ESTABLISHMENT OF AN INDIRECT ORGANOGENESIS PROTOCOL FOR *Eucalyptus grandis* SPECIES AND HYBRIDS

by

ELLIOSHA HAJARI

Submitted in fulfilment of the academic requirements for the degree of Master of Science in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban.

December, 2004

PREFACE

The experimental work described in this thesis was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Durban, from March 2003 to December 2004, under the supervision of Professor M.P. Watt and Professor D.J. Mycock (University of the Witwatersrand).

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.

Elliosha Hajari
December 2004

ACKNOWLEDGEMENTS

I would like to extend a special word of thanks to my supervisor Prof. Paula Watt for her constant guidance and encouragement throughout the duration of this study. I would also like to thank Mrs Marzena Banasiak for her assistance in the laboratory and to Mr Oscar Mokotedi for assisting me with aspects relating to the rooting and acclimatization studies. Thanks also to my co-supervisor Prof. Dave Mycock for always being able to provide a fresh perspective.

I would like to extend thanks to Mondi Forests for supplying plant material and the National Research Foundation for funding this work.

Finally, I want to express my heartfelt thanks to my family for their unflagging support throughout the course of this study.

ABSTRACT

The prospect of integrating transgenic eucalypts with conventional breeding programmes is of value to the Plantation Forestry and Forest Products Industries. However, significant progress in this regard has still to be reported, one constraint is the lack of appropriate high yielding regeneration culture methods for clonal material. Such was the main aim of the present study. The strategy was to develop a suitable protocol using *in vitro* shoots of an *E. grandis* x *E. urophylla* clone (GU185) and thereafter to test its applicability to other clones. Explants from greenhouse-established cuttings provided the *in vitro* shoots, which were multiplied via axillary bud proliferation either on semi-solid medium or using a RITA system. To determine the best conditions for callus and shoot regeneration, parameters such as vessels (Petri dishes and tubes) and types and levels of plant growth regulators were tested. The best callus production (100%) and shoot regeneration (78.9 – 100% callus with shoots) for GU185 occurred on MS, 30 g l^{-1} sucrose, 4 g l^{-1} Gelrite, 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. Parameters tested to identify the most suitable explants for indirect organogenesis were the age of parent plants, different systems to generate *in vitro* shoots, elongation status of explants, 1st and 2nd generation *in vitro* shoots and the use of hyperhydric shoots. Of these, the most suitable explants for indirect organogenesis were shoots from axillary bud multiplication of 3-month-old parent plants using the semi-solid system (33 shoots/dish). Up to 90% rooting was achieved on ¼ MS (Murashige and Skoog, 1962), 15 g l^{-1} sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 4 g l^{-1} Gelrite and IBA. The highest rooting was obtained when regenerated shoots were first multiplied and then placed on medium without plant growth regulators for one week, before transfer to root induction medium containing 0.1 – 0.5 mg l^{-1} IBA. Acclimatization success was 95% when rooted shoots were placed in pots with a rooting mix (2 perlite: 1 coir) enclosed in plastic bags and the humidity was gradually reduced over four weeks. The developed indirect organogenesis protocol appeared to have a broad general application, although the tested clones exhibited a genotype-dependent response, with GU180, GU177 and TAG31 producing fewer shoots (9, 6 and 7 shoots/dish) than ZG14 and GU185 (24 and 18 shoots/dish). Similarly high levels of rooting were obtained for TAG31 (93.8%) and ZG14 (90%) and for hardening-off (90.7% for TAG31 and 91.4% for ZG14).

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LIST OF ABBREVIATIONS

°C	degrees Celsius
$g\ l^{-1}$	grams per litre
$ml\ l^{-1}$	millilitres per litre
Fe	iron
Cu	copper
Zn	zinc
B	boron
Mo	molybdenum
N	nitrogen
P	phosphorous
K	potassium
$HgCl_2$	mercuric chloride
$Ca(OCl)_2$	calcium hypochlorite
v/v	volume by volume
%	percent
min	minutes
Tween 20	polyoxyethylene sorbitan monolaurate
ml	millilitres
KH_2PO_4	potassium dihydrogen orthophosphate
$MgSO_4.7H_2O$	magnesium sulphate heptahydrate
NAA	α-napthaleneacetic acid
BAP	6-benzylaminopurine
$\mu g\ ml^{-1}$	microgram per millilitre
KPA	kilopascal
pH	hydrogen ion concentration
$\mu mol\ m^{-2}\ s^{-1}$	micromoles per metre squared per second
PPFD	photosynthetic photon flux density
ZG	*Eucalyptus grandis*
TAG	*Eucalyptus grandis*

GU	*Eucalyptus grandis* x *urophylla*
MS	Murashige and Skoog (1962) basal nutrients
RITA	Recipient for Automated Temporary Immersion
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
cm	centimetre
$mg\ l^{-1}$	milligrams per litre
mm	millimetres
h	hours
d	days
CO_2	carbon dioxide gas
m^3	metres cubed
km	kilometres
kan	kanamycin
rif	rifampicin

1. INTRODUCTION AND LITERATURE REVIEW

1.1 Brief history of eucalypt forestry in South Africa

The genus *Eucalyptus* belongs to the family Myrtaceae. Eucalypts can occur in one of three sub-genera *viz.* Symphomyrtus, Monocalyptus or Corymbia (Muralidharan and Mascarenhas, 1995). It is estimated that there are between 450 – 700 *Eucalyptus* species (Turnbull, 1991). According to the latter, *Eucalyptus* trees were first discovered by the French botanist Charles Louis L'Heritier de Brutelle over 200 years ago in Australia, they were initially regarded as botanical curiosities and were popular as attractions in botanical gardens and private arboreta in Europe. During the nineteenth century, seeds were dispersed to all parts of the world by travellers, traders, goldminers, soldiers, priests and botanists (Turnbull, 1991). At the time of their discovery, the potential of eucalypts as a major source of forest products was not recognised, as the wood was deemed to be problematic to saw and season and many believed that the only value of this species was as a source of firewood (Turnbull, 1991). Since then, eucalypts have emerged as one of the most widely planted hardwood species in the world (Turnbull, 1991; Campinhos, 1999).

Eucalyptus trees are indigenous to Australia. They are widely planted in other parts of the world as exotics. It has been estimated that there are approximately 120 000 km^2 of eucalypt plantations established worldwide, and large plantations exist in Brazil, India, China, Chile, South Africa, Morocco, Portugal and Spain (Turnbull, 1999). *Eucalyptus* trees are versatile and can be used to produce sawn timber, mine props, railroad sleepers, paper pulp, fibreboard, furniture, firewood, charcoal, essential oils, honey, tannin, shade and shelter (Zacharin, 1978; Campinhos, 1999; Turnbull, 1999; Smit and Pitcher, 2003).

Eucalypts were introduced into South Africa as early as 1803 and by 1820, a number of these trees were growing in the Cape region (Zacharin, 1978). During World War I, South Africa experienced timber shortages when overseas supplies were suspended, and existing plantations and indigenous forests were unable to meet the demand. Temporary

relief at the time was provided by a stand of 734 000 cubic feet of *Pinus radiata* trees grown at Tokai near Cape Town. However, the situation was still critical; timber prices soared, building operations were suspended and great difficulties were experienced in the marketing of agricultural and other products (King, 1951). This critical timber shortage prompted government as well as the private sector to make greater efforts to promote the development of the forestry sector. Consequently, during World War II, the timber shortage was not as dire since substantial amounts of timber were available from government and privately owned plantations (King, 1951).

The discovery of gold in the Witwatersrand severely depleted indigenous forests, as timber was required for mining purposes (King, 1951). In order to meet this demand, the South African government experimented with developing plantations of exotic trees such as pines and eucalypts. Initial work was done using indigenous trees, but these trees grew too slowly to accommodate the increasing demand for timber (King, 1951). Therefore, exotic trees were planted to provide a source of fast-growing trees to meet timber demands (Smith, 1996; Anon, 2003a). Forestry began in the Sabie and Pilgrims Rest districts. In 1904, the Transvaal Gold Mining Estates planted the first *Eucalyptus* trees on Driekop farm.

Eucalypts exhibit a number of features that make them desirable exotic plantation species including fast growth rates, good growth under a range of soil types and various climatic conditions. Since they are exotic species disease and pest problems are manageable, they coppice readily, seeds are orthodox and can be stored and transported easily, are relatively easy to clone, species can be crossed readily to produce hybrids and they provide a range of valuable wood and non-wood products (Zacharin, 1978; Gupta and Mascarenhas, 1987; Campinhos, 1999; Turnbull, 1999; Bouillet *et al.*, 2004; Whitehead and Beadle, 2004).

Despite the advantages offered by the use of *Eucalyptus* trees, there exists strong opposition to the establishment of *Eucalyptus* plantations (King, 1951; Zacharin, 1978; Zobel, 1993; Chaste, 2004). Major criticisms relate to the ecological impacts of

Eucalyptus plantations (Pereira, 2004). The most prominent argument is that *Eucalyptus* plantations utilise too much water (Campinhos, 1999; Pereira, 2004). It has also been suggested that eucalypt plantations have negative impacts on soil nutrients and biodiversity, and that plantations are not aesthetically pleasing (Turnbull, 1991; Campinhos, 1999). A number of workers have suggested that it is the indiscriminate planting of eucalypts on sites that have not been adequately assessed that has prompted much of the criticism levelled against this species (Adlard, 1987; Turnbull, 1991). Adlard (1987) suggests that *Eucalyptus* trees should be planted on suitable land that can sustain the fast growth rate of these trees and that this can only be done if sound land-use planning is enforced.

1.2 Importance of hybrid intensive forestry in South Africa

Initially *Eucalyptus* plantations were established in South Africa predominantly for the production of mining timber however, emphasis has now shifted to pulp production (Smit and Pitcher, 2003). A range of *Eucalyptus* species are currently utilised in this country (Table 1.1). The most widely planted species in the warmer regions is *E. grandis* (Denison and Quaile, 1987; van Wyk, 1990), which also occurs in hybrid combinations, e.g. with *E. camaldulensis*, *E. tereticornis*, *E. urophylla* and *E. nitens* (Anon, 1999a). Hybridisation (the crossing of diverse species) manipulates the existing genetic variation within populations thereby allowing greater flexibility of species. It has been shown that hybrid vigour (heterosis) can greatly benefit the productivity of *Eucalyptus* and other plantation species (Denison and Kietzka, 1993; Anon, 1999b; Malan, 2000). More importantly, hybrid eucalypts can expand afforested areas by allowing plantations to be established on marginal sites. This implies that growing areas can be extended to include hotter, drier areas and also colder, more frost susceptible areas (Denison and Quaile, 1987; Denison and Kietzka, 1993; Anon, 1999b). Further, greater disease resistance can be obtained and wood properties can be targeted to produce specific end products (Denison and Quaile, 1987; Anon, 1999b).

The use of hybrids in plantation forestry is therefore of great potential value to South Africa as suitable land for forestry (in areas of good rainfall, climate and soil) is scarce (Dye, 2000). Hence, in order for greater areas to be afforested, the less suitable marginal lands must be used (Dye, 2000; Bouillet *et al.*, 2004) and hybrid species can facilitate this (Denison and Kietzka, 1993). The hybrids that have been identified for use in sub-tropical areas are *E. grandis* x *E. urophylla*, *E. grandis* x *E. camaldulensis* and *E. grandis* x *E. tereticornis*. In temperate locations use can be made of *E. grandis* x *E. nitens* and *E. grandis* x *E. macarthurii* hybrids (Denison and Kietzka, 1993).

Table 1.1: Record of *Eucalyptus* species used in South Africa by Mondi Forests (Anon, 1999b).

Species	
Eucalyptus grandis	*Eucalyptus dunnii*
Eucalyptus urophylla	*Eucalyptus camaldulensis*
Eucalyptus saligna	*Eucalyptus tereticornis*
Eucalyptus nitens	*Eucalyptus benthamii*
Eucalyptus macarthurii	*Eucalyptus nobilis*
Eucalyptus elata	*Eucalyptus dorrigoensis*
Eucalyptus fastigata	*Eucalyptus bicostata*
Eucalyptus smithii	*Eucalyptus cypellocarpa*

1.3 Impact of the forestry industry on the economy of South Africa

The forestry industry in South Africa makes a significant contribution to the economy of the country. The industry is a strong net exporter of products and this contributes towards earning valuable foreign exchange (Cellier, 1993; Edwards, 2000; Smit and Pitcher, 2003). In 2001, the industry was valued at approximately R 12 billion (Harvett, 2001). In addition, the forestry and forest products industries provide employment opportunities for a large sector of the labour market (Anon, 1997). In 2003, the industry employed approximately 46 000 forestry workers, 106 000 workers in the forests products sector and more than 15 000 workers were employed by contractors (Smit and Pitcher, 2003).

