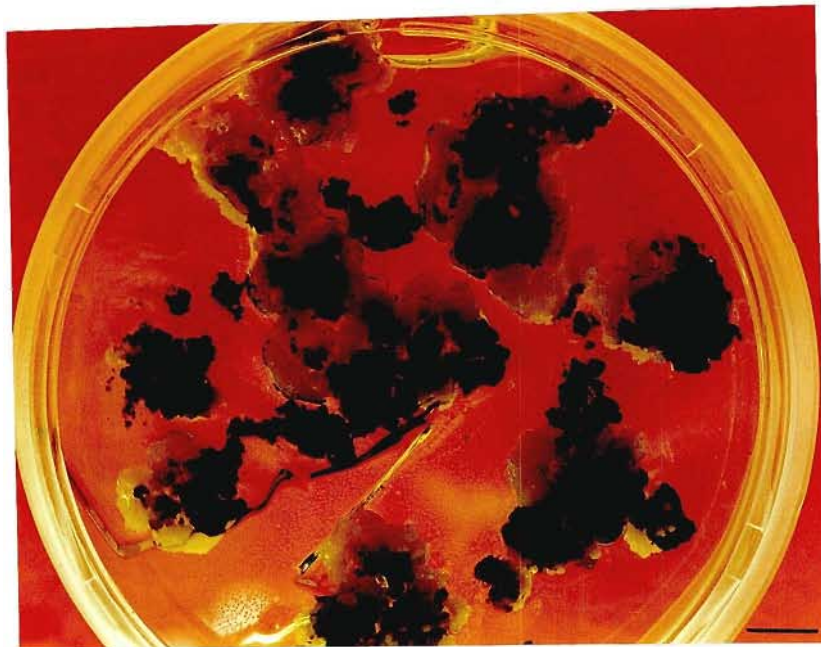


Figure 3.11: Callus produced from transformed cell suspension cultures and selected on medium containing $50 \mu\text{g.ml}^{-1}$ sulfadiazine. Calli were produced on solid callus induction medium (MS nutrients, 3 mg.l^{-1} 2,4-D, 30 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite) and were then subcultured onto fresh medium supplemented with $50 \mu\text{g.ml}^{-1}$ sulfadiazine, for 4 weeks (bar = 9 mm).

3.3.4 Characterisation of transformed calli

Since the production of transgenic plantlets from transformed calli was not successful, analysis of the transformed callus was undertaken in order to determine whether integration of the DNA of interest had occurred. This type of verification was important in the present situation as it would give an indication of the success of the protocol thus far. Although the callus had been selected on both kanamycin ($50 \mu\text{g.ml}^{-1}$) and sulfadiazine ($50 \mu\text{g.ml}^{-1}$), it was necessary to determine whether the foreign DNA had been successfully incorporated into the host genome. However, it must be stressed that because there are likely to be both transformed and non-transformed callus cells being tested simultaneously, it is not an ideal situation for carrying out such tests.

3.3.4.1 Isolation and purification of DNA

In order to analyse whether DNA integration had occurred, DNA was extracted from the cell suspension culture-derived callus that had been selected on both kanamycin and sulfadiazine as described above (Section 3.3.3.2). Since most published DNA extraction protocols are based on isolation of DNA from leaf tissue (Rogers and Bendich, 1985; Cheung *et al.*, 1993), it was necessary to modify the available protocols for use with callus cells. This is due to the difference between leaves and callus with respect to number of cells per fresh mass. Since callus cells are approximately 2 - 3 times larger than leaf cells (F. Blakeway, pers. comm.), for the same amount of plant material there is likely to be less DNA in callus than in leaf material. Therefore, various masses of callus were used to determine the mass of callus required for subsequent DNA extractions. From the bands obtained on the agarose gel (Figure 3.12), 2.5 g of callus was found to be adequate and was thus used in all further DNA extractions (Figure 3.12, lane 3).

In conjunction with the investigation into the mass of callus to be used, the addition of PVP-40 to the extraction buffer was investigated, since *Eucalyptus* spp. have been

reported to produce large quantities of phenolics (Watt *et al.*, 1991; Blakeway *et al.*, 1993; Jones and Van Staden, 1994; Lainé and David, 1994). Phenolics act as contaminants of the extracted DNA solution and cause an unacceptable darkening of the DNA extract. Their presence interferes with the quantification of nucleic acids by spectrophotometric methods and may also cause anomalous hybridisation kinetics (Draper and Scott, 1988). The presence or absence of a phenol-chloroform extraction as part of the DNA isolation procedure was also investigated. This process, which denatures and then precipitates proteins from the extract (Draper and Scott, 1988), usually forms part of DNA isolation procedures (Razin, 1988), but its possible exclusion was investigated, since a chloroform-isoamyl alcohol extraction, which has a similar function, was also part of the protocol used. The DNA fractions extracted using the various combinations of treatments described above, were run on an agarose gel (Figure 3.12) in order to verify yield and purity.

The addition of PVP-40 did not reduce the darkening of the DNA solution or appear to influence DNA yield or purity (Figure 3.12, lanes 3 and 4) and was therefore omitted in further DNA isolations. The exclusion of the phenol-chloroform extraction did not seem to markedly affect DNA purity (Figure 3.12, lanes 5 and 6), and its incorporation into the protocol was therefore deemed not necessary. DNA yield was estimated spectrophotometrically and the results confirm that the optimal extraction procedure involved using 2.5 g callus tissue and no PVP-40 addition to the extraction buffer (Figure 3.12, lane 3). (DNA yield of $45 \mu\text{g.ml}^{-1}$)

The DNA extracted, as described above, was then used to investigate different methods of purification, as DNA purity can influence the binding specificity and resolution obtained on the Southern blot. In this regard, the GENE CLEAN™ method (Bio 101 Inc., U.S.A.) was compared with the use of spun column chromatography (Sambrook *et al.*, 1989) followed by mini-dialysis (S. MacRae, pers. comm.) (Figure 3.13). The clarity of the bands obtained on the agarose gel were used

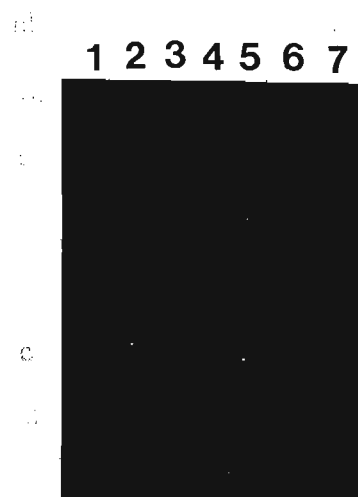


Figure 3.12: Agarose gel electrophoresis of DNA from cell suspension culture-derived callus was used to determine the optimal mass of callus to use, as well as the effect of inclusion of either PVP-40 (lane 4) or phenol-chloroform (Ph/Ch) extraction (lane 6).

Lanes: 1 - Lambda molecular weight markers III

2 - DNA from 1 g callus

3 - DNA from 2.5 g callus

4 - DNA from 2.5 g callus

5 - DNA from 5 g callus

6 - DNA from 5 g callus

7 - Lambda molecular weight markers III

as an indication of DNA purity; as the DNA band obtained in lane 4 (Figure 3.13) was the most distinct, this seemed to indicate that the GENECLAN™ method was the most efficient, and was used in subsequent investigations.

3.3.4.2 Analysis of gene integration

Probe preparation

DNA was extracted from the bacterial cells and purified using cesium chloride (CsCl) density gradient centrifugation (Armitage *et al.*, 1988) (Figure 3.14). The concentration of this plasmid DNA was $50 \mu\text{g.ml}^{-1}$ and the ratio of A_{260}/A_{280} was 1.82. This DNA was then restricted using the restriction endonuclease HindIII (Guerineau *et al.*, 1990), which cleaves the DNA insert from the rest of the pJIT119 plasmid (Figure 3.1). A low melting point agarose gel was used to separate the small (6 kb) and large (12 kb) DNA fragments produced as a result of the digestion (Figure 3.15). The small fragment, containing the DNA insert of interest, was then isolated from the gel, labeled with ^{32}P (Feinberg and Vogelstein, 1984) and used as the DNA probe for Southern blotting (Southern, 1975). The result obtained using Southern blotting, discussed below, indicated that the preparation of the probe was successful.

Southern blotting

It is generally accepted that an initial analysis of the organisation of foreign DNA integrated into the plant genome can best be done by Southern blotting (Draper and Scott, 1988; Scott *et al.*, 1988). Southern blotting is used to analyse the callus DNA, since it provides a simple way of detecting DNA fragments after they have been separated by agarose gel electrophoresis (Southern, 1975). Southern analysis yields information on the copy number of the integrated DNA sequences and whether any multiple inserts are tandemly linked or dispersed (Draper and Scott, 1988). These authors also point out that it is important to know as much as possible about the structure and location of the transferred genes in the transformed plant genome, since such factors have a great effect on their expression and inheritance. Southern blotting

is commonly used by numerous workers to assess transformation efficiency (De Block, 1990; Miljuš-Djukic *et al.*, 1992; Pawlicki *et al.*, 1992; Chan *et al.*, 1993; Delbreil *et al.*, 1993; Grevelding *et al.*, 1993; Han *et al.*, 1993; Hassan *et al.*, 1993; McGranahan *et al.*, 1993; Ottaviani *et al.*, 1993; Confalonieri *et al.*, 1994; Vallés and Lasa, 1994) and the factors that affect this efficiency (Beilmann *et al.*, 1992; Goto *et al.*, 1993; Hobbs *et al.*, 1993).

In this study, transgenic plants were not available for Southern analysis, and therefore DNA extracted from the putatively transformed callus cells was used. Thus, the purified callus DNA fractions (Section 3.3.4.1) were restricted using BamHI (Miljuš-Djukic *et al.*, 1992; Pawlicki *et al.*, 1992; Hassan *et al.*, 1993; Vallés and Lasa, 1994) and subjected to agarose gel electrophoresis (Figure 3.16). This gel was blotted onto a nitrocellulose membrane which was then hybridised with the radioactively-labeled probe (Southern, 1975). The autoradiograph that was obtained (Figure 3.17) was indicative of successful transformation, since the positive reaction obtained on the blot was in the area representing approximately 6 kb, which was the expected size of the DNA probe (Figure 3.1). This result also reflects the success of the probe preparation mentioned previously, since there did not seem to be a problem with non-specific binding of the probe to other areas of the blot.

The analysis of callus DNA using Southern blotting would need to be repeated several times. However, building alterations and the demolition of the radioisotope laboratory at the time of this undertaking, prevented this work from being repeated or extended. In future research the use of a modification of the Southern blotting procedure, described by Scott *et al.* (1988), could also be utilised to give an indication of the copy number, multiple insertion patterns and stability of the DNA.



Figure 3.13: A comparison of different methods for the purification of DNA extracted from cell suspension culture-derived callus using agarose gel electrophoresis. GC = GENE CLEANTM; SCD = spun column chromatography + mini-dialysis.

Lanes: 1 - Lambda molecular weight markers III

2 - unpurified callus DNA

3 - BamHI-restricted unpurified callus DNA

4 - GC-purified callus DNA

5 - BamHI-restricted GC-purified callus DNA

6 - SCD-purified callus DNA

7 - BamHI-restricted SCD-purified callus DNA

8 - Lambda molecular weight markers III



Figure 3.14: Banding of plasmid and chromosomal DNA in a CsCl density gradient. (1 = chromosomal band; 2 = plasmid band)

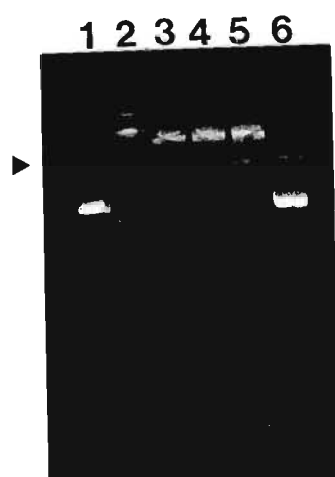


Figure 3.15: Isolation of the small fragment of plasmid DNA using a preparative low melting point agarose gel. (arrowhead = small fragment of plasmid DNA)

Lanes: 1 - pBR322 standard

2 - undigested plasmid DNA

3 - digested plasmid DNA

4 - digested plasmid DNA

5 - digested plasmid DNA

6 - pBR322 standard

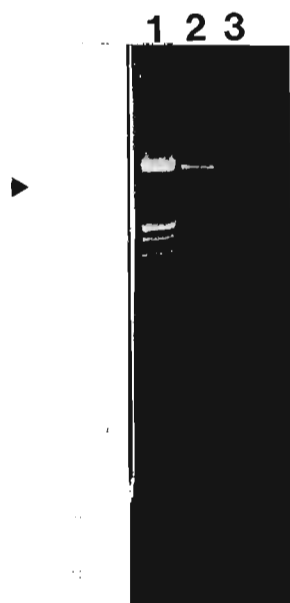


Figure 3.16: Agarose gel electrophoresis of BamHI-digested DNA extracted from cell suspension culture-derived callus. (arrowhead = position of DNA band of interest)

Lanes: 1 - Lambda molecular weight markers III

2 - DNA from control callus

3 - DNA from transformed callus

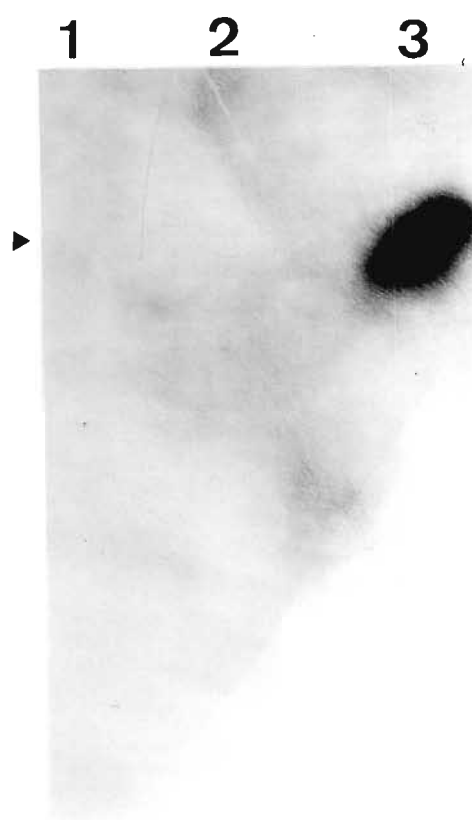


Figure 3.17: Autoradiogram of Southern blot hybridisation of BamHI-digested genomic DNA probed with the 6 kb HindIII fragment of the pJIT119 plasmid.

(arrowhead = position of DNA fragment of interest)

Lanes: 1 - Lambda molecular weight markers III

2 - DNA from control callus

3 - DNA from transformed callus

3.4 Conclusions

In this study, *Agrobacterium*-mediated transformation of *Eucalyptus* was attempted with two bacterial strains, C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119). The production of the binary vector LBA4404 (pJIT119) was successfully carried out using a triparental mating procedure, while the vector C58C1 (pMP90) (pJIT119) was obtained from Dr S. MacRae (Forestek, CSIR, Durban). The use of these two vectors for the transformation of different explant systems of *Eucalyptus* revealed that both strains are adequate for transformation, since there were no differences observed between the two strains.

The transformation of both cell suspension cultures and leaf discs of *Eucalyptus* with *A. tumefaciens* strains C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119) have resulted in the production of putatively transformed calli, but plantlet regeneration was not achieved. For both transformation systems, conditions for efficient transformation with both strains of *Agrobacterium*, curing manipulations for both bacterial strains, callus production, selection of callus on kanamycin and/or sulfadiazine and transient gus expression were established.

In conclusion, the protocol for the production of transgenic plants of *Eucalyptus* is impeded by the lack of methods for plant regeneration. However, recent investigations into both indirect organogenesis and indirect somatic embryogenesis have opened potential avenues to solve this problem and should be pursued. Towards this end, the recent observation on the production of embryoids from cell suspension culture-derived callus indicates that the possibility of producing somatic embryos from cell suspension cultures of *Eucalyptus* does exist.

CHAPTER 4: CONCLUDING REMARKS AND FUTURE RESEARCH STRATEGIES

4.1 Progress towards the production of transgenic *Eucalyptus*

The investigation into the micropropagation of *Eucalyptus* via indirect organogenesis and somatic embryogenesis achieved some success. Indirect organogenesis was achieved from leaf explants (Table 4.1, Part 1), while cell suspension cultures remained recalcitrant to regeneration using this method (Table 4.1, Part 2.1). On the other hand, while numerous media formulations for the induction of indirect somatic embryogenesis from cell suspension cultures were unsuccessful, results obtained towards the end of this investigation (Section 2.3.4.5) were encouraging, since embryogenic structures were produced on media containing ABA (12 mg.l⁻¹) and/or PEG (40 g.l⁻¹) (Table 4.1, Part 2.2). However this work needs to be extended to achieve embryo germination and subsequent plantlet regeneration.

As summarised in Table 4.1 (Part 3), the development of a model system for the *Agrobacterium*-mediated transformation, using both C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119), of both cell suspension cultures (Table 4.1, Part 3.2) and leaf explants (Table 4.1, Part 3.3) of *Eucalyptus* resulted in the formation of putatively transformed calli, which were GUS-positive and exhibited resistance to the selective antibiotic kanamycin. Sulfadiazine was also used for the selection of cell suspension culture-derived callus. These results indicated that both *Agrobacterium* strains are appropriate for the transformation of *Eucalyptus*, since differences in transformation efficiency between the two strains were not observed. However, plantlet regeneration from these transgenic calli has not been achieved.

The model system developed in this study for the genetic modification of *Eucalyptus* via *Agrobacterium* can be applied to the insertion of genes which are economically important for the forestry industry, when they become available.

Table 4.1: Summary of successes achieved during this investigation, and areas where further research is required.

PROTOCOL		RESULTS
1	Plant regeneration from leaf discs via indirect organogenesis	Achieved (Section 2.3.2)
2	Production of cell suspension cultures	Achieved (Section 2.3.3)
2.1	Plant regeneration from cell suspension cultures via indirect organogenesis	<u>Not achieved (Section 2.3.4.1)</u>
2.2	Plant regeneration from cell suspension cultures via indirect somatic embryogenesis	Achieved (Section 2.3.4.5)
	<u>production of embryos</u>	Awaiting results
	<u>germination of embryos and plantlet establishment</u>	
3	<i>Agrobacterium</i>-mediated transformation	
3.1	Production of <i>Agrobacterium</i> vector [LBA4404 (pJIT119)] for transformation of <i>Eucalyptus</i>	Achieved (Section 3.3.1)
3.2	Transformation of cell suspension cultures using both C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119) vectors	Protocol established (Section 3.3.3.1)
	<u>Establishment of conditions for selection of transformed cells</u>	Achieved (Section 3.3.3.2)
	<u>Production of putatively transformed callus</u>	Achieved (Section 3.3.3.2)
	<u>Production of transgenic plants</u>	Not achieved
3.3	Transformation of leaf discs using both C58C1 (pMP90) (pJIT119) and LBA4404 (pJIT119) vectors	Protocol established (Section 3.3.2.1)
	<u>Establishment of conditions for selection of transformed cells</u>	Achieved (Section 3.3.2.3)
	<u>Production of putatively transformed callus</u>	Achieved (Section 3.3.2.2)
	<u>Production of transgenic plants</u>	Not achieved

4.2 Potential applications of micropropagation to *Eucalyptus*

As previously mentioned, the propagation of tree species is an especially favourable target for *in vitro* regeneration techniques, and, although there are many applications of micropropagation *per se* (Figure 4.1), only those pertaining to the potential application of the protocols established in this study will be discussed here.

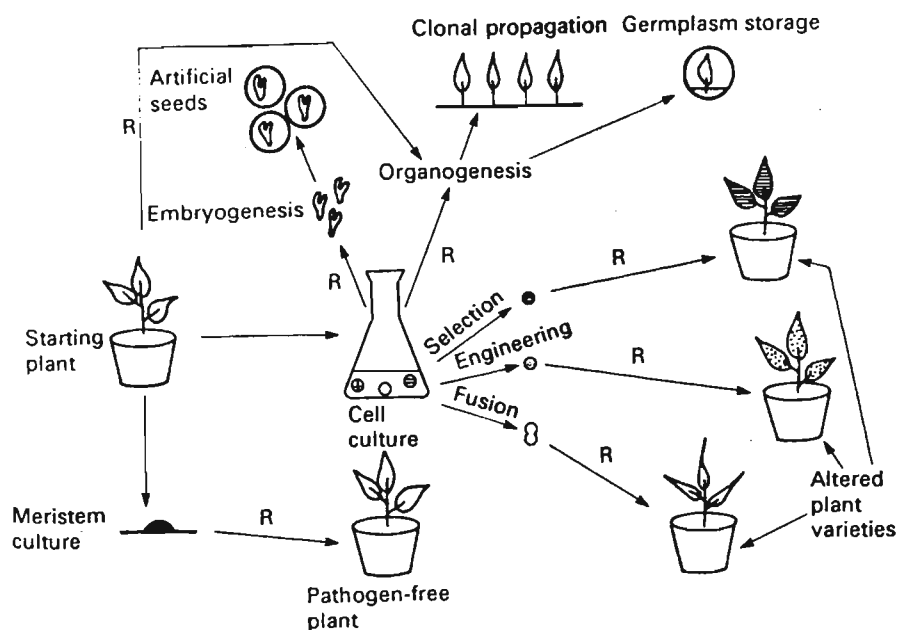


Figure 4.1: The exploitation of regeneration techniques for the improvement of plant species (reproduced from Warren, 1991).

4.2.1 Large-scale micropropagation

One of the major aspects of plant biotechnology is the ability to produce large numbers of identical individuals via *in vitro* cloning techniques. The recent advances in this field have led to the increased acceptance of *in vitro* propagation technology by the commercial sector (Prakash, 1989). The ability to produce many identical clones of a favourable genotype in a relatively short period of time allows for the selection and large-scale micropropagation of eucalypts with desirable attributes such as growth form or wood characteristics. This is economically beneficial when the long generation time and heterozygosity encountered with tree species is taken into account

(Warren, 1991). Mass propagation of superior high-yielding trees may contribute to improving the productivity of plantations so that the same amount of wood is produced in a smaller area (Turnbull, 1991). However, the major difficulties in expanding propagation techniques to new species are the high production costs and the problems encountered in developing efficient systems to deal with a large number of plants (Ammirato, 1986; Prakash, 1989; Warren, 1991). The use of direct organogenesis would be the method of choice for mass propagation, since somaclonal variation would be minimised. Therefore an investigation into the direct organogenic procedure would be a viable alternative to the indirect method of organogenesis developed in this investigation. However, the indirect organogenic protocol does have potential for applications such as genetic transformation studies.

Another possibility for large-scale micropropagation of *Eucalyptus* is the production of artificial or synthetic seeds by the encapsulation of somatic embryos (Redenbaugh *et al.*, 1987), which would dramatically reduce the cost of seedling production (Gupta *et al.*, 1993). This is achieved by encapsulating the somatic embryo in an artificial coat such as calcium alginate, which contains all the nutrients and hormones which would normally be supplied by the seed endosperm in nature (Warren, 1991). The synthetic seeds are then used as normal seeds. In an example given by Nashar (1989), a few hundred grams of initial material will be able to produce one million embryos within a few weeks in a 50 litre fermenter. This has the potential to revolutionise the forestry industries, provided that protocols for the production of somatic embryos of economically important forest species, such as *Eucalyptus* are available. In this regard, the progress towards the production of somatic embryos achieved in this study is the first step which would make this possible. However, in order to make the procedure economically feasible and to meet the high production costs, the finished plants must individually command a high enough price (Ammirato, 1986). Therefore, investigations into the mechanisation of propagation techniques and the use of bioreactor systems for liquid cultures must be undertaken to minimise the cost and

labour required for large-scale micropropagation of *Eucalyptus* (Constantine, 1986; Gupta *et al.*, 1991; Ziv, 1991b).

4.2.2 Germplasm conservation and storage

The preservation of genetically stable tissue for future propagation is of fundamental importance to plant breeders, in order to maintain the existing biodiversity (Wilkins *et al.*, 1982). The irretrievable loss of naturally occurring germplasm is a problem of great concern to tree breeders (Chen and Kartha, 1987). With the increasing demand for forest products and continuing deforestation, conservation of genetic resources is a vital area for consideration, since the available germplasm needs to be preserved for use in future tree improvement programmes (Chen and Kartha, 1987; Ahuja, 1989).

Maintaining active collections as living plants in the field or greenhouse is labour-intensive and expensive and may result in the loss of material due to pests and natural disasters (Kartha, 1981; Dodds and Roberts, 1985c; Krogstrup *et al.*, 1992). For forest species, such as *Eucalyptus*, other major problems are the large area required for a field collection and, due to the heterogeneity of forest trees, the large sample collection necessary in order to conserve the genetic variation within a population (Chen and Kartha, 1987). Consequently, alternative strategies to the conventional preservation of woody species, are required. This has led to the increasing interest in the potential use of *in vitro* techniques for plant genetic conservation (Wilkins *et al.*, 1982). However, as the ultimate goal of a tissue culture system employed for genetic conservation is to preserve specific and unique genotypes, and hence clonal fidelity, tissue culture-induced variation must be minimised by the selection of a suitable explant (Section 2.1.2.4). For this reason, meristem culture seems to be the most suitable for purposes of germplasm conservation, since it is genetically stable and plants regenerated from meristem cultures contain very little genetic variation (Chen and Kartha, 1987). Towards this end, axillary bud proliferation and (possibly) somatic embryos, could be employed.

Germplasm can be stored *in vitro* in two ways: in tissue culture conditions and in liquid nitrogen. The former method is suitable for short to medium term storage and reduces subculture intervals. This method can be achieved by changes in the composition of the culture medium, such as nutrient depletion, or changes in the physical storage conditions of the cultures, such as a decrease in temperature (Wilkins *et al.*, 1982; Krogstrup *et al.*, 1992). Storage of plant tissue in liquid nitrogen is called cryopreservation. This involves the controlled freezing of the tissue to the temperature of liquid nitrogen, with or without prior use of a cryoprotectant, and is a method that has been successful for the long-term storage of forest tree germplasm *in vitro* (Kantha *et al.*, 1988; Krogstrup *et al.*, 1992), and could be applied to the cryopreservation of *Eucalyptus* germplasm. However, cryopreservation relies on methods being available to multiply and regenerate plantlets from the small explants stored in liquid nitrogen.

The advantages of *in vitro* conservation include the fact that these methods are extremely convenient in terms of labour costs and space utilisation since they require little or no maintenance (Wilkins *et al.*, 1982), which is especially important for *Eucalyptus* spp. which would, as already mentioned, take up vast areas of space if storage was in the form of field collections. It is also advantageous to have an alternative storage method available for species and hybrids where seeds are not set or are not readily available for storage (Krogstrup *et al.*, 1992).

4.2.3 Production of secondary metabolites

In addition to the application of *in vitro* techniques to propagation and breeding, the potential value of these techniques to the production of secondary metabolites was recently addressed (Fujita and Tabata, 1987; Cresswell *et al.*, 1989; Wilson, 1990; Orihara *et al.*, 1991; Taticek *et al.*, 1991; Verpoorte *et al.*, 1993). Success in this area has been limited, probably due to the method used, where cells or tissues are cultured

and then the induction process for production of the chemical of interest begins. A better approach would involve the detection of naturally-occurring or mutant cell lines that produce the compound at high levels could be undertaken (Widholm, 1988; Wilson, 1990). The production of secondary metabolites is important in the forestry industry for the production of substances like tannins and aromatics. For example, one of the main reasons that certain *Eucalyptus* species are grown, is for the essential oils extracted from their leaves (Lakshmi Sita, 1986). The ease and speed with which the production of secondary metabolites could be achieved, would be facilitated by the availability of cell suspension cultures and/or callus systems developed in this study.

4.3 Prospects for the production of transgenic plants of *Eucalyptus*

The potential exists to improve eucalypts by the insertion of agronomically useful genes such as resistance to herbicides (Chaleff, 1988; Hathaway, 1989a,b; Schulz *et al.*, 1990; Smith and Chaleff, 1990; Cao *et al.*, 1992; Chowdry and Vasil, 1992; Vasil *et al.*, 1992), insects (Raffa, 1989; Smigocki *et al.*, 1993) and diseases (Grumet, 1990; Bejarano and Lichtenstein, 1992; van den Bulk, 1991; Harms, 1992; Broglie and Broglie, 1993), thereby producing transgenic plants. However, the ability to regenerate plants from transformed cells or tissues is an essential prerequisite for the successful application of gene transfer to crop improvement programmes (Debeaujon and Branchard, 1993). The lack of plant regeneration from cultured cells, such as those from *Eucalyptus*, is the limiting factor in the use of *in vitro* genetic manipulation techniques such as genetic transformation (White, 1984). Hence, the objectives of this study were the development of methods for plant regeneration, gene insertion and manipulation, and the production of transgenic eucalypts.

With a reliable means of regenerating plants from tissue culture (Chapter 2) and an appropriate system to deliver novel genetic information (Chapter 3), researchers are provided with the means to deliberately alter the genome of a species (Cheliak and Rogers, 1990). In this way, biotechnology is expected to reduce the long time-period

required by the conventional breeding technology for tree improvement (Nel, 1985; Riemenschneider *et al.*, 1987; Ikemori *et al.*, 1994), and breeding and biotechnology are being integrated into some tree genetic improvement programmes (Riemenschneider *et al.*, 1987; Ewald, 1992), including those for *Eucalyptus* (Ikemori *et al.*, 1994).

Chimeric genes have been constructed and transferred to plants, but recombinant DNA technologies at present are confined to single gene-controlled traits (Ahuja, 1987b; Schuch, 1991), such as resistance to diseases (Olsen, 1988), herbicides (Fillatti *et al.*, 1987) and pests (Raffa, 1989). As pointed out by Ahuja (1987b), most of the commercially important traits for the forestry industry, such as yield, vigour, growth, height and biomass, are controlled by multiple genes which are not well understood at the molecular level. Advances in the understanding of the molecular basis of gene action is important if further progress in this field is to be economically beneficial (Von Arnold *et al.*, 1990; Schuch, 1991). Progress in the elucidation of factors such as those controlling lignin biosynthesis (Dean and Eriksson, 1992) are already underway, and may be among the earliest applications to be of benefit to the forestry industry (Whetten and Sederoff, 1991). The development of protocols for the genetic modification of forest species, such the method for *Agrobacterium*-mediated transformation of *Eucalyptus* documented in this study, are vital so that when the genes controlling these desirable traits are cloned, the techniques for their application exist. Engineering resistances, for example to pests and diseases, can therefore serve as a model for other transgenic applications (Raffa, 1989).

The application of genetic engineering to *Eucalyptus* and other forest trees does, however, also raise certain concerns. Forest trees have long generation cycles, with an extended vegetative phase, and foreign genes may be expressed immediately or remain active for a long time (Ahuja, 1988). The foreign genes may thus cause genetic changes by position effects or rearrangements after long periods of time, since

it is difficult to predict how stable or unstable foreign genes would be in long-lived tree species (Ahuja, 1988). Whichever strategy of genetic modification is used, it is therefore important that material is properly evaluated in field trials (Simons, 1992), prior to release into the environment. However, Gasser and Fraley (1992) point out that crops modified by molecular and cellular means should not be subject to additional federal regulations, since they pose risks no different from those plants modified by classical methods for similar traits.

The development of protocols for the production of plants with superior genotypes, using biotechnology, would assist plant breeders with applied objectives to increase productivity and extend the plantation of economically important forest species to marginal areas.

REFERENCES

- Afele, J.C., Senaratna, T., McKersie, B.D., Saxena, P.K. (1992) Somatic embryogenesis and plant regeneration from zygotic embryo culture in blue spruce (*Picea pungens* Engelman.). *Plant Cell Reports*. **11**, 299-303.
- Agrawal, D.C., Gebhardt, K. (1994) Rapid micropropagation of hybrid willow (*Salix*) established by ovary culture. *Journal of Plant Physiology*. **143**, 763-765.
- Ahuja, M.R. (1987a) Somaclonal variation. In: *Cell and Tissue Culture in Forestry, Volume 1, General Principles and Biotechnology*, pp. 272-285, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.
- Ahuja, M.R. (1987b) Gene transfer in forest trees. In: *Genetic Manipulation of Woody Plants*, pp. 25-41, Hanover, J.W., Keathley, D.E., (eds), Plenum Press, New York London.
- Ahuja, M.R. (1988) Gene transfer in woody plants: perspectives and limitations. In: *Somatic Cell Genetics of Woody Plants*, pp. 83-101, Ahuja, M.R., (ed.), Kluwer Academic Publishers, Dordrecht Boston London.
- Ahuja, M.R. (1989) Storage of forest tree germplasm at sub-zero temperatures. In: *Applications of Biotechnology in Forestry and Horticulture*, pp. 215-228, Dhawan, V., (ed.), Plenum Press, New York London.
- Allan, E. (1991) Plant cell culture. In: *Plant Cell and Tissue Culture*, pp.1-23, Stafford, A., Warren, G., (eds), Open University Press, Milton Keynes.
- Aly, M.A.M., Owens, L.D. (1987) A simple system for plant cell microinjection and culture. *Plant Cell, Tissue and Organ Culture*. **10**, 159-174.
- Ammirato, P.V. (1983) Embryogenesis. In: *Handbook of Plant Cell Culture: Techniques for Propagation and Breeding*, pp. 82-123, Evans, D.A., Sharp, W.R., Ammirato, P.V., Yamada, Y., (eds), Macmillan Publishing Company, New York London.
- Ammirato, P.V. (1984) Induction, maintenance and manipulation of development in embryonic suspension cultures. In: *Cell Culture and Somatic Cell Genetics of Plants, Volume 1, Laboratory Procedures and their Applications*, pp. 9-29, Vasil, I.K., (ed.), Academic Press, Orlando New York.
- Ammirato, P.V. (1986) Control and expression of morphogenesis in culture. In: *Plant Tissue Culture and its Agricultural Applications*, pp. 23-45, Withers, L.A., Alderson, P.G., (eds), Butterworths, London Boston Durban Singapore Sydney Toronto Wellington.
- Ammirato, P.V. (1987) Organisational events during somatic embryogenesis. In: *Plant Tissue and Cell Culture*, pp. 57-81, Green, C.E., Somers, D.A., Hackett, W.P., Biesboer, D.D., (eds), Alan R. Liss, Inc., New York.
- An, G., Ebert, P.R., Mitra, A., Ha, S.B. (1988) Binary Vectors. In: *Plant Molecular Biology Manual A3*, pp. 1-19, Gelvin, S.B., Schilperoort, R.A., Verma, D.P.S., (eds), Kluwer Academic Publishers, Belgium.