From the mid 1980's the pulp and paper sector of the industry emerged as the most prominent and profitable sector (Anon, 2004; Louw, 2004). This trend has persisted as can be seen in Table 1.2, which illustrates the major uses and sales of roundwood harvested from South African plantations in 2001/2002.

Table 1.2: Sales of roundwood harvested from South African plantations for the year 2001/2002 by volume and value (Anon, 2003b).

Product	Sales by volume ('000m^3/Tons)	Sales by value (Rand million)
Sawn timber	1. 689 628	1 867. 7
Pulp	1. 876 818	8 641. 7
Mining timber	364 990	108. 5
Panel products	685 985	594. 7
Poles	275 759	164. 4
Charcoal	40 307	101. 0
Chips/Mill residues	3. 360 538	1 508. 1
Other	11. 213	820. 7
Total	8. 305 238	13 806.8

In 2002, the tree species planted in South Africa were comprised of pine 52.2%, *Eucalyptus* 38.9%, wattle 8.3% and other species 0.6% (Godsmark, 2003). *Eucalyptus* trees are grown in the low-lying areas of KwaZulu-Natal Midlands and Mpumalanga, coastal regions of KwaZulu-Natal, Eastern and Western Cape and in Tzaneen (Figure 1.1) (Godsmark, 2003; Smit and Pitcher, 2003; Anon, 2004). Most of the plantation forests are owned by private companies (62.3%). The remaining areas are owned by the State including SAFCOL (23.6%), municipalities (0.3%) and individuals (13.8%) (Anon, 2004). It has been reported that South African plantations are among the best managed plantations in the world. The South African forestry industry has the largest plantation area in the world (80.5%) and it has been certified by the Forestry Stewardship Council or ISO 14001 as being sustainably managed (Barnes, 2001; Kasrils, 2001; Edwards, 2002).

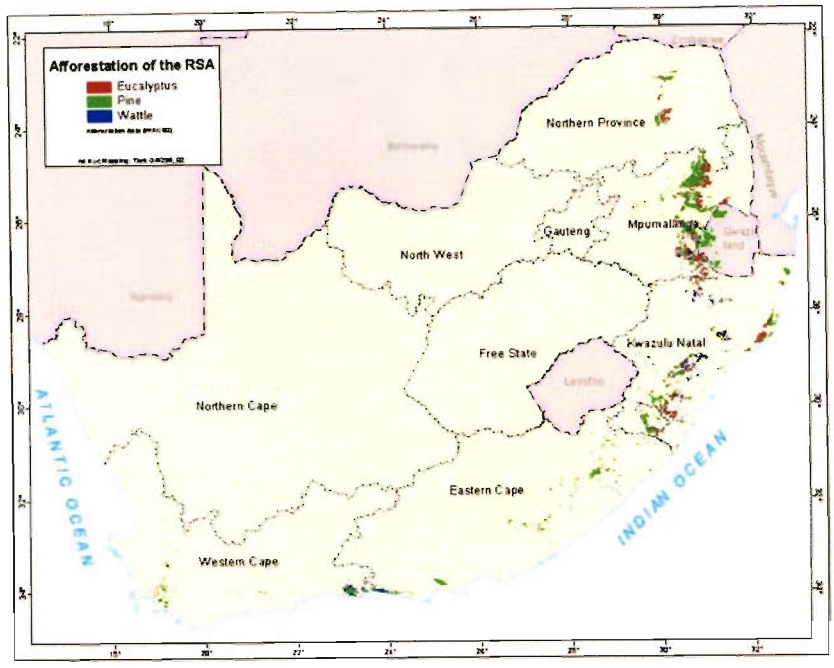

Figure 1.1: Geographic location of plantations in South Africa. (Source: Godsmark, 2003).

1.4 Breeding and propagation strategies for *Eucalyptus* species and hybrids

1.4.1 Sexual propagation

Traditional methods of regeneration relied upon growth of seedlings from bulked seed collected in nature. More recent methods involved the use of seeds collected from randomly pollinated plus-trees (Ahuja, 1993). In natural forests, trees are removed by selective felling and natural regeneration from seed occurs. In areas where trees are felled, stands of good quality trees may be left behind to re-seed the cleared area or the area may be aerially sown or established with seedlings obtained from nurseries (McComb and Bennett, 1986). However, these methods often resulted in large variation in growth, form and vigour of planted trees (Ahuja, 1993) due to the reported heterozygosity of seeds (Tibok *et al.*, 1995). This is not ideal in plantation forestry, where uniform trees with predictable growth characteristics are required for specific end-uses

(Schuch, 1991; Bell *et al.*, 1993; MacRae and Cotterill, 1997). It must be emphasised however, that sexual propagation will always be of importance in breeding programmes as it provides a source of much needed genetic variation that forms the basis of any tree improvement programme (Harvett, 2001).

1.4.2 Vegetative propagation

An alternative propagation method is vegetative propagation. This method results in the production of genetically similar individuals thereby overcoming the problems associated with variation in the growth of progeny produced by sexual propagation. A number of vegetative propagation methods have been developed. One such method involves the grafting of scions from selected plus-trees onto seedling stocks of the same species (Konar and Nagmani, 1973; Biondi and Thorpe, 1981; Gardner, 1997). Care must be exercised when using this method to ensure that graft incompatibility does not arise between the scion and stock (McComb and Bennett, 1986). Another propagation method is air layering (Mantell *et al.*, 1985) but as with grafting, this method is labour-intensive and it has been reported that eucalypts are slow to root when they have been air layered (Cresswell and de Fossard, 1974; McComb and Bennett, 1986). Cuttings are the preferred method of vegetative propagation for most eucalypts (McComb and Bennett, 1986). A disadvantage in the use of this method, however, is that cuttings from most mature eucalypts generally do not root well (McComb and Bennett, 1986), although intensive research worldwide has been addressing this problem successfully (Araujo *et al.*, 2004; Canas *et al.*, 2004).

A proven advantage of vegetative propagation is that the genetic potential of superior trees can be transferred to its asexually reproduced progeny (this is the basis of clonal forestry), thereby ensuring that desirable features are perpetuated in the species concerned (Cresswell and de Fossard, 1974).

1.4.3 Biotechnological approaches

In a traditional tree breeding programme, germplasm (provenances and families) is collected and subsequently tested, selected and crossed to produce new genotypes. The production of hybrid crosses and the use of rooted cuttings to produce desired genotypes fall within the scope of traditional tree breeding. Aspects of silviculture and tree nutrition are also closely associated with traditional breeding (Dvorak, 2001). The global demand for forest products is increasing rapidly (Durzan, 1988; Hammatt, 1992; Watt *et al.*, 1997). Traditional breeding programmes offer only limited opportunities to meet this growing demand due to the long life cycle of trees (Cresswell and de Fossard, 1974; Gunn and Day, 1986) and difficulties experienced by breeders in distinguishing between genotypic and environmental effects (Riemenschneider *et al.*, 1990; Hammatt, 1992; Watt *et al.*, 1997). Further, there is limited suitable land available for afforestation. Hence, there is an urgent need to increase the productivity of planted forests with regard to the production of short rotation trees with a high production index, and to improve the quality of trees (superior wood, optimal stem form and uniformity and increased resistance to environmental stresses) (Bajaj, 1986; Hammatt, 1992; Watt *et al.*, 1997).

Biotechnology offers opportunities to address these issues (Riemenschneider *et al.*, 1990; Ahuja, 1993; Watt *et al.*, 1997; Campbell *et al.*, 2003). Biotechnology has been defined as the management of biological systems for the benefit of humanity (Nel, 1985), and encompasses a collection of techniques that can be used to enhance the impact of existing biological programmes (Cheliak and Rogers, 1990; Riemenschneider *et al.*, 1990). Associated techniques include tissue culture, genome analysis, molecular markers, gene cloning and the genetic modification of organisms (e.g. Glick *et al.*, 1991; Schuch, 1991). It is important to emphasise that biotechnology cannot replace existing conventional breeding and vegetative (clonal) programmes. The full benefits of biotechnological approaches can only be realised if it is used in conjunction with such established programmes (Timmis *et al.*, 1987; Cheliak and Rogers, 1990; Ahuja, 1993).

1.4.3.1 Proven biotechnological approach: in vitro micropropagation via axillary bud multiplication

Plant tissue culture is a biotechnological approach that has been successfully applied to clonal programmes (McComb and Bennett, 1982; Mascarenhas *et al.*, 1989; Watt *et al.*, 1997; Anon, 1999b; Chen *et al.*, 2001; Araujo *et al.*, 2004). Plants can be produced *in vitro* through one of two developmental pathways, i.e. embryogenesis or organogenesis (Vasil, 1987; Ramage and Williams, 2002). Somatic (asexual) embryogenesis is the production of embryo-like structures from somatic cells (Haissig, 1989; Emons, 1994; Watt *et al.*, 1995; Hansen and Wright, 1999), and is similar to the process of zygotic embryogenesis. Embryogenesis can either be direct (embryos originate directly from the cells of an explant *in vitro* without a callus phase) or indirect (embryos originate after a callus phase) (Cheliak and Rogers, 1990; Watt *et al.*, 1995). Somatic embryogenesis will be discussed later in this chapter.

Organogenesis is a developmental process where organ primordia are initiated on explants in response to the application of plant growth regulators. Like embryogenesis, organogenesis can proceed either directly from explants or indirectly via a callus stage (Cheliak and Rogers, 1990; George, 1993). Axillary bud proliferation (direct organogenesis) is a micropropagation technique that has proven to be of value to clonal forestry programmes (Watt *et al.*, 1997). At Mondi Forests, micropropagation is used to multiply selected (superior) genotypes of *Eucalyptus* species and hybrids. Explants from seed (provenance trials), hedges or potted adult plants are induced to multiply *in vitro* via axillary bud proliferation. Production of roots occurs either *in vitro* or *ex vitro* and acclimatization occurs in the greenhouse (Watt *et al.*, 1997). Those authors reported that in 1997, 30 – 50 selected *Eucalyptus* clones of superior genotype were included annually in the *in vitro* programme at Mondi Forests. From these, approximately 4 200 *Eucalyptus* plants were produced monthly that were used to establish clonal hedges (Watt *et al.*, 1997). Currently Mondi deploys approximately 15 million vegetatively propagated *Eucalyptus* trees annually from their clonal programme, including plants produced from cuttings, hydroponics (Alpoim *et al.*, 2004) and *in vitro* sources (Blakeway *et al.*, 2004).

The use of vegetative propagation to multiply genotypes for clonal testing increases the genetic gain in a tree improvement programme as it provides a mechanism for the characterisation and exploitation of additive and non-additive genetic variation (Snedden and Verryn, 2004). Although this can be done via cuttings, the main advantage of axillary bud multiplication over traditional methods is the large number of plants that can be produced quickly (Hu and Wang, 1983; Hartney and Kabay, 1984; Nashar, 1989). Further, this method produces genetically uniform individuals as they arise from pre-existing or newly formed meristems without any intervening callus stage (Vasil and Vasil, 1980; Thorpe *et al.*, 1991). Therefore, this approach is of importance to the forestry industry for the rapid production of selected uniform, superior genotypes (Nel, 1985; Cocking, 1986; Mavituna, 1988; Burley, 1989; Thorpe *et al.*, 1991; Haines and Martin, 1997). In this manner, axillary bud multiplication can accelerate breeding programmes as, it has been reported that it would take twice as long to produce a sufficient number of ramets for use as cuttings for commercial production if traditional propagation methods were used (Denison and Kietzka, 1993).

Micropropagated *Eucalyptus* plants are comparable or superior to plants produced by conventional methods in terms of their growth rates, uniformity and quality (Watt *et al.*, 1995). It has been suggested that if the observed growth rate of tissue cultured plants could be maintained, then it may be possible to harvest *Eucalyptus* trees before seven years which is the standard full rotation period for *Eucalyptus* trees in South Africa (Watt *et al.*, 1995; 1997). This would accelerate breeding programmes and facilitate significant financial gains for breeders.

In vitro axillary bud proliferation can also be used to enhance macropropagation programmes. For example, such techniques can be used to produce a source of material for stock and hedge plants from which cuttings may be harvested (Watt *et al.*, 1997). In this regard, it has been reported that *Eucalyptus* cuttings from micropropagated shoots were easier to root and in some instances produced more shoots than conventional adult sources (Denison and Kietzka, 1993; Watt *et al.*, 1997). Denison and Kietzka (1993) suggested that this application holds great promise for difficult-to-root clones. This

highlights that it is through the integrated use of new methods with established conventional methods that the full benefits of new technologies can be realised (Jauhar, 2001).