- Anderson, W.C. (1984) A revised medium for shoot proliferation of *Rhododendron*. *Journal of the American Society for Horticultural Science*. **109**, 343-347.
- Anon (1993) South African Forestry. Promotion Committee, Forestry Council, Rivonia, 4 pages.
- Antonetti, P.L.E., Pinon, J. (1993) Somaclonal variation within poplar. *Plant Cell, Tissue and Organ Culture*. **35**, 99-106.
- Aragao, F.L.J., Grossi de Sa, M-F., Davey, M.R., Brasileiro, A.C.M., Faria, J.C., Rech, E.L. (1993) Factors influencing transient gene expression in bean (*Phaseolus vulgaris* L.) using an electrical particle acceleration device. *Plant Cell Reports*. **12**, 483-490.
- Armitage, P., Walden, R., Draper, J. (1988) Vectors for the transformation of plant cells using *Agrobacterium*. In: *Plant Genetic Transformation and Gene Expression: a Laboratory Manual*, pp. 3-57, Draper, J., Scott, R., Armitage, P., Walden, R., (eds), Blackwell Scientific Publications, Oxford.
- Arokiaraj, P., Jones, H., Cheong, K.F., Coomber, S., Charlwood, B.V. (1994) Gene insertion into *Hevea brasiliensis*. *Plant Cell Reports*. **13**, 425-431.
- Arrillaga, I., Tobolski, J.J., Merkle, S.A. (1994) Advances in somatic embryogenesis and plant production of black locust (*Robinia pseudoacacia* L.). *Plant Cell Reports*. **13**, 171-175.
- Atanossov, A., Brown, D.C.W. (1984) Plant regeneration from suspension culture and mesophyll protoplasts of *Medicago sativa* L. *Plant Cell, Tissue and Organ Culture*. **3**, 149-162.
- Atkinson, R.G., Gardner, R.C. (1993) Regeneration of transgenic tamarillo plants. *Plant Cell Reports*. **12**, 347-351.
- Balan, M. (1994) Callus production, regeneration and evaluation of plants from immature inflorescences of orchardgrass (*Dactylis glomerata* L.). Abstracts VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Abstract No. S1-141, p. 41.
- Baribault, T.J., Skene, K.G.M., Cain, P.A., Scott, N.S. (1990) Transgenic grapevies: regeneration of shoots expressing β -glucuronidase. *Journal of Experimental Botany*. **41**, 1045-1049.
- Batty, N.P., Evans, J.M. (1992) Biological ballistics - no longer a shot in the dark. *Transgenic Research*. **1**, 107-113.
- Baulcombe, D. (1994) Novel strategies for engineering virus resistance in plants. *Current Opinion in Biotechnology*. **5**, 117-124.
- Becwar, M.R., Wann, S.R., Johnson, M.A., Verhagen, S.A., Feirer, R.P., Nagmani, R. (1988) Development and characterisation of *in vitro* embryogenic systems in conifers. In: *Somatic Cell Genetics of Woody Plants*, pp. 1-18, Ahuja, M.R., (ed.), Kluwer Academic Publishers.
- Beijersbergen, A., Dulk-Ras, A.D., Schilperoort, R.A., Hooykaas, P.J.J. (1992) Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. *Science*. **256**, 1324-1327.
- Beilmann, A., Albrecht, K., Schultze, S., Wanner, G., Pfitzner, U.M. (1992) Activation of a truncated PR-1 promotor by endogenous enhancers in transgenic plants. *Plant Molecular Biology*. **18**, 65-78.

- Bejarano, E.R., Lichtenstein, C.P. (1992) Prospects for engineering virus resistance in plants with antisense RNA. *Tibtech.* **10**, 383-387.
- Bergmann, L. (1977) Plating of plant cells. In: *Plant Tissue Culture and its Biotechnological Applications*, pp. 213-225, Barz, W., Reinhard, E., Zenk, M.H., (eds), Springer-Verlag, Berlin New York.
- Beringer, J.E., Bale, M.J., Hayes, P.K., Lazarus, C.M. (1992) Assessing and monitoring the risks of releasing genetically manipulated plants. *Proceedings of the Royal Society of Edinburgh.* **99**, 135-140.
- Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Research.* **22**, 8711-8721.
- Bevan, M.W., Flavell, R.B., Chilton, M.D. (1983) A chimeric antibiotic resistance gene as a selectable marker for plant cell transformation. *Nature.* **304**, 184-187.
- Bhansali, R.R. (1993) Bud culture for shoot multiplication and plantlet formation of *Tecomella undulata* (Rohida), a woody tree of the arid zone. *Tropical Science.* **33**, 1-8.
- Bhat, S.R., Kackar, A., Chandel, K.P.S. (1992) Plant regeneration from callus cultures of *Piper longum* L. by organogenesis. *Plant Cell Reports.* **11**, 525-528.
- Bhattacharya, P., Dey, S., Bhattacharyya, B.C. (1994) Use of low-cost gelling agents and support matrices for industrial scale plant tissue culture. *Plant Cell, Tissue and Organ Culture.* **37**, 15-23.
- Bhojwani, S.S., Dhawan, V. (1989) Acclimatisation of tissue culture-raised plants for transportation to the field. In: *Applications of Biotechnology in Forestry and Horticulture*, pp. 249-256, Dhawan, V., (ed.), Plenum Press, New York London.
- Bidney, D., Scelonge, C., Martich, J., Burrus, M., Sims, L., Huffman, G. (1992) Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. *Plant Molecular Biology.* **18**, 301-313.
- Bilang, R., Zhang, S., Leduc, N., Iglesias, V.A., Gisel, A., Simmonds, J., Potrykus, I., Sautter, C. (1993) Transient gene expression in vegetative shoot apical meristems of wheat after ballistic microtargeting. *The Plant Journal.* **4**, 735-744.
- Binh, D.Q., Heszky, L.E., Gyulai, G., Csillag, A. (1992) Plant regeneration of NaCl-pretreated cells from long-term suspension culture of rice (*Oryza sativa* L.) in high saline conditions. *Plant Cell, Tissue and Organ Culture.* **29**, 75-82.
- Binns, A.N. (1990) *Agrobacterium*-mediated gene delivery and the biology of host range limitations. *Physiologia Plantarum.* **79**, 135-139.
- Biondi, S., Thorpe, T.A. (1981) Clonal propagation of forest tree species. In: *Proceedings of the COSTED Symposium on Tissue Culture of Economically Important Plants*, pp. 197-204, Rao, A.N., (ed.), Singapore.
- Blakeway, F.C. Mondi Forests, P. O. Box 12, Hilton, 3245, South Africa.

- Blakeway, F.C. (1992) Development of *in vitro* culture systems for *Eucalyptus grandis*. MSc Thesis, Faculty of Science, University of Natal, Durban.
- Blakeway, F.C., Herman, B., Watt, M.P. (1993) Establishment of cell suspension cultures of *Eucalyptus grandis* and *E. grandis x camaldulensis*. South African Forestry Journal. **166**, 17-26.
- Blomstedt, C., Cameron, J., Whiteman, P., Chandler, S.F. (1991) Micropropagation of juvenile *Eucalyptus regnans* (Mountain Ash). Australian Journal of Botany. **39**, 179-186.
- Bolyard, M.G., Srinivasan, C., Cheng, J., Sticklen, M. (1991) Shoot regeneration from leaf explants of American and Chinese elm. HortScience. **26**, 1554-1555.
- Bommineni, V.R., Chibbar, R.N., Datla, R.S.S., Tsang, E.W.T. (1993) Transformation of white spruce (*Picea glauca*) somatic embryos by microprojectile bombardment. Plant Cell Reports. **13**, 17-23.
- Bonneau, L., Beranger-Novat, N., Monin, J. (1994) Somatic embryogenesis and plant regeneration in a woody species: the European Spindle Tree (*Euonymus europaeus* L.). Plant Cell Reports. **13**, 135-138.
- Botha, F. Biotechnology Department, SASEX, Private Bag X02, Mount Egdecombe, 4300, South Africa.
- Bright, S.W.J., Ooms, G., Foulger, D., Karp, A., Evans, N. (1986) Mutation and tissue culture. In: Plant Tissue Culture and its Agricultural Applications, pp. 431-447, Withers, L.A., Alderson, P.G., (eds), Butterworths, London Boston Durban Singapore Sydney Toronto Wellington.
- Brogliè, R., Brogliè, K. (1993) Production of disease-resistant transgenic crops. Current Opinion in Biotechnology. **4**, 148-151.
- Brown, D.C.W., Tian, L., Buckley, D.J., Lefebvre, M., McGrath, A., Webb, J. (1994) Development of a simple particle bombardment device for gene transfer into plant cells. Plant Cell, Tissue and Organ Culture. **37**, 47-53.
- Buchanan-Wollaston, V., Snape, A., Cannon, F. (1992) A plant selectable marker gene based on the detoxification of the herbicide dalapon. Plant Cell Reports. **11**, 627-631.
- Burger, D.W. (1987) *In vitro* micropropagation of *Eucalyptus sideroxylon*. HortScience. **22**, 496-497.
- Burr, B., Burr, F.A. (1985) Towards a molecular characterisation of multiple factor inheritance. In: Biotechnology in Plant Science, pp. 277-284, Zaitlin, M., Day, P., Hollaender, A. (eds), Academic Press, Inc., San Diego New York Berkeley Boston London Sydney Tokyo Toronto.
- Büter, B., Pescitelli, S.M., Berger, K., Schmid, J.E., Stamp, P. (1993) Autoclaved and filter sterilised liquid media in maize anther culture: significance of activated charcoal. Plant Cell Reports. **13**, 79-82.
- Caboche, M. (1980) Nutritional requirements of protoplast-derived haploid tobacco cells grown at low cell densities in liquid medium. Planta. **149**, 7-18.

- Campbell, M.A., Kinlaw, C.S., Neale, D.B. (1992) Expression of luciferase and β -glucuronidase in *Pinus radiata* suspension cells using electroporation and particle bombardment. *Canadian Journal of Forestry Research*. **22**, 2014-2018.
- Capecchi, M.R. (1980) High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell*. **22**, 479-488.
- Cardarelli, M., Mariotti, D., Pomponi, M., Spano, L., Capone, I., Constantino, P. (1987) *Agrobacterium rhizogenes* T-DNA capable of inducing hairy root phenotype. *Molecular and General Genetics*. **209**, 475-480.
- Carlberg, I., Glimelius, K., Eriksson, T. (1984) Nuclear-DNA-content during the initiation of callus formation from isolated protoplasts of *Solanum tuberosum*. *Plant Science Letters*. **35**, 225-230.
- Cao, J., Duan, X., McElroy, D., Wu, R. (1992) Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. *Plant Cell Reports*. **11**, 586-591.
- Cardi, T., Iannamico, V., D'Ambrosio, F., Filippone, E., Lurquin, P.F. (1992) *Agrobacterium*-mediated genetic transformation of *Solanum commersonii* Dun. *Plant Science*. **87**, 179-189.
- Carrer, H., Hockenberry, T.N., Svab, Z., Maliga, P. (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. *Molecular and General Genetics*. **241**, 49-56.
- Chaleff, R.S. (1988) Herbicide-resistant plants from cultured cells. In: *Applications of Plant Cell and Tissue Culture*, Ciba Foundation Symposium 137, pp. 3-20, Bock, G., Marsh, J., (eds), John Wiley and Sons, Chichester New York Brisbane Toronto Singapore.
- Chan, M-T., Chang, H-H., Ho, S-L., Tong, W-F., Yu, S-M. (1993) *Agrobacterium*-mediated production of transgenic rice plants expressing a chimeric α -amylase/ β -glucuronidase gene. *Plant Molecular Biology*. **22**, 491-506.
- Chan, M-T., Lee, T-M., Chang, H-H. (1992) Transformation of Indica Rice (*Oryza sativa* L.) mediated by *Agrobacterium tumefaciens*. *Plant Cell Physiology*. **33**, 577-583.
- Charest, P.J., Caléro, N., Lachance, D., Datla, R.S.S., Duchêsne, L.C., Tsang, E.W.T. (1993) Microprojectile-DNA delivery in conifer species: factors affecting assessment of transient gene expression using the β -glucuronidase reporter gene. *Plant Cell Reports*. **12**, 189-193.
- Cheliak, W.M., Rogers, D.L. (1990) Integrating biotechnology into tree improvement programs. *Canadian Journal of Forest Research*. **20**, 452-463.
- Cheliak, W.M., Klimaszewska, K. (1990) Cell, protoplast and tissue culture of short-rotation species. *Biomass*. **22**, 63-73.
- Chen, T-H., Lam, L., Chen, S-C. (1985) Somatic embryo and plant regeneration from cultured young inflorescences of *Oryza sativa* L. (rice). *Plant Cell, Tissue and Organ Culture*. **4**, 51-54.
- Chen, T., Kartha, K.K. (1987) Cryopreservation of woody species. In: *Cell and Tissue Culture in Forestry*, Volume 2, Specific Principles and Methods: Growth and Developments, pp. 305-319, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.

- Cheng, B., Peterson, C.M., Mitchell, R.J. (1992) The role of sucrose, auxin and explant source on *in vitro* rooting of seedling explants of *Eucalyptus sideroxylon*. *Plant Science*. **87**, 207-214.
- Cheung, W.Y., Hubert, N., Landry, B.S. (1993) A simple and rapid DNA microextraction method for plant, animal, and insect suitable for RAPD and other PCR analyses. *PCR Methods and Applications*. **3**, 69-70.
- Chopra, V.L., Sharma, R.P. (1991) Biotechnology in crop improvement. *Current Science*. **60**, 543-547.
- Chowdhury, M.K.U., Vasil, I.K. (1992) Stably transformed herbicide resistant callus of sugarcane via microprojectile bombardment of cell suspension cultures and electroporation of protoplasts. *Plant Cell Reports*. **11**, 494-498.
- Chriqui, D., Adam, S., Caissard, J-C., Noin, M., Azmi, A. (1991) Shoot regeneration and *Agrobacterium*-mediated transformation of *Eucalyptus globulus* and *E. gunnii*. In: Symposium on Intensive Forestry: the Role of Eucalypts, pp. 70-80, Schönau, A.P.G., (ed.), International Union of Forestry Research Organisations.
- Christianson, M.L. (1987) Causal effects in morphogenesis. In: *Plant Tissue and Cell Culture*, pp. 45-55, Green, C.E., Somers, D.A., Hackett, W.P., Biesboer, D.D., (eds), Alan R. Liss, Inc., New York.
- Christou, P. (1991) Particle-bombardment-mediated transformation of organized tissue and its impact on agricultural biotechnology. *International Association for Plant Tissue Culture Newsletter*. **66**.
- Christou, P. (1993) Particle gun mediated transformation. *Current Opinion in Biotechnology*. **4**, 135-141.
- Christou, P., Ford, T.L., Kofron, M. (1991) Production of transgenic rice (*Oryza sativa* L.) plants from agronomically important *indica* and *japonica* varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. *Bio/Technology*. **9**, 957-962.
- Chupeau, M-C., Pautot, V., Chupeau, Y. (1994) Recovery of transgenic trees after electroporation of poplar protoplasts. *Transgenic Research*. **3**, 13-19.
- Cocking, E.C., Davey, M.R. (1987) Gene transfer in cereals. *Science*. **236**, 1259-1262.
- Colby, S.M., Juncosa, A.M., Meredith, C.P. (1991) Cellular differences in *Agrobacterium* susceptibility and regenerative capacity restrict the development of transgenic grapevines. *Journal of the American Society for Horticultural Science*. **116**, 356-361.
- Collin, H.A., Dix, P.J. (1990) Culture systems and selection procedures. In: *Plant Cell Line Selection - Procedures and Applications*, pp. 3-18, Dix, P.J., (ed.), VCH, Weinheim New York Basel Cambridge.
- Comai, L., Facciotti, D., Hiatt, W.R., Thompson, G., Rose, R.E., Stalker, D.M. (1985) Expression in plants of a mutant *aroA* gene from *Salmonella typhimurium* confers tolerance to glyphosate. *Nature*. **317**, 741-744.
- Confalonieri, M., Balestrazzi, A., Bisoffi, S. (1994) Genetic transformation of *Populus nigra* by *Agrobacterium tumefaciens*. *Plant Cell Reports*. **13**, 256-261.

- Conger, B.V. (1981) Agronomic crops. In: Cloning of Agricultural Plants via *in vitro* Techniques, pp. 165-216, Conger, B.V., (ed.), CRC Press, Inc., Florida.
- Constabel, F. (1984) Callus culture: induction and maintenance. In: Cell Culture and Somatic Cell Genetics of Plants, Volume 1, Laboratory Procedures and their Applications, pp. 27-35, Vasil, I.K., (ed.), Academic Press, Orlando San Diego New York London Toronto Montreal Sydney Tokyo.
- Constantin, M.J., Henke, R.R., Mansur, M.A. (1977) Effect of activated charcoal on callus growth and shoot organogenesis in tobacco. *In Vitro*. **13**, 293.
- Constantine, D.R. (1986) Micropropagation in the commercial environment. In: Plant Tissue Culture and Its Agricultural Applications, pp. 175-186, Withers, L.A., Alderson, P.G., (eds), Butterworths, London Boston Durban Singapore Sydney Toronto Wellington.
- Cortezzi Graça, M.E., Mendes, S. (1989) Micropropagation of *Eucalyptus dunnii* Maid. *Ann. Sci. For.* **46**, 140-144.
- Cresswell, R.C., Fowler, M.W., Stafford, A., Stephank-Sarkissian, G. (1989) Inputs and outputs: primary substrates and secondary metabolism. In: Primary and Secondary Metabolism of Plant Cells II, pp. 14-26, Kurz, G.W., (ed.), Springer-Verlag, Berlin New York.
- Cresswell, R. (1991) Improvement of plants via plant cell culture. In: Plant Cell and Tissue Culture, pp. 101-123, Stafford, A., Warren, G., (eds), Open University Press, Milton Keynes.
- Cuozzo, M., O'Connell, K.M., Kaniewski, W., Fang, R-X., Chua, N-H., Tumer, N.E. (1988) Viral protection in transgenic tobacco plants expressing the cucumber mosaic virus coat protein or its antisense RNA. *Bio/Technology*. **6**, 549-557.
- Dale, P.J. (1993) The release of transgenic plants into agriculture. *Journal of Agricultural Science*. **120**, 1-5.
- Das, P., Rout, G.R., Das, A.B. (1993) Somatic embryogenesis in callus culture of *Mussaenda erythrophylla* cvs. Queen Sirikit and Rosea. *Plant Cell, Tissue and Organ Culture*. **35**, 199-201.
- Das. T., Mitra, G.C. (1990) Micropropagation of *Eucalyptus tereticornis* Smith. *Plant Cell, Tissue and Organ Culture*. **22**, 95-103.
- Davey, M.R., Rech, E.L., Mulligan, B.J. (1989) Direct DNA transfer to plant cells. *Plant Molecular Biology*. **13**, 273-285.
- David, H., Jarlet, E., David, A. (1984) Effect of nitrogen source, calcium concentration and osmotic stress on protoplasts and protoplast-derived cell cultures of *Pinus pinaster* cotyledons. *Physiologia Plantarum*. **61**, 477-482.
- De Block, M. (1990) Factors influencing the tissue culture and the *Agrobacterium tumefaciens*-mediated transformation of hybrid aspen and poplar clones. *Plant Physiology*. **93**, 1110-1116.
- De Block, M. (1993) The cell biology of plant transformation: Current state, problems, prospects and the implications for the plant breeding. *Euphytica*. **71**, 1-14.

- De Fossard, R.A., Barker, P.K., Bourne, R.A. (1977) The organ culture of nodes of four species of *Eucalyptus*. *Acta Horticulturae*. **78**, 157-165.
- De Framond, A., Barton, K.A., Chilton, M-D. (1983) Mini-Ti: a new vector strategy for plant genetic engineering. *Bio/Technology*. **1**, 262-269.
- Dean, C., Jones, J., Favreau, M., Dunsmuir, P., Bedbrook, J. (1988) Influence of flanking sequences on variability in expression levels of an introduced gene in transgenic tobaccoplants. *Nucleic Acids Research*. **16**, 9267-9283.
- Dean, J.F.D., Eriksson, K-E.L. (1992) Biotechnological modification of lignin structure and composition in forest trees. *Holzforschung*. **46**, 135-147.
- Debeaujon, I., Branchard, M. (1993) Somatic embryogenesis in *Cucurbitaceae*. *Plant Cell, Tissue and Organ Culture*. **34**, 91-100.
- Debergh, P.C., De Meester, J., De Riek, J., Gillis, S., Van Huylenbroeck, J. (1992) Ecological and physiological aspects of tissue-cultured plants. *Acta Botanica Neerl*. **41**, 417-423.
- Delbreil, B., Guerche, P., Jullien, M. (1993) *Agrobacterium*-mediated transformation of *Asparagus officinalis* L. long-term embryogenic callus and regeneration of transgenic plants. *Plant Cell Reports*. **12**, 129-132.
- Denison, N.P., Quaile, D.R. (1987) The applied clonal eucalypt programme in Mondi forests. *South African Forestry Journal*. **142**, 60-66.
- Denison, N.P., Kietzka, J.E. (1993) The development and utilisation of vegetative propagation in Mondi for commercial afforestation programmes. *South African Forestry Journal*. **165**, 47-60.
- Deroles, S.C., Gardner, R.C. (1988) Analysis of the T-DNA structure in a large number of petunias generated by *Agrobacterium*-mediated transformation. *Plant Molecular Biology*. **11**, 365-377.
- Dillen, W., Buysens, S. (1989) A simple technique to overcome vitrification in *Gypsophylla paniculata*. *Plant Cell, Tissue and Organ Culture*. **19**, 181-188.
- Dixon, R.A., (ed.) (1985) *Plant Cell Culture: a Practical Approach*, p. 236, IRL Press, Oxford Washington DC.
- Dodds, J.H., Roberts, L.W., (eds) (1985a) Cell suspension cultures. In: *Experiments in Plant Tissue Culture*, Second Edition, pp. 104-112, Cambridge University Press, Cambridge New York New Rochelle Melbourne Sydney.
- Dodds, J.H., Roberts, L.W., (eds) (1985b) Anther and pollen culture. In: *Experiments in Plant Tissue Culture*, Second Edition, pp. 157-171, Cambridge University Press, Cambridge New York New Rochelle Melbourne Sydney.
- Dodds, J.H., Roberts, L.W., (eds) (1985c) Storage of plant genetic resources. In: *Experiments in Plant Tissue Culture*, Second Edition, pp. 172-179, Cambridge University Press, Cambridge New York New Rochelle Melbourne Sydney.
- Dodds, J.H., Roberts, L.W., (eds) (1985d) Aseptic Techniques. In: *Experiments in Plant Tissue Culture*, Second Edition, pp. 21-34, Cambridge University Press, Cambridge New York New Rochelle Melbourne Sydney.

- Donald, D.G.M., Newton, D.J. (1991) *In vitro* propagation of *Eucalyptus radiata*. South African Forestry Journal. **157**, 66-68.
- Dong, J-Z., McHughen, A. (1993) An improved procedure for production of transgenic flax plants using *Agrobacterium tumefaciens*. Plant Science. **88**, 61-71.
- Douglas, C., Halperin, W., Gordon, M., Nester, E. (1985) Specific attachment of *Agrobacterium tumefaciens* to bamboo cells in suspension cultures. Journal of Bacteriology. **161**, 764-766.
- Draper, J., Scott, R., Hamil, J. (1988) Transformation of dicotyledonous plant cells using the Ti plasmid of *Agrobacterium tumefaciens* and the Ri plasmid of *A. rhizogenes*. In: Plant Genetic Transformation and Gene Expression: a Laboratory Manual, pp. 69-198, Draper, J., Scott, R., Armitage, P., Walden, R., (eds), Blackwell Scientific Publications, Oxford.
- Draper, J., Scott, R. (1988) The isolation of plant nucleic acids. In: Plant Genetic Transformation and Gene Expression: a Laboratory Manual, pp. 199-261, Draper, J., Scott, R., Armitage, P., Walden, R., (eds), Blackwell Scientific Publications, Oxford.
- Druart, P., De Wulf, O. (1993) Activated charcoal catalyses sucrose hydrolysis during autoclaving. Plant Cell, Tissue and Organ Culture. **32**, 97-99.
- Drummond, M. (1979) Crown Gall Disease. Nature. **281**, 343-347.
- Duchesne, L.C., Lelu, M-A., Von Aderkas, P., Charest, P.J. (1993) Microprojectile-mediated DNA delivery in haploid and diploid embryogenic cells of *Larix* spp. Canadian Journal of Forest Research. **23**, 312-316.
- Duncan, D.R., Widholm, J.M. (1986) Cell selection for crop improvement. Plant Breeding Reviews. **4**, 153-173.
- Dunsmuir, P., Bond, D., Lee, K., Gidoni, D., Townsend, J. (1988) Stability of introduced genes and stability in expression. In: Plant Molecular Biology Manual, C1, pp. 1-7, Gelvin, S.B., Schilperoort, R.A., Verma, D.P.S., (eds), Kluwer Academic Publishers, Belgium.
- Dunstan, D.I., Bethune, T.D., Bock, C.A. (1993) Somatic embryo maturation from long-term suspension cultures of white spruce (*Picea glauca*). *In Vitro Cellular and Developmental Biology*. **29**, 109-112.
- Dunstan, D.I., Bethune, T.D., Schnaider, J.M., Bock, C.A. (1994) PEG 4000 stimulates somatic embryo maturation and endogenous ABA in *Picea glauca x engelmannii*. Abstracts VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Abstract No. S11-57, p. 189.
- Durand-Cresswell, R.J., Nitsch, C. (1977) Factors influencing the regeneration of *Eucalyptus grandis* by organ culture. Acta Horticulturae. **78**, 149-155.
- Durand-Cresswell, R.J., Boulay, M., Franclet, A. (1982) Vegetative propagation of *Eucalyptus*. In: Tissue Culture in Forestry, pp. 150-181, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff, The Hague.
- Durzan, D.J. (1984) Special problems: adult vs. juvenile explants. In: Handbook of Plant Cell Culture, Volume 2, Crop Species, pp. 471-503, Sharp, W.R., Evans, D.A., Ammirato, P.V., Yamada, Y., (eds), Macmillan Publishing Company, New York.

- Durzan, D.J. (1988) Applications of cell and tissue culture in tree improvement. In: Applications of Plant Cell and Tissue Culture, Ciba Foundation Symposium 137, pp. 36-58, Bock, G., Marsh, J., (eds), John Wiley and Sons, Chichester New York Brisbane Toronto Singapore.
- Earle, D.E., Torrey, J.G. (1965a) Morphogenesis in cell colonies grown from *Convolvulus* cell suspensions plated on synthetic media. *American Journal of Botany*. **52**, 891-899.
- Earle, D.E., Torrey, J.G. (1965b) Colony formation by isolated *Convolvulus* cells plated on defined media. *Plant Physiology*. **40**, 520-528.
- Ellis, D.D., McCabe, D.E., McInnis, S., Ramachandran, R., Russell, D.R., Wallace, K.M., Martinell, B.J., Roberts, D.R., Raffa, K.F., McCown, B.H. (1993) Stable transformation of *Picea glauca* by particle acceleration. *Bio/Technology*. **11**, 84-89.
- Etienne, H., Sotta, B., Montoro, P., Miginiac, E., Carron, M-P. (1993) Comparison of endogenous ABA and IAA contents in somatic and zygotic embryos of *Hevea brasiliensis* (Müll. Arg.) during ontogenesis. *Plant Science*. **92**, 111-119.
- Evans, D.A., Sharp, W.R., Flick, C.E. (1981) Growth and Behaviour of cell cultures: embryogenesis and organogenesis. In: *Plant Tissue Culture: Methods and Applications in Agriculture*, pp: 45-113, Thorpe, T.A., (ed.), Academic Press, New York London Toronto Sydney San Francisco.
- Evans, D.A., Sharp, W.R. (1986) Applications of somaclonal variation. *Bio/Technology*. **4**, 528-532.
- Ewald, D. (1992) Aims and results of basic research in the institute of forest tree breeding in Waldsiefersdorf, Germany. I. The development of biotechnological research. *Silvae Genetica*. **41**, 196-201.
- Feinberg, A:P., Vogelstein, B. (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry*. **132**, 6-13.
- Fillatti, J.J. (1990) Strategies for plant transformation. In: *Horticultural Biotechnology*, pp. 51-69, Bennett, A.B., O'Neill, S.D., (eds), John Wiley and Sons, Inc. Publication, New York Chichester Brisbane Toronto Singapore.
- Fillatti, J.J., Haissig, B., McCown, B., Comai, L., Riemenschneider, D. (1987) Development of glyphosate-tolerant *Populus* plants through expression of a mutant *aroA* gene from *Salmonella typhimurium*. In: *Genetic Manipulation of Woody Plants*, pp. 243-249, Hanover, J.W., Keathley, D.E., (eds), Plenum Press, New York London.
- Flick, C.E., Evans, D.A., Sharp, W.R. (1983) Organogenesis. In: *Handbook of Plant Cell Culture, Volume 1, Techniques for Propagation and Breeding*, pp. 13-81, Evans, D.A., Sharp, W.R., Ammirato, P.V., Yamada, Y., (eds), MacMillan, New York.
- Fraley, R.T., Rogers, S.G., Horsch, R.D., Sanders, P.R., Flick, J.S. (1983) Expression of bacterial genes in plant cells. *Proceedings of the National Academy of Science. USA*. **80**, 4803-4807.
- Fraley, R.T., Rogers, S.G., Horsch, R.B. (1986) Genetic transformation of higher plants. *CRC Critical Reviews in Plant Science*. **4**, 1-46.
- Francllet, A., Boulay, T. (1982) Micropropagation of frost resistant eucalypt clones. *Australian Forestry Research*. **13**, 83-89.

- Frett, J.J., Dirr, M.A. (1983) Tissue culture propagation of *Liriope muscari* and *Ophiopogon jaburan*. HortScience. **18**, 431-432.
- Fromm, M.E., Taylor, L.P., Walbot, V. (1986) Stable transformation of maize after gene transfer by electroporation. Nature. **319**, 791-793.
- Fuchs, R.L., Perlak, F.J. (1992) Commercialisation of genetically engineered plants. Current Opinion in Biotechnology. **3**, 181-184.
- Fujita, Y., Tabata, M. (1987) Secondary metabolites from plant cells - pharmaceutical applications and progress in commercial production. In: Plant Tissue and Cell Culture, pp. 169-185, Green, C.E., Somers, D.A., Hackett, W.P., Biesboer, D.D., (eds), Alan R. Liss, Inc., New York.
- Furze, M.J., Cresswell, C.F. (1985) Micropropagation of *Eucalyptus grandis* and *nitens* using tissue culture techniques. South African Forestry Journal, **135**, 20-23.
- Gallie, D.R. (1993) Introduction of mRNA to plant protoplasts using polyethylene glycol. Plant Cell Reports. **13**, 119-122.
- Gallo-Meagher, M., Irvine, J.E. (1993) Effects of tissue type and promotor strength on transient GUS expression in sugarcane following particle bombardment. Plant Cell Reports. **12**, 666-670.
- Gamborg, O.L., Miller, R.A., Ojima, K. (1968) Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research. **50**, 151-158.
- Gao, J., Lee, J.M., An, G. (1991) The stability of foreign protein production in genetically modified plant cells. Plant Cell Reports. **10**, 533-536.
- Gaspar, T., Kevers, C., Debergh, P., Maene, L., Paques, M., Boxus, P. (1987) Vitrification: morphological, physiological and ecological aspects. In: Cell and Tissue Culture in Forestry, Volume 1, General Principles and Biotechnology, pp. 152-166, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.
- Gasser, C.S., Fraley, R.T. (1992) Transgenic crops. Scientific American. **266**, 62-69.
- Gendloff, E.H., Bowen, B., Buchholz, W.G. (1990) Quantitation of chloramphenicol acetyl transferase in transgenic tobacco plants by ELISA and correlation with gene copy number. Plant Molecular Biology. **14**, 575-583.
- Godon, C., Caboche, M., Daniel-Vedele, F. (1993) Transient plant gene expression: a simple and reproducible method based on flowing particle gun. Biochimie. **75**, 591-595.
- Goldfarb, B., Strauss, S.H., Howe, G.T., Zaerr, J.B. (1991) Transient expression of microprojectile-introduced DNA in Douglas-fir cotyledons. Plant Cell Reports. **10**, 517-521.
- Goodman, R.M. (1990) Gene transfer in crop improvement - an introduction and overview. Journal of the Iowa Academy of Sciences. **97**, 1-8.
- Gordon, A.M., Brown, I.R. (1988) The occurrence of endogenous bacteria in birch shoot cultures. In: Somatic Cell Genetics of Woody Plants, pp. 191-193, Ahuja, M.R., (ed.), Kluwer Academic Publishers, Dordrecht Boston London.