Over the years most of the research relating to *in vitro* culture of *Eucalyptus* species and hybrids have focused on direct methods of regeneration (Table 1.3). The multiplication of genotypes of interest by axillary bud proliferation (direct organogenesis) represents a proven biotechnological approach that has been successfully applied to clonal programmes. McComb and Bennett (1986), Le Roux and van Staden (1991a) and Watt *et al.* (2003b) provide extensive reviews of literature relating to organogenesis in *Eucalyptus*. There exist additional biotechnological approaches that have immense potential benefits when applied to clonal forestry programmes. These approaches will be discussed in subsequent sections.

Table 1.3: List of reported successful studies of micropropagation carried out on *Eucalyptus* species and hybrids via direct organogenesis.

Species	Explant	Reference
E. grandis	Epicormic shoots and shoots	Ikemori, 1987
E. gunnii, *E. coccifera*, *E. pauciflora*, *E. darympleana*, *E. delegatensis*	Rejuvenated nodes	Boulay, 1983
E. radiata	Nodal explants	Chang *et al.*, 1992
E. grandis, *E. nitens*	Nodal explants	Furze and Cresswell, 1985
E. grandis	Nodal explants	Sankara Rao and Venkateswara, 1985
E. tereticornis	Nodal explants	Patil and Kuruvinashetti, 1998
E. nitens	Nodal explants	Gomes and Canhoto, 2003
E. grandis hybrids	Nodal explants	Warrag *et al.*, 1990
E. grandis	Nodal explants	Wachira, 1997
E. grandis	Seeds	Lubrano, 1991
E. grandis x *E. urophylla*	Nodal explants	Jones and van Staden, 1994
E. dunnii	Nodal explants	Fantini and Cortezzi-Graca, 1989
E. grandis, *E. grandis* x *E. camaldulensis*, *E. grandis* x *E. urophylla*	Nodal explants	Watt *et al.*, 2003a
E. camaldulensis	Nodal explants	Sreedhar *et al.*, 1998
E. macarthurii, *E. smithii*, *E. macarthurii* x *E. grandis*	Nodal explants	Le Roux and van Staden, 1991b

Table 1.3 (continued)

E. tereticornis, *E. camaldulensis*	Nodal explants	Yasodha *et al.*, 1997
E. marginata	Nodal explants	McComb and Bennett, 1982
E. globulus	Nodal explants	Trindade *et al.*, 1990
E. grandis	Nodal explants	Watt *et al.*, 1995
E. citriodora	Nodal explants	Koriesh *et al.*, 2004
E. tereticornis x *E. grandis*	Nodal explants	Joshi *et al.*, 2003
E. torelliana, *E. camaldulensis*	Nodal explants	Gupta *et al.*, 1983
E. nova-anglica, *E. viminalis*	Shoot tips	Mehra-Palta, 1982
E. gunnii, *E. darympleana*, *E. pauciflora*, *E. delegatensis*	Nodal explants	Franclet and Boulay, 1982
E. sideroxylon	Nodal explants	Burger, 1987
E. tereticornis	Nodal explants	Das and Mitra, 1990
E. dunnii	Nodal explants	Cortezzi-Graca and Mendes, 1989
E. tereticornis	Nodal explants	Sharma and Ramamurthy, 2000
E. resinifera, *E. maculata*	Nodal explants	McComb and Wroth, 1986
E. ficifolia	Nodal explants	de Fossard *et al.*, 1978
E. globulus	Nodal explants	Bennett *et al.*, 1994
E. citriodora	Nodal explants	Mascarenhas *et al.*, 1981
E. radiata	Nodal explants	Donald and Newton, 1991
E. regnans	*In vitro* nodal explants	Blomstedt *et al.*, 1991
E. viminalis	Nodal explants	Wiecheteck *et al.*, 1989
E. citriodora	Terminal and axillary buds	Gupta *et al.*, 1981
E. grandis	Nodal explants	Lakshmi Sita and Shobha Rani, 1985
E. camaldulensis, *E. globulus*, *E. tereticornis*, *E. torelliana*, *E. citriodora*	Nodal explants	Gupta and Mascarenhas, 1987
E. microcorys	Seeds	Niccol *et al.*, 1994
E. grandis x *E. urophylla*	Nodal explants and shoot tips	Yang *et al.*, 1995
E. globulus, *E. sargentii*, *E. occidentalis*, *E. marginata*	Nodal explants	Bennett *et al.*, 1992
E. tereticornis	Nodal explants	Gill and Gosal, 1996
E. citriodora, *E. torelliana*, *E. grandis*, *E. camaldulensis*, *E. urophylla*, *E. alba*	Nodal explants	Grattapaglia *et al.*, 1990
E. grandis	Epicormic shoots	Teixeira and da Silva, 1990
E. camaldulensis	Shoot tips and epicormic shoots	Yang *et al.*, 2002
E. globulus	Nodal explants	Oller *et al.*, 2004

As with most techniques, there are disadvantages associated with the use of micropropagation in clonal forestry programmes. One such disadvantage is the high cost

of micropropagated plantlets (Constantine, 1986; Thorpe *et al.*, 1991). The major component contributing to the high operational costs of micropropagated plants is labour (Hartney and Kabay, 1984; Constantine, 1986; Standaert-de Metsenaere, 1991). Such costs can be reduced by the automation of certain steps in the tissue culture process (Hu and Wang, 1983; Hartney and Kabay, 1984; Constantine, 1986; Thorpe *et al.*, 1991; Hvoslef-Eide *et al.*, 2003). Gorst and Teasdale (1999), Watad *et al.* (1999) and Gross and Levin (1999) describe innovative methods for reducing the cost of micropropagation by the use of automated devices. Further, the use of bioreactors with liquid media for scaling-up micropropagation (Hvoslef-Eide *et al.*, 2003; Ziv *et al.*, 2003) and the use of photoautotrophic cultures (e.g. Kozai *et al.*, 1987; Kozai, 1991a; 1991b; Zobayed *et al.*, 2000) can contribute to reductions in the cost of micropropagated plants by producing more micropropagated plants per unit time and by reducing or eliminating the use of certain expensive media components.

At Mondi Forests, investigations are being performed to increase the productivity of *Eucalyptus* clones by using a temporary immersion bioreactor system (RITA) to mass produce selected genotypes for use in trials and clonal hedges for commercial production (McAlister *et al.*, 2002). McAlister *et al.* (2000; 2002) reported that *Eucalyptus* plants produced in RITA bioreactors were of a higher quality than those produced in the semi-solid system. Admittedly, the initial outlay of money for the acquisition of RITA bioreactors is high, but this expense is offset by reduced labour and media costs together with high rooting and survival percentages (McAlister *et al.*, 2002). Further, the high multiplication rates and the shorter time required to bulk up *Eucalyptus* plants in RITA bioreactors make them a cost effective option for use in clonal programmes. A number of workers have investigated the use of RITA bioreactors for the rapid multiplication of useful genotypes of other species e.g. St. John's Wort (Zobayed and Saxenam, 2003), apple (Chakrabarty *et al.*, 2003), calabash tree (Murch *et al.*, 2004) and banana (Alvard *et al.*, 1993).

1.4.3.2 Potential applications of other biotechnological approaches: production of 'new' genotypes of interest

Tree improvement involves the management of genetic resources (Cheliak and Rogers, 1990); and in any such programme, it is essential to ensure that genetic gain is conserved and that genetic erosion is minimised (Burley, 1989). Through the process of tree breeding, the amount and organisation of genetic variation is managed by recurrent cycles of selection and breeding in order to produce superior trees with desired properties (Cheliak and Rogers, 1990; Haines, 1994; Harvett, 2001).

Intrinsic to any tree improvement programme is a reliable source of genetic variation (Bajaj, 1986) and for *Eucalyptus* species, a large untapped gene pool is available in the form of wild populations (Gunn and Day, 1986; Ahuja, 1993; Eldridge, 1995; Hansen and Kjaer, 1999; Wilson, 1999). A broad genetic base is essential to ensure that new genotypes can be selected to deal with possible future threats in the form of pests or diseases and changing market objectives (Harvett, 2001). Further, a large source of genetic variation would allow for the identification of genotypes that possess desirable physical attributes such as fast growth rates, specific wood and fibre qualities, drought resistance and the ability to grow on marginalised sites with less than ideal climatic and soil conditions (Denison and Kietzka, 1993; Anon, 1999b). These authors have argued that this approach is essential in order to meet the growing demand for wood products.

However, a significant drawback in tree breeding is the long time period required to identify and select desirable genotypes (Bajaj, 1986) since selected genotypes would have to be identified and then grown to half or full rotation age (Grattapaglia *et al.*, 1996) to determine whether they can tolerate the negative environmental conditions. Marker-assisted selection and breeding using diagnostic tools offer a mechanism to identify useful genotypes earlier (Grattapaglia *et al.*, 1995; 1996) but much work remains to be done in this area before it can be routinely applied to forestry programmes. Further, traditional tree breeding relies on genes available in the gene pool. An alternative method

to produce 'new' genotypes of interest is through the use of certain biotechnological approaches.

In vitro culture systems offer opportunities to produce 'new' genotypes of interest for the forestry industry through the processes of mutagenesis (Al-Safadi and Simon, 1996; Charbaji and Nabulsi, 1999; Predieri and Zimmerman, 2001), somaclonal variation (Bright *et al.*, 1986; Reisch, 1988; Ezhova *et al.*, 1995; Karp, 1995; Barnum, 1998; Jain and de Klerk, 1998; Jayasankar, 2000) and genetic engineering (Nel, 1985; Hammatt, 1992).

A) Mutagenesis

Mutations can be induced *in vitro* through the application of chemicals or radiation (Al-Safadi and Simon, 1996; Charbaji and Nabulsi, 1999; Predieri and Zimmerman, 2001) in order to create new genotypes that can be used in breeding programmes. Mutagenesis used in conjunction with other breeding techniques, can directly improve genotypes for specific traits and can provide material for further hybridisation work (Predieri and Zimmerman, 2001). However, care must be exercised with the use of mutagenesis to generate novel genotypes, as it has been reported that promising mutant genotypes often disappear with the passage of time (Predieri and Zimmerman, 2001).

B) Somaclonal variation

Somaclonal variation is the term used to describe the variation that occurs during the tissue culture process (Ammirato, 1986) in particular with the indirect routes of regeneration (i.e. via a callus stage). Somaclonal variation has been described as being an advantage in plant tissue culture as it can be used to generate genetic variation (Karp, 1995), and a disadvantage when it is undesirable such as in clonal multiplication systems where genetically uniform propagules are required (Ammirato, 1986; Yeoman, 1986; Jain and de Klerk, 1998). The major factors that affect the nature and frequency of variation

are genotype, ploidy, tissue source, tissue culture procedure, culture environment and media composition (Karp, 1989; 1995).

It is generally accepted that the greater the departure from organised growth (such as callus formation), the greater the chances of generating somaclonal variants (Bayliss, 1980; Karp, 1995). The exact mechanism of somaclonal variation is not clearly understood however, the genetic changes associated with somaclonal variants have been identified as point mutations, karyotype changes (chromosome number and structure), chromosome rearrangements, altered sequence copy number, transposable elements, somatic crossing over, sister chromatid exchange, gene amplification and deletion (Cocking, 1986; Scowcroft *et al.*, 1987; Phillips *et al.*, 1994; Karp, 1995; Jain and de Klerk, 1998; Kaeppler *et al.*, 2000).

The advantage of using somaclonal variation as a means to generate a rapid, novel source of variation is that it represents a cheap form of biotechnology that is perceived to be less risky by the public compared with genetic engineering. Further, no gene cloning methods or containment procedures are required (Karp, 1995). The disadvantage is that somaclonal variation is not a precise tool since it is not possible to predict the outcome of a somaclonal programme. There is no guarantee that a particular trait will be altered in a positive manner and that the trait will be stably heritable (Reisch, 1988; Karp, 1995). Further, a large number of inferior lines may be created in the process. Karp (1995) suggested that somaclonal variation is most likely to be used as a source of variation in crops with limited genetic systems or a narrow genetic base. For these reasons, somaclonal variation may not represent the ideal system to generate new genotypes of interest for *Eucalyptus* species.

C) Genetic engineering

Genetic engineering involves the insertion of specific DNA sequences (transgenes) via nonsexual processes to an unrelated organism. The modified host (transgenic organism) typically expresses the new transgene and thereby possesses a new trait or, the introduced

transgene may function to regulate the levels of expression of existing genes. The entire process of gene isolation, modification and transfer to a host organism is known as genetic engineering (Brunner *et al.*, 1998; Burdon, 2003). An advantage of this technique is that it allows for rapid genetic gain. Hence, genetic engineering represents a method for introducing a source of genetic variation within breeding programmes (Yeoman, 1986).