- Gorinova, N., Atanasov, A., Alexandrova, K., Velkova, R., Kasachka, A. (1993) Comparison of agar and microcrystal cellulose as gelling agents for *in vitro* culture of *Nicotiana tabacum* stem explants. *Biologia Plantarum*. **35**, 217-221.
- Goto, F., Toki, S., Uchimiya, H. (1993) Inheritance of a co-transferred foreign gene in the progenies of transgenic rice plants. *Transgenic Research*. **2**, 300-305.
- Graz, F.P., von Gadow, K. (1990) Forecasting the *Eucalyptus grandis* resource in South Africa. In: Management of *Eucalyptus grandis* in South Africa, pp. 121-131, von Gadow, K., Breidenkamp, B.V., (eds), Stellenbosch.
- Grevelding, C., Fantes, V., Kemper, E., Schell, J., Masterson, R. (1993) Single-copy T-DNA insertions in *Arabidopsis* are the predominant form of integration in root-derived transgenics, whereas multiple insertions are found in leaf discs. *Plant Molecular Biology*. **23**, 847-860.
- Grierson, D., Covey, S.N. (1984a) Prospects for the genetic engineering of plants. In: *Plant Molecular Biology*, First Edition, pp. 147-159, Blackie & Son Limited, Glasgow London.
- Grierson, D., Covey, S.N. (1984b) Genetic colonization of plants by *Agrobacterium*. In: *Plant Molecular Biology*, First Edition, pp. 112-125, Blackie & Son Limited, Glasgow London.
- Grierson, D., Covey, S.N. (1988) Genetic engineering of plants. In: *Plant Molecular Biology*, Second Edition, pp. 182-211, Blackie, Glasgow London.
- Griga, M. (1993) Some factors affecting somatic embryogenesis efficiency in soybean (*Glycine max* (L.) Merr.). *Biologia Plantarum*. **35**, 179-189.
- Grosser, J.W., Collins, G.B. (1984) Isolation and culture of *Trifolium rubens* protoplasts with whole plant regeneration. *Plant Science Letters*. **37**, 165-170.
- Grumet, R. (1990) Genetically engineered plant virus resistance. *HortScience*. **25**, 508-513.
- Guerineau, F., Brooks, L., Mullineaux, P.M. (1989) A bacterial sulfonamide resistance gene as a selectable marker for plant transformation. *Plant Genetics and Breeding Research Annual Report*, pp. 13-14.
- Guerineau, F., Brooks, L., Meadows, J., Lucy, A., Robinson, C., Mullineaux, P. (1990) Sulfonamide resistance gene for plant transformation. *Plant Molecular Biology*. **15**, 127-136.
- Guo, J.H., Liang, G.H. (1993) Callus induction and plant regeneration of cultivated and wild sorghums. *Cytologia*. **58**, 203-210.
- Gupta, P.K., Mehta, U.J., Mascarenhas, A.F. (1983) A tissue culture method for rapid clonal propagation of mature trees of *Eucalyptus torelliana* and *Eucalyptus camaldulensis*. *Plant Cell Reports*. **2**, 296-299.
- Gupta, P.K., Mascarenhas, A.F. (1987) *Eucalyptus*. In: *Cell and Tissue Culture in Forestry*, Volume 3, Case Histories: Gymnosperms, Angiosperms and Palms, pp. 385-399, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.
- Gupta, P.K., Timmis, R., Mascarenhas, A.F. (1991) Field performance of micropropagated forestry species. *In Vitro Cellular and Developmental Biology*. **27**, 159-164.

- Gupta, P.K., Pullman, G., Timmis, R., Kreitinger, M., Carlson, W.C., Grob, J., Welty, E. (1993) Forestry in the 21st century: the biotechnology of somatic embryogenesis. *Bio/Technology*. **11**, 454-459.
- Gupta, P.K., Mehta, U.J., Mascarenhas, A.F. (1983) A tissue culture method for rapid clonal propagation of mature trees of *Eucalyptus torelliana* and *Eucalyptus camaldulensis*. *Plant Cell Reports*. **2**, 296-299.
- Haissig, B.E. (1989) Status of forest tree vegetative regeneration for biotechnology. *American Biotechnology Laboratory*. **7**, 48-51.
- Hammatt, N. (1992) Progress in the biotechnology of trees. *World Journal of Microbiology and Biotechnology*. **8**, 369-377.
- Hammatt, N., Ridout, M.S. (1992) Micropropagation of common ash (*Fraxinus excelsior*). *Plant Cell, Tissue and Organ Culture*. **13**, 67-74.
- Han, K-H., Keathley, D.E., Davis, J.M., Gordon, M.P. (1993) Regeneration of a transgenic woody legume (*Robinia pseudoacacia* L., black locust) and morphological alterations induced by *Agrobacterium rhizogenes*-mediated transformation. *Plant Science*. **88**, 149-157.
- Harms, C.T. (1992) Engineering genetic disease resistance into crops: biotechnological approaches to crop protection. *Crop Protection*. **11**, 291-306.
- Harrigan, W.F., McCance, M.E. (1966) *Laboratory Methods in Microbiology*, pp. 8-10, Academic Press, London.
- Harris, R.E., Mason, E.B.B. (1983) Two machines for *in vitro* propagation of plants in liquid media. *Canadian Journal of Plant Science*. **63**, 311-316.
- Hartney, V.J. (1980) Vegetative propagation of eucalypts. *Australian Forestry Research*. **10**, 191-211.
- Hassan, M.A., Swartz, H.J., Inamine, G., Mullineaux, P. (1993) *Agrobacterium tumefaciens*-mediated transformation of several *Rubus* genotypes and recovery of transformed plants. *Plant Cell, Tissue and Organ Culture*. **33**, 9-17.
- Hathaway, D.E., (ed.) (1989a) Genetic resistance to herbicides. In: *Molecular Mechanisms of Herbicide Selectivity*, pp. 133-145, Oxford Science Publications, Oxford University Press, Oxford New York Tokyo.
- Hathaway, D.E., (ed.) (1989b) Improved resistance through genetic engineering. In: *Molecular Mechanisms of Herbicide Selectivity*, pp. 146-158, Oxford Science Publications, Oxford University Press, Oxford New York Tokyo.
- Hathaway, D.E., (ed.) (1989c) Herbicide metabolism in plants. In: *Molecular Mechanisms of Herbicide Selectivity*, pp. 43-132, Oxford Science Publications, Oxford University Press, Oxford New York Tokyo.
- Haughn, G.W., Smith, J., Mazur, B., Somerville, C. (1988) Transformation with a mutant *Arabidopsis* acetolactate synthase gene renders tobacco resistant to sulfonylurea herbicides. *Molecular and General Genetics*. **211**, 266-271.

- Hensgens, L.A.M., Fornerod, M.W.J., Rueb, S., Winkler, A.A., van der Veen, S., Schilperoort, R.A. (1992) Translation controls the expression level of a chimaeric reporter gene. *Plant Molecular Biology*. **20**, 921-938.
- Herman, B. Mondi Forests, P.O. Box 12, Hilton, 3245, South Africa.
- Herrera-Estrella, L., Simpson, J. (1988) Foreign gene expression in plants. In: *Plant Molecular Biology - a Practical Approach*, pp. 131-159, Shaw, C.H., (ed.), IRL Press, Oxford Washington DC.
- Herrera-Estrella, L., Teeri, T.H., Simpson, J. (1988) Use of reporter genes to study gene expression in plant cells. In: *Plant Molecular Biology Manual B1*, pp. 1-22, Gelvin, S.B., Schilperoort, R.A., Verma, D.P.S., (eds), Kluwer Academic Publishers, Belgium.
- Ho, R.H. (1989) Adventitious root and shoot regeneration in cultured explants of mature black spruce. *Plant Science*. **62**, 129-135.
- Hobbs, S.L.A., Kpodar, P., DeLong, C.M.O. (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. *Plant Molecular Biology*. **15**, 851-864.
- Hobbs, S.L.A., Warkentin, T.D., DeLong, C.M.O. (1993) Transgene copy number can be positively or negatively associated with transgene expression. *Plant Molecular Biology*. **21**, 17-26.
- Hodal, L., Bocharadt, A., Nielsen, J.E., Mattsson, O., Okkels, F.T. (1992) Detection, expression and specific elimination of endogenous β -glucuronidase activity in transgenic and non-transgenic plants. *Plant Science*. **87**, 115-122.
- Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J., Schilperoort, R.A. (1983) A binary plant vector strategy based on separation of vir- and T-region of the *A. tumefaciens* Ti plasmid. *Nature*. **310**, 115-120.
- Hooykaas, P.J.J., Schilperoort, R.A. (1992) *Agrobacterium* and plant genetic engineering. *Plant Molecular Biology*. **19**, 15-38.
- Hope, B.A., Blakeway, F.C., MacRae, S., Herman, B., Watt, M.P. (1994) Plant regeneration and transformation systems for Eucalyptus. Abstracts VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Abstract No. S7-106, p. 153.
- Horsch, R.B., Fry, J., Hoffmann, N., Neidermeyer, J., Rogers, S.G., Fraley, R.T. (1988) Leaf Disc Transformation. In: *Plant Molecular Biology Manual A5*, pp. 1-9, Gelvin, S.B., Schilperoort, R.A., Verma, D.P.S., (eds), Kluwer Academic Publishers, Belgium.
- Howard, B.H., Jones, O.P., Vasek, J. (1989) Long-term improvement in the rooting of plum cuttings following apparent rejuvenation. *Journal of Horticultural Science*. **64**, 147-156.
- Hu, H., Zeng, J.Z. (1984) Development of new varieties via anther culture. In: *Handbook of Plant Cell Culture*, Volume 3, Crop Species, pp. 65-90, Ammirato, P.V., Evans, D.A., Sharp, W.R., Yamada, Y., (eds), Macmillan Publishing Company, New York London.
- Huckett, B. Biotechnology Department, SASEX, Private Bag X02, Mount Edgecombe, 4300, South Africa.

- Hughes, K.W. (1981) *In vitro* ecology: exogenous factors affecting growth and morphogenesis in plant culture systems. *Environmental and Experimental Botany*. **21**, 281-288.
- Hull, R. (1991) Impact of biotechnology on agriculture. *Biologist*. **38**, 143-146.
- Hussain, S., Lane, S.D., Price, D.N. (1994) A preliminary evaluation of the use of microbial culture filtrates for the control of contaminants in plant tissue culture systems. *Plant Cell, Tissue and Organ Culture*. **36**, 45-51.
- Ikemori, Y.K., Penchel, R.M., Bertolucci, F.L.G. (1994) Integrating biotechnology into eucalypt breeding. *International Symposium of Wood Biotechnology*, Fuchu, Tokyo 183, Japan.
- Jacq, B., Lesobre, O., Sangwan, R.S., Sangwan-Norreel, B.S. (1993) Factors influencing T-DNA transfer in *Agrobacterium*-mediated transformation of sugarbeet. *Plant Cell Reports*. **12**, 621-624.
- Jäger, A.K., Schottländer, B., Smitt, U.W., Nyman, U. (1993) Somatic embryogenesis in cell cultures of *Thapsia garganica*. *Plant Cell Reports*. **12**, 517-520.
- Jain, A.K., Datta, R.K. (1992) Shoot organogenesis and plant regeneration in mulberry (*Morus bombycis* Koidz): Factors influencing morphogenetic potential in callus cultures. *Plant Cell, Tissue and Organ Culture*. **29**, 43-50.
- Jain, S.M., Oker-Blom, C., Pehu, E., Newton, R.J. (1992) Genetic engineering: an additional tool for plant improvement. *Agricultural Science of Finland*. **1**, 323-337.
- Jang, J.C., Tainter, F.H. (1991) Micropropagation of shortleaf, Virginia and loblolly x shortleaf pine hybrids via organogenesis. *Plant Cell, Tissue and Organ Culture*. **25**, 61-67.
- Jefferson, R.A., Wilson, K.J. (1991) The GUS gene fusion system. In: *Plant Molecular Biology Manual B14*, pp. 1-33, Gelvin, S.B., Schilperoort, R.A., Verma, D.P.S., (eds), Kluwer Academic Publishers, Belgium.
- Jefferson, R.A., Kavanaugh, T.A., Bevan, M.W. (1987) GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**, 3901-3907.
- Joersbo, M., Brunstedt, J. (1990) Direct gene transfer to plant protoplasts by mild sonication. *Plant Cell Reports*. **9**, 207-210.
- Joersbo, M., Brunstedt, J. (1991) Electroporation: mechanism and transient expression, stable transformation and biological effects in plant protoplasts. *Physiologia Plantarum*. **81**, 256-264.
- Jones, J.D.G., Gilbert, D.E., Grady, K.L., Jorgensen, R.A. (1987) T-DNA structure and gene expression in *Petunia* plants transformed by *Agrobacterium tumefaciens* C58 derivatives. *Molecular and General Genetics*. **207**, 478-485.
- Jones, J.D.G., Shlumukov, L., Carland, F., English, J., Scofield, S.R., Bishop, G.J., Harrison, K. (1992) Effective vectors for transformation, expression of heterologous genes, and assaying transposon excision in transgenic plants. *Transgenic Research*. **1**, 285-297.
- Jones, M.G.K., Lindsey, K. (1988) Plant biotechnology. In: *Molecular Biology and Biotechnology*, Second Edition, pp. 117-147, Walker, J.M., Gingold, E.B., (eds), Royal Society of Chemistry.

- Jones, N.B., Van Staden, J. (1994) Micropropagation and establishment of *Eucalyptus grandis* hybrids. South African Journal of Botany. **60**, 122-126.
- Jones, O.P., Hadlow, W.C.C. (1989) Juvenile-like character of apple trees produced by grafting scions and rootstocks produced by micropropagation. Journal of Horticultural Science. **64**, 395-401.
- Jones, S. (1992) Engineering for horticulture. Outlook on Agriculture. **21**, 183-188.
- Kado, C.I., Liu, S.T. (1981) Rapid procedure for detection and isolation of large and small plasmids. Journal of Bacteriology. **145**, 1365-1373.
- Kaeppler, H.F., Gu, W., Somers, D.A., Rines, H.W., Cockburn, A.F. (1990) Silicon carbide fiber-mediated DNA delivery into plant cells. Plant Cell Reports. **9**, 415-418.
- Kaeppler, S.M., Phillips, R.L. (1993) Tissue culture-induced DNA methylation variation in maize. Proceedings of the National Academy of Sciences USA. **90**, 8773-8776.
- Kalia, P., Crisp, P. (1994) *In vitro* propagation of cauliflower from cotyledon and inflorescence explants. Abstracts VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Abstract No. S1-138, p. 40.
- Kartha, K.K. (1981) Meristem culture and cryopreservation - methods and applications. In: Plant Tissue Culture: Methods and Applications in Agriculture, pp. 181-211, Thorpe, T.A., (ed.), Academic Press Inc., New York London Toronto Sydney San Francisco.
- Kartha, K.K., Fowke, L.C., Leung, N.L., Caswell, K.L., Hakman, I. (1988) Induction of somatic embryos and plantlets from cryopreserved cell cultures of white spruce (*Picea glauca*). Journal of Plant Physiology. **132**, 529-539.
- Kavathekar, A.K., Ganapathy, P.S., Johri, B.M. (1977) Chilling induces development of embryoids into plantlets in *Eschscholzia*. Z. Pflanzenphysiol. **81**, 358-363.
- Kim, Y.W., Lee, B.C., Lee, S.K., Jang, S.S. (1994) Somatic embryogenesis and plant regeneration in *Quercus acutissima*. Plant Cell Reports. **13**, 315-318.
- King, P.J. (1984) Induction and Maintenance of cell suspension cultures. In: Cell Culture and Somatic Cell Genetics of Plants, Volume 1, Laboratory Procedures and their Applications, pp. 130-137, Vasil, I.K., (ed.), Academic Press, Orlando San Diego New York London Toronto Montreal Sydney Tokyo.
- Kirby, E.G., Leustek, T., Lee, M.S. (1987) Nitrogen nutrition. In: Cell and Tissue Culture in Forestry, Volume 1, General Principles and Biotechnology, pp. 67-89, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.
- Kitto, S.L., Young, M.J. (1981) *In vitro* propagation of Carrizo citrange. HortScience. **16**, 305-306.
- Klein, T.M., Wolf, E.D., Wu, R., Sanford, J.C. (1987) High-velocity microprojectiles for delivering nucleic acids into living cells. Nature. **327**, 70-73.
- Klein, T.M., Fromm, M.E., Gradziel, T., Sanford, J.C. (1988) Factors influencing gene delivery into *Zea mays* cells by high velocity microprojectiles. Bio/Technology. **6**, 923-926.