Genetic engineering can be applied to *Eucalyptus* clonal forestry programmes to produce genotypes that can tolerate environmental stresses in order to allow for the afforestation of marginal land in South Africa. Eucalypts are vulnerable to sudden frosts, therefore using a genetic engineering approach, extra frost tolerance could be conferred to specific genotypes (Haines, 1994; Edwards *et al.*, 1995; Altman, 2003; Burdon, 2003). These cold-tolerant genotypes can be planted in areas that would traditionally not be ideal for commercial forestry. In addition, genetic engineering technology holds promise to confer resistance to plants against drought, salinity and heavy metal toxicity (Altman, 2003; Burdon, 2003). Tolerance to these abiotic stresses is of perceived value to the forestry industry, as this would allow for the establishment of plantations on marginal lands. Burdon (2003) suggests that it is through abiotic stress tolerance that genetic engineering could potentially make its greatest contribution to commercial forestry productivity.

Genetic engineering also has the potential to improve the efficiency of management practices through the production of insect and herbicide-tolerant genotypes (Owusu, 1999; Williamson, 2002; Watt *et al.*, 2003b). The use of insect-resistant genotypes would reduce the costs associated with repeated application of expensive insecticides and avoid the environmental implications involved in the use of such toxic chemicals (Harcourt *et al.*, 1995; Altman, 2003; Burdon, 2003). In addition, the concept of herbicide resistance is of relevance to commercial eucalypt forestry as these plants are particularly sensitive to weed competition and herbicide damage during establishment (Griffin, 1995; Walter *et al.*, 1995; Burdon, 2003). Therefore herbicide resistance offers greatly reduced establishment costs and gains in productivity for commercial eucalypt breeding (Burdon, 2003).

Another avenue where genetic engineering may be of use to the forestry industry is with regard to product improvement (Watt *et al.*, 2003b). For example, lignin is a component of plant cell walls that is removed during chemical pulping. There are substantial costs associated with the removal of lignin during the pulping process, therefore significant cost reductions could be achieved if *Eucalyptus* trees could be engineered to have reduced levels of lignin or could synthesise lignin that is easier and cheaper to dissolve (Whetten and Sederoff, 1991; Haines, 1994; Edwards *et al.*, 1995; Owusu, 1999; Burdon, 2003). A number of key enzymes have been identified and targeted towards this end (Boudet and Grima-Pettenati, 1996; Altman, 2003; Poke *et al.*, 2004). Indeed, Pilate *et al.* (2002) have reported on the production of transgenic poplar trees with altered lignification that has yielded wood that could be more easily processed by chemical pulping, incurred reduced energy and pollutant chemical costs and produced pulp with improved properties. In 2001, it was reported that there were approximately 12 field trials in progress with transgenic *Eucalyptus* species using *E. camaldulensis*, *E. globulus* and *E. grandis*. The traits being tested were herbicide resistance (four trials), virus resistance (one trial), lignin alteration (two trials), marker genes (three trials) and other traits (two trials) (Anon, 2001).

No technology is completely risk-free. The risks associated with the use of genetically engineered plants include horizontal gene transfer (transfer of genes from one organism to another by means other than classical sexual reproduction) thereby promoting the contamination of non-genetically engineered species, the creation of 'super weeds' that could be potentially difficult to control by the use of available herbicides, adverse effects on non-target species, negative impacts on biodiversity, creation of pests resistant to pesticides and ethical concerns (Pimentel *et al.*, 1989; Anon, 1999c; Robinson, 1999; Wolfenbarger, 2000; Altieri, 2001; Burdon and Walter, 2001; Jauhar, 2001; Burdon, 2003). The risks associated with the use of this technology cannot be totally avoided, but efforts can be made to minimise them (Altman, 2003). Strategies such as the engineering of sexual sterility in trees (Haines, 1994; Brunner *et al.*, 1998), creation of refuges (Altieri, 2001) and containment procedures have been investigated to minimise some of the abovementioned risks.

In order for genetic engineering technology to be applied to plant systems, a protocol for the regeneration of plants from individual transformed cells is essential (Evans *et al.*, 1981; Heberle-Bors, 1991; Hammatt, 1992; Cid *et al.*, 1999; Sharma *et al.*, 2002). Although it should be possible to transform cells of an explant and regenerate them directly into a plant, and this has been reported in a number of crops e.g. sugarcane (Snyman *et al.*, 2000), an indirect method of regeneration of plants (i.e. via a callus stage) is usually needed, either indirect somatic embryogenesis or indirect organogenesis.

a) Indirect somatic embryogenesis

As previously mentioned, somatic embryogenesis can be direct or indirect. In all cases, the characteristic embryo development stages are the globular, heart and torpedo stages (Emons, 1994). As with zygotic development, somatic embryos develop in a bipolar manner (Ahuja, 1993), and produce propagules with both a shoot and root meristem. This is desirable since plantlets regenerated from somatic embryos generally produce a tap root system, which is of value to the commercial forestry industry (Watt *et al.*, 1995). The presence of root and shoot meristems also means that a separate root induction stage during propagation is not necessary (White, 2001). Other advantages in the use of somatic embryogenesis to regenerate plants is that rapid and easy scale-up of embryos can be achieved by the use of liquid media in bioreactors (Ahuja, 1993; Merkle, 1995), somatic embryos are suitable targets for genetic transformation work (Hansen and Wright, 1999) and there exists the possibility of encapsulation of embryos and the potential for the induction of dormancy and long-term storage via cryopreservation (Ford-Lloyd and Jackson, 1991; Blakesley *et al.*, 1996; Withers and Engelmann, 1998).

There are major limitations to the application of somatic embryogenesis to forest tree programmes. With very few exceptions, success has only been achieved with germinated seedlings or hypocotyls (Table 1.4). This is not ideal for eucalypt clonal forestry since *Eucalyptus* trees can only be identified as superior when they reach maturity. Therefore for somatic embryogenesis to be carried out, explants from these selected identified mature trees must be used. *In vitro* shoots can be obtained from mature trees to be used as

explants for somatic embryogenesis but seedlings represent an 'unknown' explant source. Further, it can be seen from Table 1.4 that only four studies reported regenerated plantlets from somatic embryos. Another limitation is with regard to low yields that have been obtained from most somatic embryogenesis systems (Watt *et al.*, 1991; Merkle, 1995; White, 2001). Further, some species may prove to be recalcitrant to somatic embryo induction (Ahuja, 1993). Other limitations include the inability to induce embryos from mature, elite trees (Merkle, 1995), problems related to maturation and conversion (germination) of somatic embryos and subsequent development of viable somatic seedlings in some forest trees (Cheliak and Rogers, 1990; Watt *et al.*, 1991; Ahuja, 1993; Muralidharan and Mascarenhas, 1995) and questions regarding the genetic stability of plants regenerated from somatic embryos (Cheliak and Rogers, 1990; Ahuja, 1993). Therefore, while somatic embryogenesis holds great potential for mass propagation and genetic improvement of *Eucalyptus* trees, much work needs to be done in order to overcome the limitations imposed by this system before it can be applied for operational use (Cheliak and Rogers, 1990; Merkle, 1995).

Table 1.4: Examples of reported studies of work conducted on somatic embryogenesis in *Eucalyptus* species and hybrids.

Species	Explant	Success	Reference
E. dunnii	Hypocotyls	Plantlets	Termignoni *et al.*, 1996
E. grandis	Seedling-derived leaves	Plantlets	Watt *et al.*, 1991
E. grandis	Zygotic embryos and hypocotyls	Bipolar 'embryo-like' structures	Major *et al.*, 1997
E. nitens	Hypocotyls, leaves, cotyledons and epicotyls	Somatic embryos	Ruaud *et al.*, 1997
E. citriodora	Cotyledons	Plantlets	Muralidharan *et al.*, 1989
E. grandis	Internodal segments	Embryoids	Lakshmi Sita, 1986
E. citriodora	Shoots	Embryoids	Lakshmi Sita, 1986
E. globulus	Cotyledons and hypocotyls	Shoots	Nugent *et al.*, 2001
E. globulus	*In vitro* leaves	Embryogenic callus	Oller *et al.*, 2004
E. nitens	Cotyledons	Somatic embryos	Bandyopadhyay and Hamill, 2000
E. urophylla	Cotyledons	Somatic embryos	Arruda *et al.*, 2000
E. globulus	Cotyledons, hypocotyls, leaves and seedling-derived stems	Plantlets	Pinto *et al.*, 2004

b) Indirect organogenesis

The process of indirect organogenesis begins with the de-differentiation of the primary explant to a less committed, more plastic developmental state (callus). This is followed by the induction phase where cells become committed to the production of either shoots or roots. The final stage is differentiation, where the actual process of morphological differentiation results in the production of organs (Schwarz and Beaty, 2000). These phases are controlled *in vitro* by manipulating the ratio of exogenously applied plant growth regulators. When the ratio of auxin to cytokinin is relatively high, then roots are initiated, and when the ratio of cytokinin to auxin is high, then shoots are initiated. Auxins are generally required for callus induction and are used in conjunction with cytokinins to maintain callus cultures (Butcher and Ingram, 1976; Minocha, 1987). Therefore, it is possible to regenerate shoots by indirect organogenesis for a number of plant species.

As previously mentioned, a protocol for the regeneration of transformed cells is of paramount importance in order for genetic engineering technology to be applied to commercial *Eucalyptus* forestry species. It is in fact the unavailability of high-yielding regeneration protocols via the indirect route of morphogenesis that represents one of the factors that limits the application of genetic engineering technology to commercial eucalypt forestry species (Watt *et al.*, 2003b). Research in this respect has not proceeded as rapidly as for direct methods of axillary bud proliferation (Table 1.3 vs. Table 1.5). Therefore, there is an urgent need for research towards the development of indirect organogenesis protocols for *Eucalyptus* species and hybrids. The small amount of literature that is available reports on protocols that have been developed predominantly using seedling-derived explants for example, Tibok *et al.* (1995), Bandyopadhyay *et al.* (1999), Cid *et al.* (1999), Kitahara and Caldas (1975), Warrag *et al.* (1991), Azmi *et al.* (1997), McComb and Bennett (1982) and de Fossard *et al.* (1974). Hence, there exists the need to develop an indirect organogenesis protocol for eucalypts using clonal material since these genotypes are uniform and have been selected for their desirable traits in

contrast to explants obtained from seedlings whose genotypes cannot be predicted (Gupta and Mascarenhas, 1987).

A review of the published literature on the development of indirect organogenesis protocols for eucalypts indicated that most are not high-yielding protocols. For example, Bandyopadhyay *et al.* (1999) reported percentage callus with regenerated shoots of 18 – 32% for *E. nitens* and 12 – 37% for *E. globulus*. Similarly, Tibok *et al.* (1995) reported percentage callus with regenerated shoots of 0 – 37.5% for *E. urophylla*. Further, Bandyopadhyay *et al.* (1999) reported a low average number of shoots per explant *viz.* 2 – 6 for *E. nitens* and 2 – 5 for *E. globulus*, whereas Tibok *et al.* (1995) reported a relatively high average number of shoots per explant for *E. urophylla* of 15 – 27.6. Cid *et al.* (1999) obtained high percentages of callus induction in *E. grandis* x *E. urophylla* (95 – 100%), and callus with shoots (11 – 98%) but did not report the average number of shoots regenerated per explant. Similarly, the protocol of Laine and David (1994) resulted in up to 64% of callus of clonal *E. grandis* that produced buds but the authors did not present the average number of shoots regenerated per explant.

Table 1.5: Examples of reported studies of work conducted on indirect organogenesis in *Eucalyptus* species and hybrids.

Species	Explant	Success	Reference
E. grandis	Hypocotyls	Plantlets	Warrag *et al.*, 1991
E. urophylla	Hypocotyls	Plantlets	Tibok *et al.*, 1995
E. nitens, E. globulus	Cotyledons and hypocotyls	Plantlets	Bandyopadhyay *et al.*, 1999
E. grandis	Leaves	Plantlets	Laine and David, 1994
E. globulus	Cotyledons, hypocotyls and *in vitro* shoots	Plantlets	Azmi *et al.*, 1997
E. camaldulensis	*In vitro* leaves	Shoots	Mullins *et al.*, 1997
E. grandis x *E. urophylla*	Cotyledons and hypocotyls	Plantlets	Cid *et al.*, 1999
E. bancroftii	Stem and lignotubers	Nodular callus	de Fossard *et al.*, 1974
E. marginata	Stamens	Plantlets	McComb and Bennett, 1982
E. camaldulensis	*In vitro* leaves	Plantlets	Muralidharan and Mascarenhas, 1987
E. grandis x *E. urophylla*	*In vitro* shoots	Plantlets	Chen *et al.*, 2002
E. alba	Hypocotyls	Shoots	Kitahara and Caldas, 1975

There are a number of disadvantages with the use of callus cultures, the most notable being the problem of somaclonal variation (see earlier; Butcher and Ingram, 1976; Bajaj, 1986; Burley, 1989; Ahuja, 1993). In addition, it has been reported that cultures tend to lose the ability to differentiate and regenerate plants with successive subcultures (Yeoman and Forche, 1980; Thorpe *et al.*, 1991). However, the advantages associated with the use of callus cultures including the enormous number of plants that can potentially be produced (Bajaj, 1986; Sha Valli Khan *et al.*, 2002) and applications of genetic engineering technology imply that indirect methods of regeneration are of significant importance to the forestry industry.