- Klein, T.M., Goff, S.A., Roth, B.A., Fromm, M.E. (1990) Applications of the particle gun in plant biology. In: Progress in Plant Cellular and Molecular Biology, pp. 56-66, Nijkamp, H.J.J., v.d. Plas, L.H.W., Aartrijk, J.V., (eds), Kluwer Academic Publishers, Dordrecht Boston London.
- Klopfenstein, N.B., McNabb, H.S., Hart, E.R., Hall, R.B., Hanna, R.D., Heuchelin, S.A., Allen, K.K., Shi, N-Q., Thornburg, R.W. (1993) Transformation of *Populus* hybrids to study and improve pest resistance. *Silvae Genetica*. **42**, 86-90.
- Kneifel, W., Leonhardt, W. (1992) Testing of different antibiotics against Gram-positive and Gram-negative bacteria isolated from plant tissue culture. *Plant Cell, Tissue and Organ Culture*. **29**, 139-144.
- Kobayashi, S., Uchimiya, H. (1989) Expression and integration of a foreign gene in orange (*Citrus sinensis* Osb.) protoplasts by direct DNA transfer. *Japanese Journal of Genetics*. **64**, 91-97.
- Komamine, A., Matsumoto, M., Tsukahara, M., Fujiwara, A., Kawahara, R., Ito, U., Smith, J., Nomura, K., Fujimura, T. (1990) Mechanisms of somatic embryogenesis in cell cultures: physiology, biochemistry and molecular biology. In: Progress in Plant Cellular and Molecular Biology, pp. 307-313, Nijkamp, H.J.J., van der Plas, L.H.W., van Aartrijk, J., (eds), Kluwer Academic Publishers, Dordrecht Boston London.
- Kooter, J.M., Mol, J.N.M. (1993) *Trans*-inactivation of gene expression in plants. *Current Opinion in Biotechnology*. **4**, 166-171.
- Kozai, T. (1991) Environmental control in plant tissue culture and its application for micropropagation. IFAC Mathematical and Control Applications in Agriculture and Horticulture, Matsuyama, Japan. pp. 99-104.
- Krens, F.H., Molendijk, L., Wullems, G., Schilperoort, R.A. (1982) *In vitro* transformation of plant protoplasts with Ti plasmid DNA. *Nature*. **296**, 72-74.
- Kristiansen, K. (1991) Post-propagation growth of cuttings from *in vitro* and *in vivo* propagated stock plants of *Ficus benjamina*. *Scientia Horticulturae*. **46**, 315-322.
- Krogstrup, P. (1990) Effect of culture densities on cell proliferation and regeneration from embryogenic cell suspensions of *Picea sitchensis*. *Plant Science*. **72**, 115-123.
- Krogstrup, P., Baldursson, S., Norgaard, J.V. (1992) Ex situ genetic conservation by use of tissue culture. *Opera Botanica*. **113**, 49-53.
- Krugman, S.L. (1988) Guidelines for research and development in biotechnology. In: Somatic Cell Genetics of Woody Plants, pp. 221-225, Ahuja, M.R., (ed.), Kluwer Academic Publishers, Dordrecht Boston London.
- Lainé, E., David, A. (1994) Regeneration of plants from leaf explants of micropropagated clonal *Eucalyptus grandis*. *Plant Cell Reports*. **13**, 473-476.
- Lakshmi Sita, G. (1986) Progress towards the *in vitro* clonal propagation of *Eucalyptus grandis*. In: Plant Tissue Culture and its Agricultural Applications, pp. 159-165, Withers, L.A., Alderson, P.G., (eds), Butterworths, London Boston Durban Singapore Sydney Toronto Wellington.

- Lakshmi Sita, G., Raghava Ram, N.V., Vaidyanathan, C.S. (1979) Differentiation of embryoids and plantlets from shoot callus of sandalwood. *Plant Science Letters*. **15**, 265-270.
- Larkin, P.J., Scowcroft, W.R. (1981) Somaclonal variation - a novel source of variability from cell culture for plant improvement. *Theoretical and Applied Genetics*. **60**, 197-214.
- Le Roux, J.J., Van Staden, J. (1991a) Micropropagation of *Eucalyptus* species. *HortScience*. **26**, 199-200.
- Le Roux, J.J., Van Staden, J. (1991b) Micropropagation and tissue culture of *Eucalyptus* - a review. *Tree Physiology*. **9**, 435-477.
- Lee, N., Wang, Y., Yang, J., Ge, K., Huang, S., Tan, J., Testa, D. (1991) Efficient transformation and regeneration of rice small cell groups. *Proceedings of the National Academy of Sciences*. **88**, 6389-6393.
- Lee, Y-I., Lee, S-J., Shin, I-C., Kim, J-S. (1994) Plantlet regeneration of cotyledon and hypocotyl culture of *Melandryum album*. Abstracts VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Abstract No. S1-50, p. 18.
- Lesney, M.S., Johnson, J.D., Korhnak, T., McCaffery, M.W. (1987) In vitro manipulation of slash pine (*Pinus elliottii*). In: Genetic Manipulation of Woody Plants, pp. 43-55, Hanover, J.W., Keathley, D.E., (eds), Plenum Press, New York London.
- Levi, A., Sink, K.C. (1992) Asparagus somatic embryos: production in suspension culture and conversion to plantlets on solidified medium as influenced by carbohydrate regime. *Plant Cell, Tissue and Organ Culture*. **31**, 115-122.
- Li, X-Q., Liu, C-N., Ritchie, S.W., Peng, J-y., Gelvin, S.B., Hodges, T.K. (1992) Factors influencing *Agrobacterium*-mediated transient expression of *gusA* in rice. *Plant Molecular Biology*. **20**, 1037-1048.
- Li, L., Qu, R., de Kochko, A., Fauquet, C., Beachy, R.N. (1993) An improved rice transformation system using the biolistic method. *Plant Cell Reports*. **12**, 250-255.
- Libby, W.J. (1991) Use of genetic variation for forest trees. In: *Plant Breeding in the 1990's, Proceedings of the Symposium on Plant Breeding in the 1990's*, pp. 101-117, Stalker, H.T., Murphy, J.P., (eds), CAB International.
- Lindsey, K., Jones, M.G.K. (1990a) Selection of transformed cells. In: *Plant Cell Line Selection - Procedures and Applications*, pp. 317-339, Dix, P.J., (ed.), VCH, Weinheim New York Basel Cambridge.
- Lindsey, K., Jones, M.G.K. (1990b) Electroporation of cells. *Physiologia Plantarum*. **79**, 168-172.
- Lipp João, K.H., Brown, T.A. (1993) Enhanced transformation of tomato co-cultivated with *Agrobacterium tumefaciens* C58C1Rif^r::pGSFR1161 in the presence of acetosyringone. *Plant Cell Reports*. **12**, 422-425.
- Lloyd, G.B., McCown, B.H. (1980) Commercially feasible micropropagation of mountain laurel (*Kalmia latifolia*) by use of shoot tip culture. *Plant Propagations Society*. **30**, 421-437.

- Luo, G., Hepburn, A., Widholm, J. (1994) A simple procedure for the expression of foreign genes in transgenic soybean callus tissue. *Plant Cell Reports*. **13**, 632-636.
- Lyznik, L.A., Peng, J., Hodges, T.K. (1991) Simplified procedure for transient transformation of plant protoplasts using polyethylene glycol treatment. *BioTechniques*. **10**, 295-300.
- MacRae S. CELBI, Quinta do Furadouro, Amoreira, 2510 Obidos, Portugal.
- MacRae, S., Van Staden, J. (1990) *In vitro* culture of *Eucalyptus grandis*: effect of gelling agents on propagation. *Journal of Plant Physiology*. **137**, 249-251.
- Mandal, A. (1989) Micropropagation of *Populus tremula* L.: condition for induction of shoots and roots. *Scandinavian Journal of Forestry Research*. **4**, 285-293.
- Mansur, E.A., Lacorte, C., de Freitas, V.G., de Oliveira, D.E., Timmerman, B., Cordeiro, A.R. (1993) Regulation of transformation efficiency of peanut (*Arachis hypogaea* L.) explants by *Agrobacterium tumefaciens*. *Plant Science*. **89**, 93-99.
- Mantell, S.H., Matthews, J.A., McKee, R.A., (eds) (1985a) Vectors for gene cloning in plants. In: *Principles of Plant Biotechnology: an Introduction to Genetic Engineering in Plants*, pp. 62-88, Blackwell Scientific Publications, Oxford London Edinburgh Boston Palo Alto Melbourne.
- Mantell, S.H., Matthews, J.A., McKee, R.A., (eds) (1985b) Cultural tools and technique. In: *Principles of Plant Biotechnology: an Introduction to Genetic Engineering in Plants*, pp. 89-129, Blackwell Scientific Publications, Oxford London Edinburgh Boston Palo Alto Melbourne.
- Manzanera, J.A., Astorga, R., Bueno, M.A. (1993) Somatic embryo induction and germination in *Quercus suber* L. *Silvae Genetica*. **42**, 90-93.
- Marks, T.R., Myers, P.E. (1994) Physiological variability arising from *in vitro* culture is induced by shoot selection and manipulation strategies. *Journal of Horticultural Science*. **69**, 1-9.
- Martin, G.C., Miller, A.N., Castle, L.A., Morris, J.W., Morris, R.O., Dandekar, A.M. (1990) Feasibility studies using β -glucuronidase as a gene fusion marker in apple, peach, and radish. *Journal of the American Society for Horticultural Science*. **115**, 686-691.
- Mascarenhas, A.F., Muralidharan, E.M. (1989) Tissue culture of forest trees in India. *Current Science*. **58**, 606-613.
- Mauney, J.R. (1961) The culture *in vitro* of immature cotton embryos. *Botanical Gazette*. **122**, 205-209.
- Mauney, J.R., Chappell, J., Ward, B.J. (1967) Effects of malic acid salts on growth of young cotton embryos *in vitro*. *Botanical Gazette*. **128**, 198-200.
- Maximova, S., Kolova, L. (1994) Anther culture of apple (*Malus pumila* Mill). Abstracts VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Abstract No. S1-183, p. 51.
- Mazur, B.J., Falco, S.C. (1989) The development of herbicide resistant crops. *Annual Review of Plant Physiology and Plant Molecular Biology*. **40**, 441-470.
- McClelland, M.T., Smith, M.A.L. (1990) Vessel type, closure, and explant orientation influence *in vitro* performance of five woody species. *HortScience*. **25**, 797-800.

- McComb, J.A., Bennett, I.J. (1986) Eucalypts (*Eucalyptus* spp.) In: Biotechnology in Agriculture and Forestry, Volume 1, Trees, pp. 340-362, Bajaj, Y.P.S., (ed.), Springer-Verlag, Berlin.
- McCown, B.H., Sellmer, J.C. (1987) General media and vessels suitable for woody plant culture. In: Cell and Tissue Culture in Forestry, Volume 1, General Principles and Biotechnology, pp. 4-17, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.
- McGranahan, G.H., Leslie, C.A., Dandekar, A.M., Uratsu, S.L., Yates, I.E. (1993) Transformation of pecan and regeneration of transgenic plants. *Plant Cell Reports*. **12**, 634-638.
- Meredith, C.P. (1990) Summary: gene transfer. In: Horticultural Biotechnology, pp. 49-50, Bennett, A.B., O'Neill, S.D., (eds), John Wiley and Sons, Inc. Publication, New York Chichester Brisbane Toronto Singapore.
- Meyer, M.M. (1982) *In vitro* propagation of *Rhododendron catawbiense* from flower buds. *HortScience*. **17**, 891-892.
- Miguel, C.M., Raquel, M.H., Pais, M.S., Oliveira, M.M. (1994) *In vitro* establishment of portuguese varieties of *Prunus dulcis* (Miller) D.A. Webb (almond) and preliminary studies of *Agrobacterium*-mediated transformation. Abstracts VIIIth International Congress of Plant Tissue and Cell Culture. Firenze. Abstract No. S7-13, p. 130.
- Miljuš-Djukic, J., Neškovic, M., Ninkovic, S., Crkvenjakov, R. (1992) *Agrobacterium*-mediated transformation and plant regeneration of buckwheat (*Fagopyrum esculentum* Moench.) *Plant Cell, Tissue and Organ Culture*. **29**, 101-108.
- Miller, L.R., Murashige, T. (1976) Tissue culture propagation of tropical foliage plants. *In Vitro*. **12**, 797-813.
- Minocha, S.C. (1987) Plant growth regulators and morphogenesis in cell and tissue culture of forest trees. In: Cell and Tissue Culture in Forestry, Volume 1, General Principles and Biotechnology, pp. 50-67, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.
- Mitra, G.C., Chaturvedi, H.C. (1972) Embryoids and complete plants from unpollinated ovaries and from ovules of *in vitro* grown emasculated flower buds of *Citrus* species. *Bulletin of the Torrey Botanical Club*. **99**, 184-189.
- Mohamed-Yasseen, Y., Splittstoesser, W.E., Litz, R.E. (1993) *In vitro* bulb formation and plant recovery from onion inflorescences. *HortScience*. **28**, 1052.
- Mozo, T., Hooykaas, P.J.J. (1992a) Factors affecting the rate of T-DNA transfer from *Agrobacterium tumefaciens* to *Nicotiana glauca* plant cells. *Plant Molecular Biology*. **19**, 1019-1030.
- Mozo, T., Hooykaas, P.J.J. (1992b) Design of a novel system for the construction of vectors for *Agrobacterium*-mediated plant transformation. *Molecular and General Genetics*. **236**, 1-7.
- Mukhopadhyay, T., Desjardins, Y. (1994) Direct gene transfer to protoplasts of two genotypes of *Asparagus officinalis* L. by electroporation. *Plant Cell Reports*. **13**, 421-424.

- Mullin, T.J., Park, Y.S. (1992) Estimating genetic gains from alternative breeding strategies for clonal forestry. *Canadian Journal of Forestry Research*. **22**, 14-23.
- Mullineaux, P.M. (1992) Genetically engineered plants for herbicide resistance. In: *Plant Genetic Manipulation for Crop Protection, Biotechnology in Agriculture Series No. 7*, pp. 73-107, Gatehouse, A.M.R., Hilder, V.A., Boulter, D., (eds), C.A.B. International, Wallingford, U.K.
- Muralidharan, E.M., Gupta, P.K., Mascarenhas, A.P. (1989) Plantlet production through high frequency somatic embryogenesis in long term cultures of *Eucalyptus citriodora*. *Plant Cell Reports*. **8**, 41-43.
- Muralidharan, E.M., Mascarenhas, A.F. (1987) *In vitro* plantlet formation by organogenesis in *E. camaldulensis* and by somatic embryogenesis in *Eucalyptus citriodora*. *Plant Cell Reports*. **6**, 256-259.
- Murashige, T. (1974) Plant propagation through tissue cultures. *Annual Review of Plant Physiology*. **25**, 135-166.
- Murashige, T., Skoog, E. (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**, 473-497.
- Nap, J-P., Bijvoet, J., Stiekema, W.J. (1992) Biosafety of kanamycin-resistant transgenic plants. *Transgenic Research*. **1**, 239-249.
- Nashar, L. (1989) Micropropagation. *Australian Journal of Biotechnology*. **3**, 288-290.
- Nel, P.M. (1985) Recent developments in biotechnology: possible applications to forest tree breeding in southern Africa. *South African Forestry Journal*. **135**, 40-42.
- Nester, E.W. (1987) Analysis of host range in transformation of higher plants by *Agrobacterium tumefaciens*. In: *Genetic Manipulation of Woody Plants*, pp. 219-229, Hanover, J.W., Keathley, D.E., (eds), Plenum Press, New York London.
- Neuhaus, G., Spangenberg, G. (1990) Plant transformation by microinjection techniques. *Physiologia Plantarum*. **79**, 213-217.
- Norton, M.E., Norton, C.R. (1985) *In vitro* propagation of Ericaceae: a comparison of the activity of the cytokinins N⁶-benzyladenine and N⁶- isopentenyladenine in shoot proliferation. *Scientia Horticulturae*. **27**, 335-340.
- Oliver, S.G., Ward, J.M. (1985) *A Dictionary of Genetic Engineering*. Cambridge University Press, England, 153 pages.
- Oliveira, M.M., Barroso, J.G., Raquel, M.H., Pais, M.S. (1994) Direct DNA transfer to *Actinidia deliciosa* cv. Hayward (Kiwifruit) protoplasts: studies on GUS transient expression and on stable transformation. Abstracts VIIIth International Congress on Plant Tissue and Cell Culture. Firenze. Abstract No. S7-11, p. 129.
- Olsen, W.L. (1988) Molecular biology in forestry research: a review. In: *Forest and Crop Biotechnology: Progress and Prospects*, pp. 315-334, Valentine, F.A. (ed.), Springer-Verlag, New York Berlin Heidelberg London Paris Tokyo.