1.5 Aims

Eucalyptus trees are plantation species of immense importance to the South African forestry industry. Shortages of wood and wood-based products are predicted in the near future unless increasing demands can be met. Biotechnological approaches in conjunction with traditional tree improvement programmes have great potential to meet projected demands. Some are already applied very successfully. Others such as genetic modification of trees are not. This is because in order for genetic engineering technology to be applied to *Eucalyptus* species, it is vital to have a reliable protocol for the regeneration of plants from transformed cells. Hence, the aim of this study was to develop an indirect organogenesis protocol for *Eucalyptus* species and hybrids. To ensure a constant supply of *in vitro* shoots for the development of the indirect organogenesis protocol, it was necessary to first generate a supply of decontaminated *in vitro* shoots using greenhouse-established inserts as the starting material. The indirect organogenesis protocol was developed using *in vitro* shoots of an *E. grandis* x *E. urophylla* hybrid (GU185) and was subsequently tested on other *E. grandis* x *E. urophylla* hybrids (GU177 and GU180) and pure *E. grandis* clones (TAG31 and ZG14).

2. MATERIALS AND METHODS

2.1 Plant material and maintenance of parent plants

Cutting-derived inserts in seedling trays (Figure 2.1) were obtained from Mountain Home Laboratory, Mondi Forests, Hilton (KwaZulu-Natal, South Africa). The *Eucalyptus grandis* x *Eucalyptus urophylla* hybrid inserts (GU177, GU178 and GU185) were maintained in a mist tent (85 - 94% humidity) in the greenhouse at the University of KwaZulu-Natal, Durban, (29°52'S, 30°59'E; 25 °C day/18 °C night). Plants were sprayed with fungicides and fertilizers on a weekly basis. The fungicides used were mixtures of 2 g l^{-1} mancozeb (Dithane; Efekto, South Africa) and 1 ml l^{-1} chlorothalonil (Bravo; Shell, South Africa) applied as a foliar spray and a mixture of 1 g l^{-1} prochloraz manganese chloride (Sporgon; Hoechst Schering AgrErvo, South Africa) and 1.25 ml l^{-1} tebuconazole (Folicur; Bayer, South Africa) applied to the soil. The fertilizers used were 2.5 ml l^{-1} trace element solution (18 g l^{-1} Fe, 4 g l^{-1} Cu, 2 g l^{-1} Zn, 1 g l^{-1} B and 0.4 g l^{-1} Mo) (Trelmix; Hubers, South Africa) applied as a foliar spray and 1 g l^{-1} Mondi Orange 1N-2P-1K (Harvest Chemicals, South Africa) applied as a soil spray, alternately once a week.

Figure 2.1: Cutting-derived inserts of an *E. grandis* x *E. urophylla* clone (GU185) maintained in the greenhouse, bar = 6.3 cm.

2.2 Establishment of *in vitro* shoot cultures

The initial decontamination protocol employed for the decontamination of GU177, GU178 and GU185 inserts consisted of two-minute dips in 0.2 g l^{-1} $HgCl_2$ followed by 10 g l^{-1} $Ca(OCl)_2$ with sterile water rinses between sterilants. No further GU177 and GU178 material was available; therefore the strategy for these clones was to bulk up all the *in vitro* uncontaminated shoots from the initial culture. A supply of GU185 material was readily available therefore this material was used for further decontamination studies. A summary of the various protocols tested for the decontamination of GU185 inserts are presented in Table 2.1. The *Eucalyptus grandis* x *E. urophylla* hybrid clone (GU180) and pure *Eucalyptus grandis* clones (TAG31 and ZG14) were also used for subsequent indirect organogenesis work but this material was already available in the laboratory as *in vitro* shoot cultures.

Table 2.1: Decontamination protocols employed for *E. grandis* x *E. urophylla* (GU185) explants. The fungicide cocktail contained 1 g l^{-1} Benlate, 1 g l^{-1} boric acid, 0.5 ml l^{-1} Bravo and two drops of Tween 20. Plants treated with 70% (v/v) ethanol were sprayed before harvesting. The antibiotics used were applied sequentially with a two-day recovery period for plants by incubation on antibiotic-free medium.

Protocol	Fungicide cocktail (minutes)	$HgCl_2$ (0.2 g l^{-1})	$Ca(OCl)_2$ (10 g l^{-1})	Ethanol (70% v/v)	Antibiotics (kan 100 µg ml^{-1} and rif 20 µg ml^{-1})
A	-	-	-	-	-
B	10	+	+	-	-
C	20	+	+	-	-
D	30	+	+	-	-
E	-	+	+	+	-
F	-	+	+	-	+
G	10	+	+	+	-
H	10	+	+	-	+
I	-	+	+	+	+

Following decontamination, explants (from 3- and 5-month-old parent plants) were trimmed into two node shoots and placed on microbial M 523 screening medium (Viss *et al.*, 1991) containing 10 g l^{-1} sucrose, 8 g l^{-1} casein hydrolysate, 4 g l^{-1} yeast extract, 2 g l^{-1}

KH_2PO_4 (potassium dihydrogen orthophosphate), 0.15 g l^{-1} $MgSO_4.7H_2O$ (magnesium sulphate heptahydrate) and 8 g l^{-1} agar for 24 hours to detect contaminants. There was one shoot/tube (10 x 2.5 cm) with 2.5 ml of microbial medium. After 24 hours, nodal shoots were removed from microbial medium and transferred to either bud break or multiplication medium depending on the age of the parent plants (explants from mature parent plants were placed on bud break and thereafter multiplication medium, while explants from young parent plants were placed directly onto multiplication medium). The tubes containing microbial medium that housed nodal shoots for 24 hours were monitored for contamination over a period of five days.

Bud break medium consisted of Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962; Highveld Biological, South Africa), 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.04 mg l^{-1} α-napthaleneacetic acid (NAA), 0.11 mg l^{-1} 6-benzylaminopurine (BAP), 0.05 mg l^{-1} kinetin, 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite (Polychem, South Africa). Shoots remained on this medium for ten to fourteen days. There was 10 ml of medium and one shoot/tube. Multiplication medium consisted of MS salts and vitamins, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.01 mg l^{-1} NAA, 0.2 mg l^{-1} BAP, 30 g l^{-1} sucrose and 4 g l^{-1} Gelrite.

In order to generate a supply of *in vitro* shoots for use as explants in subsequent indirect organogenesis work, shoots were multiplied either in the semi-solid (Figure 2.2A) or RITA (Recipient for Automated Temporary Immersion System) systems (Figure 2.2B). There was one shoot/tube with 20 ml multiplication medium in the semi-solid system or fifteen shoots/vessel with 200 ml liquid multiplication medium in the RITA system (30 seconds flush and 10 minutes rest). *In vitro* shoots were subcultured onto fresh medium every four to six weeks in the semi-solid system and every two to three weeks in the RITA system. A diagrammatic representation of the operation of a RITA bioreactor is provided in Figure 2.3. When required, shoots were elongated on semi-solid medium comprising MS salts and vitamins, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.35 mg l^{-1} NAA, 0.1 mg l^{-1} kinetin, 0.05 mg l^{-1} indole-3-butyric acid (IBA), 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite for four to six weeks.

All media were adjusted to pH 5.6 – 5.8 (except media containing the antibiotics kanamycin and rifampicin which were adjusted to pH 6.5) (Reed *et al.*, 1995) prior to autoclaving for 20 minutes at 120 °C and 121 KPa.

All cultures were maintained in the growth room under a 16-h photoperiod at a photosynthetic photon flux density (PPFD) of 37 μmol m^{-2} s^{-1} (sideways lighting) provided by Biolux tubes (Osram L58W) and 23 °C day/21 °C night unless otherwise stated.

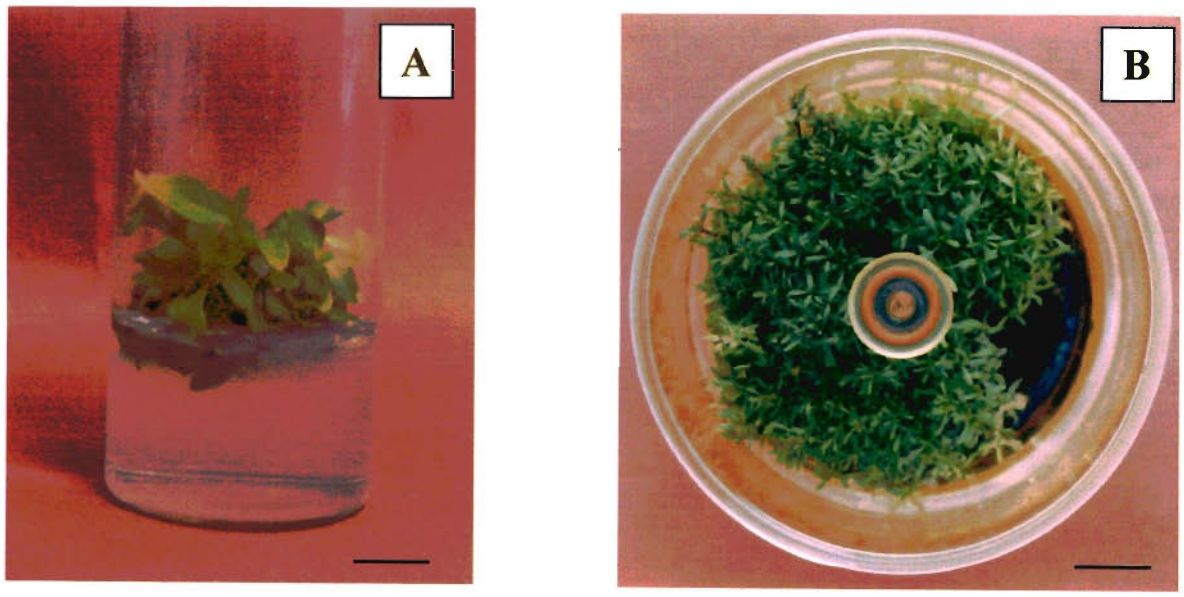

Figure 2.2: *In vitro* shoots of an *E. grandis* x *E. urophylla* clone (GU185). A) Shoots produced in the semi-solid system, bar = 2.3 cm; and B) shoots produced in the RITA system, bar = 1.6 cm.

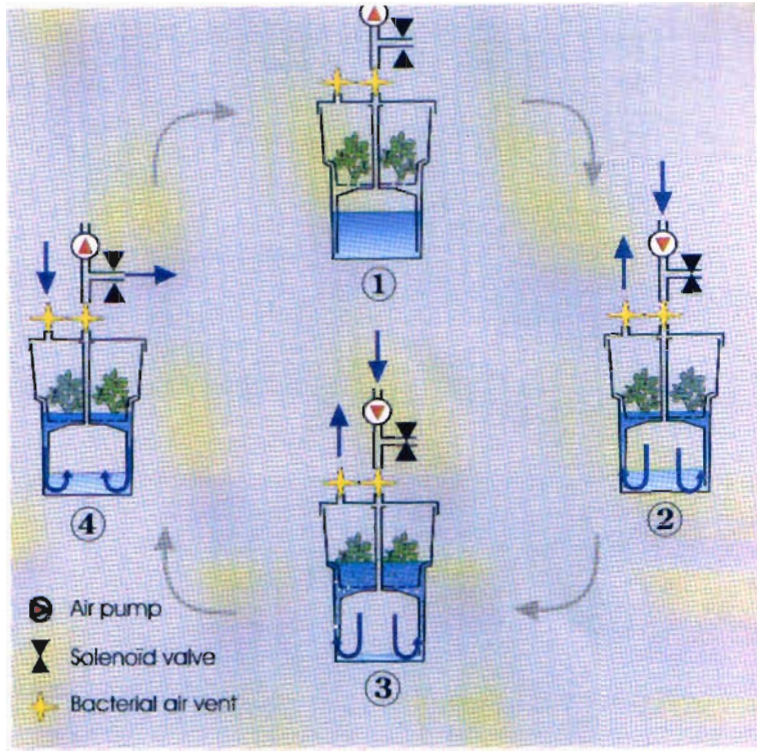

Figure 2.3: Operating cycle of a RITA bioreactor.