- Orihara, Y., Saiki, K., Furuya, T. (1991) Biotransformation of steviol by cultured cells of *Eucalyptus perriniana* and *Coffea arabica*. *Phytochemistry*. **30**, 3989-3992.
- Ormerod, A.J., Caligari, P.D.S. (1994) Anther and microspore culture of *Lupinus albus* in liquid culture medium. *Plant Cell, Tissue and Organ Culture*. **36**, 227-236.
- Ottaviani, M-P., Smits, T., Hänisch ten Cate, H. (1993) Differential methylation and expression of the β -glucuronidase and neomycin phosphotransferase genes in transgenic plants of potato cv. Bintje. *Plant Science*. **88**, 73-81.
- Owens, L., Galun, E. (1983) Morphogenesis of transformed cells from octopine-type crown gall. In: Genetic Engineering: Applications to Agriculture, pp. 229-235, Owens, L.D., (ed.), Rowman & Allanheld Publishers, Granada London Toronto Sydney.
- Palmgren, G., Mattson, O., Okkels, F.T. (1993) Treatment of *Agrobacterium* or leaf discs with 5-azacytidine increases transgene expression in tobacco. *Plant Molecular Biology*. **21**, 429-435.
- Papachatzi, M., Hammer, P.A., Hasegawa, P.M. (1981) *In vitro* propagation of *Hosta decorata* 'Thomas Hogg' using cultured shoot tips. *Journal of the American Society for Horticultural Science*. **106**, 232-236.
- Park, Y.G., Son, S.H. (1988) Regeneration of plantlets from cell suspension culture derived callus of white poplar (*Populus alba* L.). *Plant Cell Reports*. **7**, 567-570.
- Park, Y.S., Pond, S.E., Bonga, J.M. (1993) Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for tree breeding. *Theoretical and Applied Genetics*. **86**, 427-436.
- Paszkowski, J., Shillito, R.D., Saul, M., Mandák, V., Hohn, T., Hohn, B., Potrykus, I. (1984) Direct gene transfer to plants. *EMBO Journal*. **3**, 2717-2722.
- Paterson, K.E., Everett, N.P. (1985) Regeneration of *Helianthus annuus* inbred plants from callus. *Plant Science*. **42**, 125-132.
- Pavingerová, D., Dostál, J., Bísková, R., Benetka, V. (1994) Somatic embryogenesis and *Agrobacterium*-mediated transformation of chrysanthemum. *Plant Science*. **97**, 95-101.
- Pawlicki, N., Sangwan, R.S., Sangwan-Norreel, B.S. (1992) Factors influencing the *Agrobacterium tumefaciens*-mediated transformation of carrot (*Daucus carota* L.). *Plant Cell, Tissue and Organ Culture*. **31**, 129-139.
- Peeters, M-C., Willems, K., Swennen, R. (1994) Protoplast-to-plant regeneration in cotton (*Gossypium hirsutum* L. cv. Coker 312) using feeder layers. *Plant Cell Reports*. **13**, 208-211.
- Perl, A., Galili, S., Shaul, O., Ben-Tzvi, I., Galili, G. (1993) Bacterial dihydrodipicolinate synthase and desensitized aspartate kinase: two novel selectable markers for plant transformation. *Bio/Technology*. **11**, 715-718.
- Pimentel, D., Hunter, M.S., LaGro, J.A., Efroymson, R.A., Landers, J.C., Mervis, F.T., McCarthy, C.A., Boyd, A.E. (1989) Benefits and risks of genetic engineering in agriculture. *BioScience*. **39**, 606-614.

- Potrykus, I., Saul, M., Petruska, J., Paszowski, J., Shillito, R.D. (1985) Direct gene transfer to cells of a graminaceous monocot. *Molecular and General Genetics*. **199**, 183-188.
- Potrykus, I. (1991) Gene transfer to plants: assessment of published approaches and results. *Annual Review of Plant Physiology and Plant Molecular Biology*. **42**, 205-225.
- Prakash, C.S., Varadarajan, U. (1992) Genetic transformation of sweet potato by particle bombardment. *Plant Cell Reports*. **11**, 53-57.
- Prakash, J. (1989) Large-scale production of plants through micropropagation: problems, prospects and opportunities for India. In: *Applications of Biotechnology in Forestry and Horticulture*, pp. 265-274, Dhawan, V., (ed.), Plenum Press, New York London.
- Pugliesi, C., Megale, P., Cecconi, F., Baroncelli, S. (1993) Organogenesis and embryogenesis in *Helianthus tuberosus* and in the interspecific hybrid *Helianthus annuus x Helianthus tuberosus*. *Plant Cell, Tissue and Organ Culture*. **33**, 187-193.
- Qiao, Y-M., Cattaneo, M., Locatelli, F., Lupotto, E. (1992) Plant regeneration from long term suspension culture-derived protoplasts of hexaploid wheat (*Triticum aestivum* L.). *Plant Cell Reports*. **11**, 262-265.
- Raffa, K.F. (1989) Genetic engineering of trees to enhance resistance to insects. *BioScience*. **39**, 524-534.
- Raghava Swamy, B.V., Himabindu, K., Lakshmi Sita, G. (1992) *In vitro* micropropagation of elite rosewood (*Dalbergia latifolia* Roxb.). *Plant Cell Reports*. **11**, 126-131.
- Raghavan, V., Torrey, J.G. (1963) Growth and morphogenesis of globular and older embryos of *Capsella* in culture. *American Journal of Botany*. **50**, 540-551.
- Raghavan, V., Torrey, J.G. (1964) Effect of certain growth substances on the growth and morphogenesis of immature embryos of *Capsella* in culture. *Plant Physiology*. **39**, 691-699.
- Rangan, T.S. (1976) Growth and plantlet regeneration in tissue cultures of some Indian millets: *Paspalum scrobiculatum* L., *Eleusine coracana* Gaert. and *Pennisetum typhoideum* Pers. *Z. Pflanzenphysiol.* **78**, 208-216.
- Rao, A.N., Lee, S.K. (1986) An overview of the *in vitro* propagation of woody plants and plantation crops. In: *Plant Tissue Culture and its Agricultural Applications*, pp. 123-138, Withers, L.A., Alderson, P.G., (eds), Butterworths, London Boston Durban Singapore Sydney Toronto Wellington.
- Rasmussen, J.L., Kikkert, J.R., Roy, M.K., Sanford, J.C. (1994) Biolistic transformation of tobacco and maize suspension cells using bacterial cells as microprojectiles. *Plant Cell Reports*. **13**, 212-217.
- Rathore, T.S., Singh, R.P., Shekhawat, N.S. (1991) clonal propagation of desert teak (*Tecomella undulata*) through tissue culture. *Plant Science*. **79**, 217-222.
- Razin, A. (1988) Assays for studying DNA methylation. In: *Plant Molecular Biology Manual*. **B3**, pp. 1-28, Gelvin, S.B., Schilperoort, R.A., Verma, D.P.S., (eds), Kluwer Academic Publishers, Belgium.

- Ream, W. (1989) *Agrobacterium tumefaciens* and interkingdom genetic exchange. *Annual Review of Phytopathology*. **27**, 583-618.
- Redenbaugh, K., Viss, P., Slade, D., Fujii, J.A. (1987) Scale-up: Artificial seeds. In: *Plant Tissue and Cell Culture*, pp. 473-495, Green, C.E., Somers, D.A., Hackett, W.P., Biesboer, D.D. (eds), Alan R. Liss, Inc., New York.
- Reynaerts, A., De Block, M., Hernalsteens, J-P., Van Montagu, M. (1988) Selectable and screenable markers. In: *Plant Molecular Biology Manual A9*, pp. 1-10, Gelvin, S.B., Schilperoort, R.A., Verma, D.P.S., (eds), Kluwer Academic Publishers, Belgium.
- Reynolds, J.F. (1987) Chemical regulation in tissue culture: an overview. *HortScience*. **22**, 1192-1207.
- Riemenschneider, D.E., Haissig, B.E., Bingham, E.T. (1987) Integrating biotechnology into woody plant breeding programs. In: *Genetic Manipulation of Woody Plants*, pp. 433-449, Hanover, J.W., Keathley, D.E., (eds), Plenum Press, New York London.
- Ritchie, S.W., Lui, C-N., Sellmer, J.C., Kononowicz, H., Hodges, T.K., Gelvin, S.B. (1993) *Agrobacterium tumefaciens*-mediated expression of *gusA* in maize tissues. *Transgenic Research*. **2**, 252-265.
- Robertson, D., Weissinger, A.K., Ackley, R., Glover, S., Sederoff, R.R. (1992) Genetic transformation of Norway spruce (*Picea abies* (L.) Karst) using somatic embryo explants by microprojectile bombardment. *Plant Molecular Biology*. **19**, 925-935.
- Rockwood, D.L., Warrag, E.I. (1994) Field performance of micropropagated, macropropagated, and seed-derived propagules of three *Eucalyptus grandis* ortets. *Plant Cell Reports*. **13**, 628-631.
- Rogers, S.O., Bendich, A.J. (1985) Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology*. **5**, 69-76.
- SAS Institute Incorporated. (1982) SAS user's guide: Statistics. SAS Institute Incorporated, Cary, North Carolina.
- Sagi, L., Remy, S., Panis, B., Swennen, R., Volckaert, G. (1994) Transient gene expression in electroporated banana (*Musa* spp., cv. "Bluggoe", ABB group) protoplasts isolated from regenerable embryogenetic cell suspensions. *Plant Cell Reports*. **13**, 262-266.
- Samartin, A., Vieitez, A.M., Vieitez, E. (1984) *In vitro* propagation of *Camellia japonica* seedlings. *HortScience*. **19**, 225-226.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbour Laboratory Press, U.S.A.
- Sanford, J.C. (1990) Biolistic plant transformation. *Physiologia Plantarum*. **79**, 206-209.
- Sangwan, R.S., Bourgeois, Y., Brown, S., Vasseur, G., Sangwan-Norreel, B. (1992) Characterisation of competent cells and early events of *agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. *Planta*. **188**, 439-456.
- Sankara Rao, K., Venkateswara, R. (1985) Tissue culture of forest trees: clonal multiplication of *Eucalyptus grandis* L. *Plant Science*. **40**, 51-55.

- Saravitz, C.H., Blazich, F.A. (1991) *In vitro* propagation of Fraser fir from embryonic explants. *Canadian Journal of Forestry Research*. **21**, 404-409.
- Sautter, C., Waldner, H., Neuhaus-Url, G., Galli, A., Neuhaus, G., Potrykus, I. (1991) Micro-targeting: high efficiency gene transfer using a novel approach for the acceleration of micro-projectiles. *Bio/Technology*. **9**, 1080-1085.
- Sawahel, W.A. (1994) Transgenic plants: performance, release and containment. *World Journal of Microbiology and Biotechnology*. **10**, 139-144.
- Sawahel, W.A., Cove, D.J. (1992) Gene transfer strategies in plants. *Biotechnology Advances*. **10**, 393-412.
- Scarpa, G.M., Pupilli, F., Damiani, F., Arcioni, S. (1993) Plant regeneration from callus and protoplasts in *Medicago polymorpha*. *Plant Cell, Tissue and Organ Culture*. **35**, 49-57.
- Schröder, R., Gärtner, F., Steinbrenner, B., Knoop, B., Beiderbeck, R. (1989) Viability factors in plant suspension cultures - some properties. *Journal of Plant Physiology*. **135**, 422-427.
- Schuch, W. (1991) Advances in plant biotechnology and their implication for forestry research. In *In Vitro Cellular and Developmental Biology*. **27**, 99-103.
- Schulz, A., Wengenmayer, F., Goodman, H.M. (1990) Genetic engineering of herbicide resistance in higher plants. *Critical Reviews in Plant Science*. **9**, 1-15.
- Scott, R. (1988) DNA restriction and analysis by Southern hybridisation. In: *Plant Genetic Transformation and Gene Expression: a Laboratory Manual*, pp. 237-262, Draper, J., Scott, R., Armitage, P., Walden, R., (eds), Blackwell Scientific Publications, Oxford.
- Scott, R., Draper, J., Jefferson, R., Dury, G., Jacob, L. (1988) Analysis of gene organization and expression in plants. In: *Plant Genetic Transformation and Gene Expression: a Laboratory Manual*, pp. 263-339, Draper, J., Scott, R., Armitage, P., Walden, R., (eds), Blackwell Scientific Publications, Oxford.
- Scowcroft, W.R., Larkin, P.J. (1988) Somaclonal variation. In: *Applications of Plant Cell and Tissue Culture*, Ciba Foundation Symposium 137, pp. 21-35, Bock, G., Marsh, J., (eds), John Wiley and Sons, Chichester New York Brisbane Toronto Singapore.
- Seki, M., Komeda, Y., Iida, A., Yamada, Y., Morikawa, H. (1991) Transient expression of β -glucuronidase in *Arabidopsis thaliana* leaves and roots and *Brassica napus* stems using a pneumatic particle gun. *Plant Molecular Biology*. **17**, 259-263.
- Shahin, E.A., Shepard, J.F. (1980) Cassava mesophyll protoplasts: isolation, proliferation and shoot formation. *Plant Science Letters*. **17**, 459-465.
- Sharma, V.K., Jethwani, V., Kothari, S.L. (1993) Embryogenesis in suspension cultures of *Datura innoxia* Mill. *Plant Cell Reports*. **12**, 581-584.
- Shirsat, A.H., Wilford, N., Croy, R.R.D. (1989) Gene copy number and levels of expression in transgenic plants of a seed specific gene. *Plant Science*. **61**, 75-80.

- Sidikou-Seyni, R., Rambaud, C., Dubois, J., Vasseur, J. (1992) Somatic embryogenesis and plant regeneration from protoplasts of *Cichorium intybus* L. x *Cichorium endivia* L. *Plant Cell, Tissue and Organ Culture*. **29**, 83-91.
- Simons, A.J. (1992) Genetic improvement of non-industrial trees. *Agroforestry Systems*. **18**, 197-212.
- Smigocki, A., Neal, J.W., McCanna, I., Douglass, L. (1993) Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the *ipt* gene. *Plant Molecular Biology*. **23**, 325-335.
- Smith, W.A., Chaleff, R.S. (1990) Herbicide resistance. In: *Plant Cell Line Selection - Procedures and Applications*, pp. 151-166, Dix, P.J., (ed.), VCH, Weinheim New York Basel Cambridge.
- Smith, M.K., Drew, R.A. (1990) Current Applications of Tissue Culture in Plant Propagation and Improvement. *Australian Journal of Plant Physiology*. **17**, 267-289.
- Smith, R.H., Murashige, T. (1970) *In vitro* development of the isolated shoot apical meristem of angiosperms. *American Journal of Botany*. **57**, 562-568.
- Snyman, S.J., Black, K.G., Hockett, B.I., Watt, M.P. (1992) Towards a gene transfer system for sugarcane (*Saccharum* spp. hybrids). *Proceedings of the South African Sugar Technologists' Association - June*. pp. 29-37.
- Sommer, H.E., Caldas, L.S. (1981) *In vitro* methods applied to forest trees. In: *Plant Tissue Culture: Methods and Applications in Agriculture*, pp. 349-358, Thorpe, T.A., (ed.), Academic Press, New York London Toronto Sydney San Francisco.
- Sommer, H.E., Wetzstein, H.Y. (1984) Hardwoods. In: *Handbook of Plant Cell Culture, Volume 3, Crop Species*, pp. 511-540, Ammirato, P.V., Evans, D.A., Sharp, W.R., Yamada, Y., (eds), Macmillan Publishing Company, New York London.
- Sood, N. (1994) Some unusual observations in cultures of *Brassica* spp. *in vitro*. Abstracts VIIIth International Congress of Plant Tissue and Cell Culture, Firenze, Abstract No. S1-11, p. 8.
- Southern, E.M. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*. **98**, 503-517.
- Spangenberg, G., Koop, H-U., Lichter, R., Schweiger, H.G. (1986) Microculture of single protoplasts of *Brassica napus*. *Physiologia Plantarum*. **66**, 1-8.
- Sriskandarajah, S., Goodwin, P.B., Speirs, J. (1994) Genetic transformation of the apple scion cultivar 'Delicious' via *Agrobacterium tumefaciens*. *Plant Cell, Tissue and Organ Culture*. **36**, 317-329.
- Srivastava, P.S., Steinhauer, A. (1982) *In vitro* culture of embryo segments of *Quercus lebanii*: organogenesis and callus growth as a differential response to experimental conditions. *Z. Pflanzenphysiol*. **106**, 93-96.
- Stachel, S.E., Messens, E., Van Montagu, M., Zambryski, P. (1985) Identification of the signal molecules produced by wounded plant cells which activate the T-DNA transfer process in *Agrobacterium tumefaciens*. *Nature*. **318**, 624-629.
- Stachel, S.E., Nester, E.W. (1986) The genetic and transcriptional organisation of the *vir* region of the A6 Ti plasmid of *Agrobacterium tumefaciens*. *EMBO Journal*. **7**, 1445-1454.