1. Plants are placed on a polyurethane foam disc in the upper compartment while the liquid medium remains in the lower compartment.
2. An over pressure of sterile air is applied in the lower compartment that forces the medium into the upper compartment, immersing the plants.
3. During the immersion period, a flow of sterile air aerates the medium, agitates the plants and replaces the atmosphere inside the vessel as the over pressure escapes through an outlet at the top of the apparatus. Optimum duration of flooding and rest must be determined empirically.
4. When the over pressure drops, the medium returns to the lower compartment by gravity. The plants remain covered by a film of medium by capillary attraction (Teisson and Alvard, 1995; VITROPIC, 2004).

2.3 Establishment of the indirect organogenesis protocol

2.3.1 Callus induction and shoot initiation

In vitro shoots of GU185 obtained from semi-solid and RITA systems were used as explants for callus induction and shoot initiation. Shoots (1.5 – 4 cm) were carefully

fragmented and all axillary buds were dissected out and discarded using a scalpel blade and then placed on callus induction media (Figure 2.4) containing MS salts and vitamins, 30 g l^{-1} sucrose and 4 g l^{-1} Gelrite supplemented with either 5 mg l^{-1} IAA (indole-3-acetic acid) and 0.25 mg l^{-1} BAP or 5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin. Callus induction media were dispensed into Petri dishes (40 ml/dish) or culture tubes (20 ml/tube). There were ten to twenty Petri dishes/treatment (five fragmented shoots/dish) or 20 tubes/treatment (one fragmented shoot/tube).

Parameters tested included: 1) age of parent plants (3 and 5 months); 2) system to generate *in vitro* shoots (semi-solid and RITA); 3) elongation status of explants; 4) 1st and 2nd generation *in vitro* shoots and; 5) hyperhydric shoots as explants for indirect organogenesis. Elongated shoots were produced by incubation on elongation medium (see section 2.2), 1st generation shoots were produced by axillary bud multiplication of explants obtained from inserts and 2nd generation shoots were produced by indirect organogenesis. All cultures were incubated in the dark at 24 – 26 °C. After five weeks, regenerated shoots were removed (to eliminate any 'escapes' from non-destroyed buds) and calli were subcultured onto fresh medium for another five weeks. At the end of ten weeks, indirect organogenic calli and shoot production was assessed. Selected parameters were tested on other clones *viz.* the system to generate *in vitro* shoots (GU180 and ZG14), elongation status of explants (GU177 and TAG31) and the use of hyperhydric shoots as explants for indirect organogenesis (TAG31). *In vitro* shoots of the *E. grandis* x *E. urophylla* hybrid (GU185) and the pure *E. grandis* clone (TAG31) were subjected to the protocol of Bandyopadhyay *et al.* (1999). This involved chopping shoots and placing them on callus induction media comprising MS salts and vitamins, 30 g l^{-1} sucrose, 4 g l^{-1} Gelrite, 1 mg l^{-1} NAA and 0.5 mg l^{-1} BAP for two subcultures of three to four weeks each in the dark.

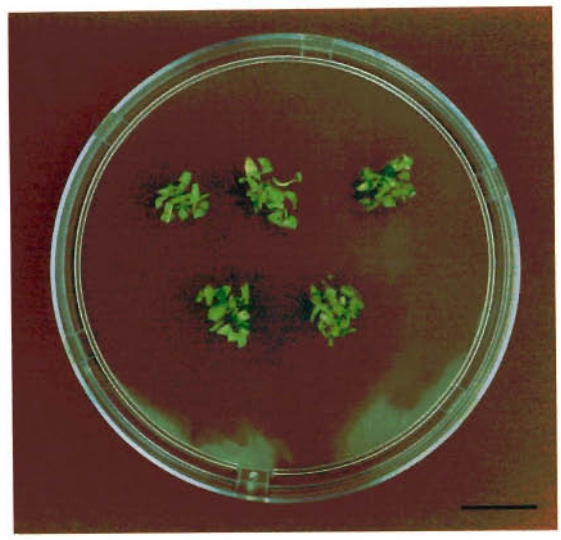

Figure 2.4: Fragmented *in vitro* shoots used as explants for callus and shoot production, bar = 1.5 cm.

2.3.2 Root production

In a preliminary study, GU185 shoots produced by indirect organogenesis were placed on root induction medium comprised of ½ MS salts and vitamins, 15 g l^{-1}sucrose and 4 g l^{-1} Gelrite for four weeks.

Subsequent studies involving all clones and *E. grandis* cultures (GU185, TAG31 and ZG14) used the rooting conditions of Mokotedi *et al.* (2000). The basic protocol involved placing shoots on root induction medium comprising ¼ MS salts and vitamins, 15 g l^{-1}sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate and 4 g l^{-1} Gelrite supplemented with various concentrations of IBA (0 – 1 mg l^{-1}). Cultures were incubated in the dark for 72 hours (24 – 26 °C), after which they were transferred to a 16-h photoperiod at 66 µmol.m^{-2}.s^{-1} PPFD (both side and overhead lighting) and 27 °C day/ 21 °C night. The entire rooting period, including the 72-h dark incubation, lasted four weeks.

An alteration to this protocol involved subjecting GU185 shoots to various pre-treatments prior to root induction, viz. shoots regenerated from calli were isolated and: 1) placed directly onto a pre-rooting medium (MS salts and vitamins, 15 g l^{-1} sucrose and 4 g l^{-1} Gelrite) for one week and then transferred to root induction medium; 2) first multiplied on semi-solid multiplication medium for four to six weeks, and 'new' shoots placed (singly) onto root induction medium and; 3) first multiplied as in 2) and then as in 1).

2.3.3 Acclimatization of regenerated plants

In a preliminary study, rooted shoots of GU185 produced by indirect organogenesis were acclimatized in sterile, moistened sand in a seedling tray (63 x 34 cm), enclosed in a large plastic bag (120 x 100 cm) and maintained in the greenhouse. In subsequent studies, shoots were acclimatized in pots (5.5 cm diameter) containing a rooting mix (2 perlite: 1 coir) and sealed individually in plastic bags (17 x 14 cm). Further, plants were provided with nutrients (1/3 MS salts) and a source of water in a Petri dish. Plants were maintained in the growth room under a 16-h photoperiod at 66 μmol m^{-2} s^{-1} PPFD and 27 °C day/21 °C night. In all studies, the humidity of the microclimate was gradually reduced by punching holes in bags after two to three weeks and by the fourth week; the plastic bags were completely removed and acclimatized plants were transferred to ambient conditions. This method was also tested on other *E. grandis* clones (TAG31 and ZG14).

2.4 Data analysis and photography

Means were determined from an average of two to three replicates with a sample size ranging from 50 – 115 explants. Data were analysed using a One Way Analysis of Variance (ANOVA) and means were contrasted using Scheffe's multiple range test (95% confidence interval), Kruskal Wallis, Mann-Whitney U or T-Tests where appropriate. Alphabetical values were assigned to the mean values recorded per treatment. Mean values that did not share the same letter were recognised as being significantly different. Photographs were recorded with a Nikon FM2 camera fitted with a 60 mm Mikro macro lens.

3. RESULTS

3.1 STRATEGIES FOR THE PRODUCTION OF DECONTAMINATED EXPLANTS

3.1.1 Decontamination of parent material

A substantial problem often encountered in plant tissue culture laboratories is the successful disinfection of plant material taken from the field or greenhouse (Danby *et al.*, 1994; Tanprasert and Reed, 1998). In the present study, explants (2 – 2.5 cm long shoots) excised from inserts maintained in the greenhouse were decontaminated and screened on microbial M 523 medium (Viss *et al.*, 1991) for 24 h to detect contamination. Thereafter, decontaminated explants were placed onto bud break followed by multiplication media in order to generate a supply of *in vitro* shoots for subsequent studies. In a preliminary study, the standard decontamination protocol that is routinely used in our laboratory for decontamination of *Eucalyptus* explants was applied to GU177, GU178 and GU185 inserts (Table 3.1). There was no significant difference in the percentage of explants contaminated by fungi at the bud break or multiplication stages (Table 3.1) for the clones tested. There was however, a significant difference in the percentage of explants contaminated by bacteria at the bud break stage (Table 3.1), as GU178 explants yielded a significantly lower percentage of explants contaminated by bacteria (6.7%) than GU177 (46.5%) and GU185 (61.7%) at this stage. However, all exhibited high levels of fungal contamination. Therefore, regardless of the specific type of contamination, the overall effect was that the proliferation of contamination made it impossible to generate an adequate supply of *in vitro* shoots for further work, hence this protocol was deemed ineffective to produce decontaminated shoots.

Table 3.1: Effect of initial decontamination method on percentage bacterial and fungal contamination, death, bud break and the number of shoots/shoot explant using GU177, GU178 and GU185 shoots. The initial decontamination method comprised two-minute dips in 0.2 g l^{-1} $HgCl_2$ followed by 10 g l^{-1} $Ca(OCl)_2$ with sterile water rinses between sterilants. Following decontamination, explants were sequentially placed on microbial M 523, bud break and multiplication media for 24 h, 10 – 14 d and 28 d, respectively. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 45 – 90).

Clone	Bud break stage				Multiplication stage		
	% bacteria	% fungi	% death	% bud break	% bacteria	% fungi	no. shoots/shoot explant
GU177	46.5[a]	34.9[a]	0[a]	27.3[a]	41.2[a]	58.8[a]	1[a]
GU178	6.7[b]	42.2[a]	0[a]	44.4[a]	20.8[a]	70.8[a]	1[a]
GU185	61.7[a]	44.7[a]	0[a]	0[b]	-	-	-

3.1.2 Production of decontaminated shoots of GU185

In order to obtain decontaminated material for the development of the indirect organogenesis protocol, alternative strategies for the production of *in vitro* shoots needed to be investigated. As GU185 material was readily available, an investigation was performed in order to obtain decontaminated material of this clone. The strategies employed for this clone do not represent a progression of work, but simply outline the attempts made to generate clean material.

Since the initial decontamination protocol (Table 3.1) was unsuccessful in producing a sufficient amount of material, a more stringent protocol was tested. This second method involved immersing shoots in a fungicide cocktail comprising 1 g l^{-1} Benlate, 1 g l^{-1} boric acid, 0.5 ml l^{-1} Bravo and two drops of Tween 20 for 10, 20 or 30 minutes followed by two-minute dips in 0.2 g l^{-1} $HgCl_2$ and 10 g l^{-1} $Ca(OCl)_2$ as in Table 3.1. Immersion in the fungicide cocktail reduced fungal contamination levels from 70.8% (Table 3.1) to 10 – 20% (Table 3.2). There was no significant difference in the percentage of explants contaminated by bacteria, fungi or percentage explant death for the immersion times tested (Table 3.2). However, there appeared to be a trend of increasingly greater levels of

bacterial contamination (15 – 62%) and death (5 – 20%) the longer explants were exposed to the fungicides (Table 3.2, protocols B – D). This result suggested a possible phytotoxic effect of the fungicides used, that has been reported by other workers (Shields *et al.*, 1984; Watt *et al.*, 1996).

From the results obtained (Table 3.2), it was concluded that immersion of explants for 20 – 30 minutes in the fungicide cocktail was detrimental to explant health (even though there was no significant difference in percentage contamination and death for the various immersion times), therefore protocols C and D were deemed inappropriate to produce decontaminated shoots. For these reasons, immersion of explants for 10 minutes in fungicides (1g l^{-1} Benlate, 1 g l^{-1} boric acid and 0.5 ml l^{-1} Bravo) was selected as the best treatment for decontamination of explants. Beck *et al.* (1998) also found that successful decontamination of *Acacia mearnsii* coppice material could be achieved by soaking explants for 10 minutes in a solution containing 2 g l^{-1} Benlate and 1 g l^{-1} boric acid.

Table 3.2: Effect of immersion time in the fungicide cocktail step of the decontamination protocol on percentage bacterial and fungal contamination and the number of shoots/shoot explant of GU185 inserts. The fungicide cocktail contained 1 g l^{-1} Benlate, 1 g l^{-1} boric acid, 0.5 ml l^{-1} Bravo and two drops of Tween 20. Other steps in the decontamination protocol as in Table 3.1. Following decontamination, explants were sequentially placed on microbial M 523 and multiplication media for 24 h and 28 d, respectively. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 20 – 50).
Protocol A: control - shoots not decontaminated;
B: shoots immersed for 10 minutes in fungicide cocktail;
C: shoots immersed for 20 minutes in fungicide cocktail; and
D: shoots immersed for 30 minutes in fungicide cocktail.