- Street, H.E. (1973) Cell (suspension) cultures-techniques. In: Tissue Culture and Plant Science: Proceedings of the Third International Congress of Plant Tissue and Cell Culture, pp. 59-99, Street, H.E., (ed.), Academic Press, London.
- Stuart, R., Street, H.E. (1969) Studies on the growth in culture of plant cells. IV. The initiation of division in suspensions of stationary phase cells of *Acer pseudoplatanus* L. *Journal of Experimental Botany*. **20**, 96-106.
- Sundberg, E., Glimelius, K. (1986) method for production of interspecific hybrids within *Brassica* via somatic hybridisation, using resynthesis of *Brassica napus* as a model. *Plant Science*. **43**, 155-162.
- Taticek, R.A., Moo-Young, M., Legge, R.L. (1991) The scale-up of plant cell culture: engineering considerations. *Plant Cell, Tissue and Organ Culture*. **24**, 139-158.
- Teixeira, J.B., Sondahl, M.R., Kirby, E.G. (1994) Somatic embryogenesis from immature inflorescences of oil palm. *Plant Cell Reports*. **13**, 247-250.
- Teng, W-L., Liu, Y-J., Soong, T-S. (1992) Rapid regeneration of lettuce from suspension culture. *HortScience*. **27**, 1030-1032.
- Teulières, C., Boudet, A.M. (1991) Isolation of protoplasts from different *Eucalyptus* species and preliminary studies on regeneration. *Plant Cell, Tissue and Organ Culture*. **25**, 133-140.
- Thengane, S.R., Joshi, M.S., Khuspe, S.S., Mascarenhas, A.F. (1994) Anther culture in *Helianthus annuus* L., influence of genotype and culture conditions on embryo induction and plant regeneration. *Plant Cell Reports*. **13**, 222-226.
- Thompson, M.R., Thorpe, T.A. (1987) Metabolic and non-metabolic roles of carbohydrates. In: Cell and Tissue Culture in Forestry, Volume 1, General Principles and Biotechnology, pp. 89-113, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.
- Thorpe, T.A. (1980) Organogenesis *in vitro*: structural, physiological, and biochemical aspects. *International Review of Cytology Supplement* **11A**, 71-111.
- Thorpe, T.A. (1983) Morphogenesis and regeneration in tissue culture. In: Genetic Engineering: Applications to Agriculture, pp. 285-303, Owens, L.D., (ed.), Rowman and Allanheld Publishers, Granada London Toronto Sydney.
- Thorpe, T.A., Murashige, T. (1968) Starch accumulation in shoot-forming tobacco callus cultures. *Science*. **160**, 421-422.
- Thorpe, T.A., Murashige, T. (1970) Some histochemical changes underlying shoot initiation in tobacco callus cultures. *Canadian Journal of Botany*. **48**, 277-285.
- Tibok, A. Plant Genetic Manipulation Group, Department of Life Science, University of Nottingham, Nottingham NG7 2RD, United Kingdom.
- Tisserat, B., Foster, G., De Mason, D. (1979) plantlet production *in vitro* from *Phoenix dactylifera* L. *Date Growers Institute Report*. **54**, 19-23.

- Tulecke, W. (1987) Somatic embryogenesis in woody perennials. In: Cell and Tissue Culture in Forestry, Volume 2, Specific Principles and Methods Growth and Developments, pp. 61-91, Bonga, J.M., Durzan, D.J., (eds), Martinus Nijhoff Publishers, Dordrecht Boston Lancaster.
- Turnbull, J.W., Boland, D.J. (1984) *Eucalyptus*. *Biologist*. **31**, 49-56.
- Turnbull, J.W. (1991) Future use of *Eucalyptus*: opportunities and problems. International Union of Forestry Research Organisations symposium on Intensive Forestry: The Role Of *Eucalypts*. Durban, South Africa, pp. 2-27.
- Uchimiyama, H., Handa, T., Brar, D.S. (1989) Transgenic plants. *Journal of Biotechnology*. **12**, 1-20.
- Vain, P., McMullen, M.D., Finer, J.J. (1993) Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. *Plant Cell Reports*. **12**, 84-88.
- Vallés, M.P., Lasa, J.M. (1994) *Agrobacterium*-mediated transformation of commercial melon (*Cucumis melo* L., cv. Amarillo Oro). *Plant Cell Reports*. **13**, 145-148.
- van den Bulk, R.W. (1991) Application of cell and tissue culture and *in vitro* selection for disease resistance breeding - a review. *Euphytica*. **56**, 269-285.
- van den Elzen, P.J.M., Huisman, M.J., Willink, D.P-L., Jongedijk, E., Hoekema, A., Cornelissen, B.J.C. (1989) Engineering virus resistance in agricultural crops. *Plant Molecular Biology*. **13**, 337-346.
- Van Telgen, H-J., Van Mil, A., Kunneman, B. (1992) Effect of propagation and rooting conditions on acclimatization of micropropagated plants. *Acta Botanica Neerl*. **41**, 453-459.
- Van Wert, S.L., Saunders, J.A. (1992) Electrofusion and electroporation of plants. *Plant Physiology*. **99**, 365-367.
- Van Wyk, G. (1985) The potential for improving forestry yields and timber quality through vegetative propagation. *Wood Southern Africa*. **10**, 55-61
- Van Wyk, G. (1990) Genetic improvement of timber yield and wood quality in *Eucalyptus grandis* (Hill) Maiden. *South African Forestry Journal*. **153**, 1-11.
- Vasil, I.K. (1990) The realities and challenges of plant biotechnology. *Bio/Technology*. **8**, 296-301.
- Vasil, I.K. (1991) Plant tissue culture and molecular biology as tools in understanding plant development and in plant improvement. *Current Opinion in Biotechnology*. **2**, 158-163.
- Vasil, V., Castillo, A.M., Fromm, M.E., Vasil, I.K. (1992) Herbicide resistant fertile transgenic wheat plants obtained by microprojectile bombardment of regenerable embryogenic callus. *Bio/Technology*. **10**, 667-674.
- Vasil, V., Vasil, I.K. (1984) Preparation of cultured tissues for scanning electron microscopy. In: Cell Culture and Somatic Cell Genetics of Plants, Volume 1: Laboratory Procedures and their Applications, pp. 738-743, Vasil, I.K., (ed.), Academic Press, Inc., London.
- Vasil, I.K., Vasil, V., Redway, F. (1990) Plant regeneration from embryogenic calli, cell suspension cultures and protoplasts of *Triticum aestivum* L. (wheat). In: Progress in Plant Cellular and Molecular Biology, pp. 33-37, Nijkamp, H.J.J., v.d. Plas, L.H.W., Aartrijk, J.V., (eds), Kluwer Academic Publishers, Dordrecht Boston London.

- Verdeil, J.-L., Huet, C., Grosdemange, F., Buffard-Morel, J. (1994) Plant regeneration from cultured immature inflorescences of coconut (*Cocos nucifera* L.): evidence for somatic embryogenesis. *Plant Cell Reports*. **13**, 218-221.
- Verma, D.C., Dougall, D.K. (1977) Influence of carbohydrates on qualitative aspects of growth and embryo formation in wild carrot suspension culture. *Plant Physiology*. **59**, 81-85.
- Verpoorte, R., Van der Heijden, R., Schripsema, J. (1993) Plant cell biotechnology for the production of alkaloids: present status and prospects. *Journal of Natural Products*. **56**, 186-207.
- Vitha, S., Beneš, K., Michalová, Ondrej, M. (1993) Quantitative β -glucuronidase assay in transgenic plants. *Biologia Plantarum*. **35**, 151-155.
- Von Arnold, S., Clapham, D., Ekberg, I. (1990) Has biotechnology a future in forest tree breeding? *Forest Tree Improvement*. **23**, 33-47.
- Von Arnold, S., Hakman, I. (1987) Plantlet regeneration in vitro via adventitious buds and somatic embryos in Norway spruce (*Picea abies*). In: *Genetic Manipulation of Woody Plants*, pp. 199-215, Hanover, J.W., Keathley, D.E., (eds), Plenum Press, New York London.
- Von Arnold, S., Eriksson, T. (1984) Effect of agar concentration on growth and anatomy of adventitious shoots of *Picea abies* (L) Karst. *Plant Cell, Tissue and Organ Culture*. **3**, 257-264.
- Walden, R. (ed.) (1988) Marker genes used to isolate plant DNA sequences. In: *Genetic Transformation in Plants*, pp. 117-118, Open University Press, Milton Keynes.
- Wang, A.S., Phillips, R.L. (1984) Synchronisation of suspension culture cells. In: *Cell Culture and Somatic Cell Genetics of Plants, Volume 1, Laboratory Procedures and their Applications*, pp. 175-181, Vasil, I.K., (ed.), Academic Press, Orlando New York London.
- Wang, Y.-C., Klein, T.M., Fromm, M., Cao, J., Sanford, J.C., Wu, R. (1988) Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. *Plant Molecular Biology*. **11**, 433-439.
- Wang, X.-H., Lörz, H. (1994) Plant regeneration from protoplasts of wild barley (*Hordeum murinum* L.). *Plant Cell Reports*. **13**, 139-144.
- Ward, E.R., Ryals, J.A., Mifflin, B.J. (1993) Chemical regulation of transgene expression in plants. *Plant Molecular Biology*. **22**, 361-366.
- Wardle, K., Dobbs, E.B., Short, K.C. (1983) *In vitro* acclimatisation of aseptically cultured plants to humidity. *Journal of the American Society for Horticultural Science*. **108**, 386-389.
- Warkentin, T.D., McHughen, A. (1992) *Agrobacterium tumefaciens*-mediated beta-glucuronidase (GUS) gene expression in lentil (*Lens culinaris* Medik.) tissues. *Plant Cell Reports*. **11**, 274-278.
- Warrag, E.I., Lesney, M.S., Rockwood, D.L. (1990) Micropropagation of field tested superior *Eucalyptus grandis* hybrids. *New Forests*. **4**, 67-79.
- Warrag, E., Lesney, M.S., Rockwood, D.J. (1991) Nodule culture and regeneration of *Eucalyptus grandis* hybrids. *Plant Cell Reports*. **9**, 586-589.

- Warren, G. (1991) The regeneration of plants from cultured cells and tissues. In: Plant Cell and Tissue Culture, pp. 82-100, Stafford, A., Warren, G., (eds), Open University Press, Milton Keynes.
- Watt, M.P. Biology Department, University of Natal, King George V Avenue, Durban, 4001, South Africa.
- Watt, M.P., Blakeway, F., Cresswell, C.F., Herman, B. (1991) Somatic embryogenesis in *Eucalyptus grandis*. South African Forestry Journal. **157**, 59-65.
- Weatherhead, M.A., Burdon, J.M., Henshaw, G.G. (1978) Some effects of activated charcoal as an additive to plant tissue culture media. Z. Pflanzenphysiol. **89**, 141-147.
- Wetzstein, H.Y., Kim, C., Sommer, H.E. (1994) Vessel volume, gelling agent, and basal salts affect pH and gel strength of autoclaved tissue culture media. HortScience. **29**, 683-685.
- Whetten, R., Sederoff, R. (1991) Genetic engineering of wood. Forest Ecology and Management. **43**, 301-316.
- White, D.W.R. (1984) Plant regeneration from long-term suspension cultures of white clover. Planta. **162**, 1-7.
- White, F.F., Taylor, B.H., Huffman, G.A., Gordon, M.P., Nester, E.W. (1985) Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes*. Journal of Bacteriology. **164**, 33-44.
- White, P.R. (ed.) (1943) A Handbook of Tissue Culture, pp. 44-47, Ronald Press Company, New York.
- Widholm, J.M. (1988) *In vitro* selection with plant cell and tissue cultures: an overview. Iowa State Journal of Research. **62**, 587-597.
- Wiecheteck, M., Cortezzi Graça, M.E., de Araújo, A.J. (1989) Micropropagation of *Eucalyptus viminalis* Labill. Ann. Sci. For. **46**, 161-164.
- Wilde, H.D., Meagher, R.B., Merkle, S.A. (1992) Expression of foreign genes in transgenic yellow-poplar plants. Plant Physiology. **98**, 114-120.
- Wilkins, C.P., Bengochea, T., Dodds, J.H. (1982) The use of *in vitro* methods for plant genetic conservation. Outlook on Agriculture. **11**, 67-72.
- Wilson, G. (1990) Screening and selection of cultured plant cells for increased yields of secondary metabolites. In: Plant Cell Line Selection: Procedures and Applications, pp. 187-216, Dix, P.J., (ed.), VCH, Cambridge.
- Wilson, S.M., Thorpe, T.A., Moloney, M.M. (1989) PEG-mediated expression of GUS and CAT genes in protoplasts from embryogenic suspension cultures of *Picea glauca*. Plant Cell Reports. **7**, 704-707.
- Wilson, H.M., Frame, B.R., Drayton, P.R., Bagnall, S.V., Lewnau, C.J., Bullock, W.P., Dunwell, J.M., Thompson, J.A., Wang, K. (1994) Production of fertile transgenic maize plants by silicon carbide fiber-mediated transformation. Abstracts VIIIth International Congress on Plant Tissue and Cell Culture. Firenze. Abstract No. S7-60, p. 141.
- Xu, X., Li, B. (1994) Fertile transgenic Indica rice plants obtained by electroporation of the seed embryo cells. Plant Cell Reports. **13**, 237-242.

- Yang, J., Ge, K., Wang, Y., Wang, B., Tan, C.C. (1993) Highly efficient transfer and stable integration of foreign DNA into partially digested rice cells using a pulsed electrophoretic device. *Transgenic Research*. **2**, 245-251.
- Yeoman, M.M. (1986) The present development and future of plant cell and tissue culture in agriculture, forestry and horticulture. In: *Plant Tissue Culture and its Agricultural Applications*, pp. 489-499, Withers, L.A., Alderson, P.G., (eds), Butterworths, London Boston Durban Singapore Sydney Toronto Wellington.
- Ying, M., Dyer, W.E., Bergman, J.W. (1992) *Agrobacterium tumefaciens*-mediated transformation of safflower (*Carthamus tinctorius* L.) cv. 'Centennial'. *Plant Cell Reports*. **11**, 581-585.
- Zaghmout, O.M-F., Trolinder, N.L. (1993) Factors affecting transient gene expression in protoplasts isolated from very slowly growing embryogenic callus cultures of wheat (*Triticum aestivum* L.). *Theoretical and Applied Genetics*. **86**, 721-730.
- Zambryski, P.C. (1992) Chronicles from the *Agrobacterium*-plant cell DNA transfer story. *Annual Review of Plant Physiology and Plant Molecular Biology*. **43**, 465-490.
- Zambryski, P., Tempe, J., Schell, J. (1989) Transfer and function of T-DNA genes from *Agrobacterium* Ti and Ri plasmids in plants. *Cell*. **56**, 193-201.
- Zel, J., Gogala, N., Camloh, M. (1988) Micropropagation of *Pinus sylvestris*. *Plant Cell, Tissue and Organ Culture*. **14**, 169-175.
- Zhou, H., Stiff, C.M., Konzak, C.F. (1993) Stably transformed callus of wheat by electroporation-induced direct gene transfer. *Plant Cell Reports*. **12**, 612-616.
- Zimmerman, R.H., Miller, S.S. (1991) Orchard growth and fruiting of micropropagated apple trees. *Journal of the American Society for Horticultural Science*. **116**, 780-785.
- Ziv, M. (1986) *In vitro* hardening and acclimatization of tissue culture plants. In: *Plant Tissue Culture and its Agricultural Applications*, pp. 187-196, Withers, L.A., Alderson, P.G., (eds), Butterworths, London Boston Durban Singapore Sydney Toronto Wellington.
- Ziv, M. (1991a) Quality of micropropagated plants - vitrification. *In Vitro Cellular and Developmental Biology*. **27**, 64-69.
- Ziv, M. (1991b) Morphogenic patterns of plants micropropagated in liquid medium in shaken flasks or large-scale bioreactor cultures. *Israel Journal of Botany*. **40**, 145-153.