Protocol	Fungicides (min)	$HgCl_2$ (0.2 g l^{-1})	$Ca(OCl)_2$ (10 g l^{-1})	% bacteria	% fungi	% dead	no. shoots/ shoot explant
A	-	-	-	72^{a}	28^{a}	-	-
B	10	+	+	15^{b}	20^{a}	5^{a}	2.2^{a}
C	20	+	+	30^{ab}	10^{a}	15^{a}	1.9^{ab}
D	30	+	+	62^{ab}	12^{a}	20^{a}	2.4^{ab}

As discussed above, when levels of fungal contamination were controlled, bacteria proliferated (Table 3.2). Therefore it was necessary to devise a strategy that would reduce these levels of bacterial contamination. Towards this end, two strategies were tested, *viz.* explants were sprayed with 70% (v/v) ethanol prior to harvesting and then subjected to the initial decontamination protocol as in Table 3.1 (Table 3.3, protocol E). In addition, shoots were decontaminated using the initial protocol and then incubated sequentially on media containing the antibiotics kanamycin (100 $\mu g\ ml^{-1}$) and rifampicin (20 $\mu g\ ml^{-1}$) for two days each with a recovery period for plants by a two-day incubation on antibiotic-free medium after exposure to each antibiotic treatment (Table 3.3, protocol F). Protocol E (spraying plants with 70% v/v ethanol) still produced levels of bacterial contamination that were similar to those of protocols B and C (Table 3.2). In addition, fungal contamination of explants (32%) still proved to be a problem (Table 3.3). A similar difficulty was encountered by Moutia and Dookun (1999), who found that fungal contamination in sugarcane plants proliferated when 70% ethanol was used for disinfection. The antibiotics kanamycin and rifampicin reduced, but did not eliminate bacterial contamination. A number of authors have suggested that a combination of antibiotics were more effective at reducing bacterial contamination than the use of antibiotics in isolation (Young *et al.*, 1984; Reed *et al.*, 1998). There was no significant difference in percentage contamination and death of explants for protocols E and F (Table 3.3). Further, the level of bacterial contamination encountered using protocol F (22%) was slightly higher than that of protocol B (Table 3.2) (15%). This clearly did not justify the use of the antibiotic treatment for decontamination purposes, even though the phytotoxicity of this treatment was minimal as only 2% of explants died (Table 3.3).

Table 3.3: Effect of 70% (v/v) ethanol or antibiotics (kanamycin and rifampicin) on percentage bacterial and fungal contamination, death and number of shoots/shoot explant of GU185 inserts. Other steps in the decontamination protocol as in Table 3.1. Following decontamination, explants were sequentially placed on microbial M 523 and multiplication media for 24 h and 28 d, respectively. Means with the same letter are not significantly different (Mann-Whitney U Test, $p \geq 0.05$, $n = 50$).

Protocol E: spray plants with 70% (v/v) ethanol before harvesting; and

F: incubation of shoots sequentially on media containing antibiotics kanamycin (100 $\mu g\ ml^{-1}$) and rifampicin (20 $\mu g\ ml^{-1}$) for two days each with a recovery period for plants by a two-day incubation on ¼ MS, 0.5 $g\ l^{-1}$ sucrose and 4 $g\ l^{-1}$ Gelrite after each antibiotic treatment.

Protocol	Fungicides (min)	$HgCl_2$ (0.2 $g\ l^{-1}$)	$Ca(OCl)_2$ (10 $g\ l^{-1}$)	% bacteria	% fungi	% dead	no. shoots/ shoot explant
E	-	+	+	32^a	32^a	0^a	2^a
F	-	+	+	22^a	32^a	2^a	1.8^a

Often a single disinfection step is insufficient in controlling both fungal and bacterial contamination (de Fossard and de Fossard, 1988). In the present study, when fungal contamination was controlled by the use of fungicides, bacterial contamination proliferated (Table 3.2) and when bacterial contamination was controlled by the use of antibiotics, fungal contamination persisted (Table 3.3). Clearly, a strategy needed to be devised that would ideally control both types of contamination simultaneously. Therefore, combinations of previously attempted treatments were tested (Table 3.4). There was no significant difference in the percentage of explants contaminated by fungi (20 – 22.9%) and percentage explant death (0 – 11.4%) for the protocols tested (Table 3.4). However, there was a significant difference in the percentage of explants contaminated by bacteria, with protocol H (Table 3.4) yielding a significantly higher percentage of explants contaminated by bacteria (60%) than protocols G and I (10.6 and 11.4% respectively). Therefore, protocol H was deemed inappropriate to produce decontaminated explants. Protocols G and I (Table 3.4) yielded comparable levels of contamination and only half the number of shoots/shoot explant than protocol B (Table 3.2). For these reasons, protocol B (10-minute immersion in fungicide cocktail

followed by standard decontamination protocol) was selected as the best decontamination method.

Table 3.4: Effect of combinations of previously attempted treatments on percentage bacterial and fungal contamination, death and number of shoots/shoot explant of GU185 inserts. Other steps in the decontamination protocol as in Table 3.1. Following decontamination, explants were sequentially placed on microbial M 523 and multiplication media for 24 h and 28 d, respectively. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, $n = 35$).

Protocol G: spray plants with 70% (v/v) ethanol before harvesting, followed by 10-minute immersion in fungicide cocktail during decontamination protocol;

H: 10-minute immersion of plants in fungicide cocktail during decontamination protocol and thereafter incubation on antibiotic-containing media (see F above); and

I: spray plants with 70% (v/v) ethanol before harvesting and thereafter incubation on antibiotic-containing media (see F above).

Protocol	Fungicides (min)	$HgCl_2$ (0.2 g l^{-1})	$Ca(OCl)_2$ (10 g l^{-1})	% bacteria	% fungi	% dead	no. shoots/ shoot explant
G	10	+	+	10.6^a	22.9^a	2.9^a	1.2^a
H	10	+	+	60^b	20^a	0^a	1^a
I	-	+	+	11.4^a	22.9^a	11.4^a	1^a

A protocol that successfully reduced contamination levels of explants was, therefore, not achieved. As stated by Reed and Tanprasert (1995), contamination will continue to be a problem in plant tissue culture. In this study, other combinations of treatments could have been tested, but it was decided that the amount of contamination achieved using protocol B was acceptable for the purpose of this study, which was to produce a supply of *in vitro* shoots for subsequent indirect organogenesis work. Therefore protocol B was used for the decontamination of shoot explants to yield *in vitro* shoots.

3.2 ESTABLISHMENT OF AN INDIRECT ORGANOGENESIS PROTOCOL: WORK WITH GU185

3.2.1 Callus induction and shoot initiation

Preliminary work done in our laboratory by another worker (Gaffoor, 2002) established the types and levels of plant growth regulators required for indirect organogenesis. In the present study, clonal *in vitro* shoots generated by axillary bud multiplication of explants obtained from greenhouse-established inserts, were carefully fragmented to ensure that all axillary buds were destroyed. The two types of plant growth regulator combinations that were initially tested were 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP, and 5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin. Further, the effect of two types of culture vessels (Petri dishes and culture tubes) on callus induction and shoot proliferation was tested. There were five fragmented shoots/ Petri dish and one fragmented shoot/tube. Cultures were incubated in the dark for two subcultures of five weeks each, as did Laine and David (1994) and Cid *et al.* (1999).

For both media and vessel types, all explants produced callus (Table 3.5). However, callus production was not extensive, as only small amounts of calli were produced. In an attempt to increase callus proliferation, calli that were produced after the first five weeks were subcultured onto fresh callus induction media for a further five weeks, but this proved unsuccessful. The amount of calli produced is critical, as this factor is assumed to have a direct effect on the subsequent yield of regenerated shoots.

Even though the amount of callus produced was relatively small, there was a significant difference in the percentage of callus with shoots for media with different plant growth regulator combinations (Table 3.5). Callus induction medium supplemented with 5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin produced a significantly lower percentage of callus with shoots (14.6 – 51.8%) (Figure 3.1A) than medium with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP (78.9 – 100%) (Figure 3.1B). In addition, the former medium also produced roots (Figure 3.1A), which were undesirable, while the latter did not (Figure 3.1B). For these reasons,

medium supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP was selected as the best medium for callus induction and shoot organogenesis. Explants incubated in Petri dishes produced a significantly lower percentage of callus with shoots (51.8 – 78.9%) than explants incubated in culture tubes (14.6 – 100%) (Figure 3.1C). However, it was decided to use Petri dishes for all subsequent work, as they are easier to handle than culture tubes. Also, although Petri dishes are expensive as they are consumable items, less media is used to culture explants (in the present study, there was one fragmented shoot per 20 ml medium in tubes, and five fragmented shoots per 40 ml medium in Petri dishes). Further, tubes need to be washed at the end of each culture stage whereas Petri dishes can simply be discarded. A commercial laboratory such as Mondi Forests will have to do a cost analysis in order to determine the expenses associated with the use of each type of container to select the most cost effective option.

Table 3.5: Effect of media composition and vessel type on callus and shoot production using 3-month-old parent plants. Two media types tested were 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP and 5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin, and the two vessels were Petri dishes and culture tubes. There were 5 fragmented shoots/Petri dish and 1 fragmented shoot/tube. Results after 2 subcultures of 5 weeks each. a – d = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 20 – 50).

Vessel	Media	% explants with callus	% callus with roots	% callus with shoots
Petri dish	IAA:BAP	100	0^{a}	78.9^{a}
	NAA:kin	100	23^{b}	51.8^{b}
Tubes	IAA:BAP	100	0^{a}	100^{c}
	NAA:kin	100	34.7^{b}	14.6^{d}

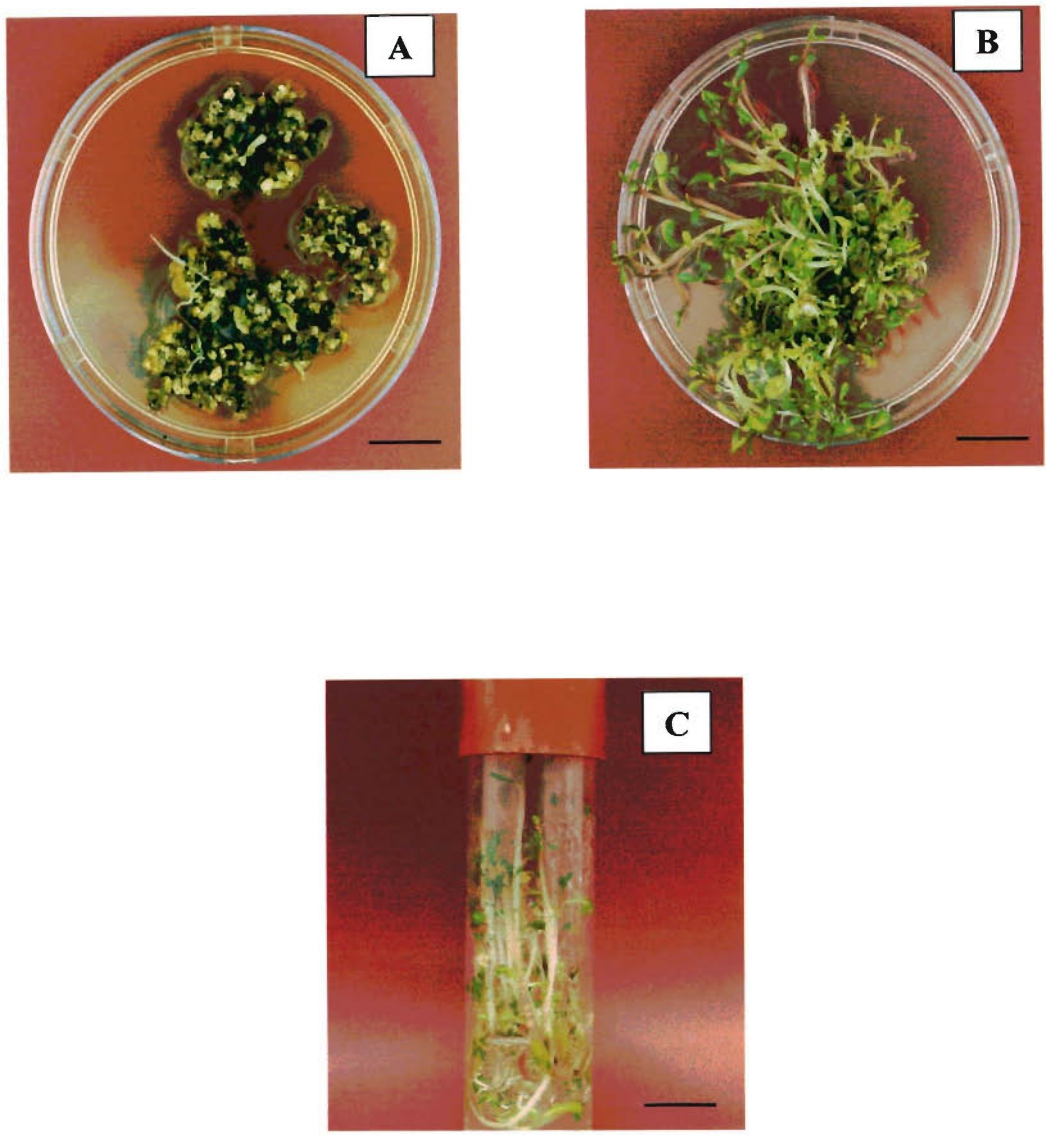

Figure 3.1: Indirect organogenic shoots produced from calli. A) shoots and roots produced in a Petri dish on medium containing 5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin, bar = 0.9 cm; B) shoots produced in a Petri dish on medium containing 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP, bar = 0.9 cm; C) shoots produced in a culture tube on medium containing 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP, bar = 1.03 cm.

3.2.2 Choice of explant

The establishment of conditions for callus and shoot organogenesis were discussed in the previous section. The next step was to refine the protocol by identifying the best explant for callus and shoot organogenesis. The first factor that was considered was the age of the parent plant that was used to generate *in vitro* shoots. Shoots from 3 and 5-month-old parent plants (i.e. 3 and 5 months from the time at which the cuttings were set) that were kept in the greenhouse were decontaminated and multiplied by axillary bud proliferation to generate a supply of *in vitro* shoots, to be fragmented and used as explants for callus induction. The results in Table 3.6 indicated that although callus production (100%) was not affected by the age of the parent plant used to produce *in vitro* shoots, subsequent shoot regeneration was affected. *In vitro* shoots obtained from 3-month-old parent plants produced a significantly higher percentage of callus with shoots (78.9%) than *in vitro* shoots from 5-month-old parent plants (46.8%). Further, *in vitro* shoots obtained from 3-month-old parent plants produced a significantly greater number of shoots per dish (33 shoots/dish) than *in vitro* shoots from 5-month-old plants (18 shoots/dish). From these results, it can be concluded that *in vitro* shoots obtained by axillary bud multiplication of 3-month-old parent plants provided a better source of explants for callus and shoot organogenesis than *in vitro* shoots obtained from 5-month-old parent plants.

Table 3.6: Effect of age of parent plant that yield *in vitro* shoots (3 and 5 months) on callus and shoot production. Results after 2 subcultures of 5 weeks each on callus induction media supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. There were 5 fragmented *in vitro* shoots/Petri dish. Means with different letters are significantly different (Mann-Whitney U Test, $p \leq 0.05$, $n = 50$).

Age of parent plant (months)	% explants with callus	% callus with shoots	no. shoots/ dish
3	100	78.9[a]	33[a]
5	100	46.8[b]	18[b]

Although the results obtained conclusively indicated that *in vitro* shoots obtained from 3-month-old parent plants provided the best material for callus and shoot organogenesis, the

only material that was available at this time was 5-month-old inserts. Hence due to such practical constraints, 5-month-old inserts had to be used for all subsequent work.

Plant material can be multiplied *in vitro* using a number of micropropagation systems. Two such systems are the semi-solid multiplication and the temporary immersion bioreactor (RITA) that utilises liquid media. In the present study, explants from inserts were multiplied by axillary bud proliferation on semi-solid medium to generate decontaminated *in vitro* shoots. In order to ensure a constant supply of *in vitro* shoots for callus and shoot organogenesis, these *in vitro* shoots were multiplied in either the semi-solid or the RITA system. *In vitro* shoots from these two systems were used to induce callus and shoot organogenesis. The results obtained (Table 3.7) indicated that there was a significant difference in the percentage of callus with shoots using explants from the two systems. Explants multiplied in the semi-solid system produced a significantly higher percentage of callus with shoots (46.8%) and number of shoots/dish (18 shoots/dish) than explants multiplied in RITA bioreactors (7.9% and 9 shoots/dish respectively). This result suggested that shoots multiplied on semi-solid medium provided the best explants for callus and shoot production.

Table 3.7: Effect of system to generate *in vitro* shoots used as explants for callus and shoot production. Results after 2 subcultures of 5 weeks each on callus induction media supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. There were 5 fragmented *in vitro* shoots/Petri dish. The two systems used to generate explants were semi-solid multiplication medium and liquid multiplication medium in RITA bioreactors. Means with different letters are significantly different (Mann-Whitney U Test, $p \leq 0.05$, $n = 50 - 60$).

System to generate explants	% explants with callus	% callus with shoots	no. shoots/ dish
Semi-solid	100	46.8[a]	18[a]
RITA	100	7.9[b]	9[b]

* Data for semi-solid as in Table 3.6.

Physiological differences exist between shoots produced in RITA bioreactors and those produced on semi-solid medium due to the different environments created by these two systems (Debergh *et al.*, 1992; Teisson and Alvard, 1995). As a result, shoots produced in RITA bioreactors are often longer than those produced from semi-solid media (Etienne and Berthouly, 2002; Murch *et al.*, 2004). This phenomenon was observed in the present study. It was hypothesised that differences in the elongation status of shoots produced by these two systems could have accounted for the results in Table 3.7, i.e. that the shorter shoots produced in the semi-solid system provided better explants for callus and shoot production than the comparatively longer shoots produced in the RITA system. It was therefore decided to test the effect of the elongation status of shoots used as explants for callus and shoot production. Since it was impossible to generate *in vitro* shoots of the same size in the two systems, the effect of internode distance was investigated as an indicator of the elongation status of shoots produced by both systems.

Although all explants produced 100% callus regardless of the elongation status of the *in vitro* shoots used as explants for callus induction (Table 3.8), there was a significant difference in the percentage of callus with shoots (Table 3.8). In the semi-solid system, shoots with the shortest internodes (0.3 cm) produced a significantly higher percentage of callus with shoots (46.8%) than those with the longer internode distance of 1.7 cm (15.4%). The former also produced a significantly higher number of shoots/dish (18 shoots/dish) than the latter (8 shoots/dish). With the RITA system, because of high and fast rates of multiplication, it was very difficult to separate shoots smaller than 2 cm. For this reason, longer shoots than those in semi-solid medium were used (2 and 5 cm). In this case, there was no significant difference in the percentage of callus with shoots (7.9 and 3.5%) and the number of shoots produced/dish (9 and 3 shoots/dish) for explants with short (2 cm) or long (5 cm) internodes (Table 3.8). The results for shoots multiplied in RITA vessels were not significantly different to the 1.7 cm shoots produced in the semi-solid system. This suggested that elongated shoots do not provide suitable explants for callus and shoot production. Even though there was no significant difference in the percentage of callus with shoots and the number of shoots/dish in the RITA system, the

absolute numbers indicated that the shoots with the shorter internode distance produced more calli with shoots and shoots/dish than shoots with the longer internode distance.

Table 3.8: Effect of elongation status (internode distance) of *in vitro* shoots used as explants for callus and shoot production. Results after 2 subcultures of 5 weeks each on callus induction media supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. There were 5 fragmented *in vitro* shoots/Petri dish. Different internodal distances were generated by incubating shoots on semi-solid multiplication or elongation medium and in RITA bioreactors for 2 or 3 weeks. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 45 – 50).

System to generate explants	Dist. bet. internodes (cm)	% explants with callus	% callus with shoots	no. shoots/ dish
Semi-solid	0.3	100	46.8[a]	18[a]
	1.7	100	15.4[b]	8[b]
RITA	2	100	7.9[b]	9[b]
	5	100	3.5[b]	3[b]

* Data for 0.3 cm semi-solid and 2 week RITA as in Table 3.7.

A number of workers have noticed that rejuvenation of plant material is often induced during micropropagation (Boulay, 1987; Raghava Swamy *et al.*, 1992) and that *in vitro* material is often more responsive to culture manipulations. Therefore it was decided to test the effect of shoots produced by axillary bud multiplication (1st generation shoots) and shoots produced by indirect organogenesis (2nd generation shoots) as explants for callus and shoot production. *In vitro* shoots produced in this manner were subjected to one round of multiplication in either the semi-solid or RITA system and were then fragmented and used as explants for callus and shoot production (Table 3.9). The results in Table 3.9 showed that when the semi-solid system was used to generate explants, there was no significant difference in the percentage of callus with shoots (46.8 and 26.4%) and number of shoots/dish (18 shoots/dish) using 1st and 2nd generation shoots respectively. However, when the RITA system was used to generate explants, there was a significant difference in the percentage of callus with shoots (7.9 and 4.8%) and the

number of shoots/dish (9 and 4 shoots/dish) for 1st and 2nd generation shoots respectively, as 1st generation shoots consistently produced significantly more indirect organogenic callus and shoots than 2nd generation shoots (Table 3.9). The higher yields obtained when explants from the semi-solid system were used may be explained by the smaller size of the internodal distance of these shoots compared to those obtained from RITA vessels, as was found in Table 3.8. The general trend that can be observed from Table 3.9 was that explants obtained from 1st generation shoots produced more callus with shoots and a greater number of shoots/dish than 2nd generation explants (even though there was no significant difference in the semi-solid system). From this result, it can be suggested that the ability of cells to de-differentiate has been reduced in the 2nd generation shoots but the potential of cells to produce 'organs' is less affected.

Table 3.9: Effect of 1st and 2nd generation shoots used as explants for callus and shoot production. Results after 2 subcultures of 5 weeks each on callus induction media supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. There were 5 fragmented *in vitro* shoots/Petri dish. The two systems used to generate explants were the semi-solid and RITA systems. The 1st generation *in vitro* shoots were produced via axillary bud multiplication from 5-month-old inserts and 2nd generation shoots from indirect organogenesis. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 45 – 50).

System to generate explants	Generation	% explants with callus	% callus with shoots	no. shoots/ dish
Semi-solid	1	100	46.8[a]	18[a]
	2	100	26.4[a]	18[a]
RITA	1	100	7.9[b]	9[b]
	2	100	4.8[c]	4[c]

* Data for 1st generation semi-solid and RITA as in Table 3.7.

3.2.3 Root production

Root production on shoots obtained by indirect organogenesis was first attempted by placing shoots on medium comprising ½ MS salts and vitamins, 15 g l^{-1} sucrose and 4 g l^{-1} Gelrite for four weeks. However, only 21% of shoots produced roots (results not shown). Consequently, all subsequent work used the rooting conditions of Mokotedi *et al.* (2000), *viz.* shoots were placed in culture tubes containing ¼ MS salts and vitamins, 15 g l^{-1} sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 4 g l^{-1} Gelrite and various concentrations of IBA. Cultures were then placed in the dark for 72 h followed by transfer to a high light intensity growth room (16-h photoperiod at 66 µmol m^{-2} s^{-1} PPFD 27 °C day/21 °C night); the entire culture period lasted four weeks. The results presented in Table 3.10 indicated that there was a significant difference in the percentage of rooting for the various IBA concentrations. Shoots placed on media with 0.1 mg l^{-1} IBA produced the significantly highest percentage rooting (53.2%). There was no significant difference in the number of roots produced/shoot. However, shoots incubated on media containing 0.1 mgl^{-1} IBA produced a higher number of roots per shoot (2.3 roots/shoot) than the other treatments (0 – 1 roots/shoot). For these reasons, 0.1 mgl^{-1} IBA was selected as the best concentration of IBA for root production.

Table 3.10: Effect of IBA (0.01 and 0.1 mg l^{-1}) on root production in shoots obtained via indirect organogenesis. Medium components were ¼ MS salts and vitamins, 15 g l^{-1} sucrose, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 4 g l^{-1} Gelrite and 0 – 0.1 mg l^{-1} IBA. Shoots were rooted under 66 µmol m^{-2} s^{-1} PPFD 27 °C day/21 °C night following an initial 72 h dark incubation period. Results after 4 weeks in culture. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30).

IBA (mg l^{-1})	% rooting	no. roots/shoot
0	0^a	0^a
0.01	13.2^a	1^a
0.1	53.2^b	2.3^a

Efforts were made to increase levels of root induction obtained by subjecting shoots to specific pre-treatments prior to placing them on root induction medium. Rooting conditions were the same as those described for Table 3.10. Shoots subjected to pre-treatment 3 (Table 3.11) produced a significantly higher percentage of rooted shoots (26.7 – 90%) than those in pre-treatments 1 (13.3 – 53.3%) and 2 (13.3 – 46.7%). Therefore, pre-treatment 3 i.e. multiplying the shoots produced via indirect organogenesis, and then placing new shoots on medium without plant growth regulators for one week, before transferring them to rooting medium containing 0 – 1 mg l^{-1} IBA was selected as the best pre-treatment for root production. Within pre-treatment 3, there was a significant difference in the percentage of rooting and the number of roots/shoot for the different IBA concentrations, as 0.5 and 1 mg l^{-1} IBA produced the significantly highest percentage rooting (83.3 – 90%) and number of roots/shoot (5.5 – 13.4) than other tested concentrations (Table 3.11) (Figure 3.2). In addition, shoots incubated on 0.5 mg l^{-1} IBA produced the significantly longest roots (3.29 cm) as well as roots of the desired morphology i.e. long, thick roots with a few well developed lateral roots. For these reasons, 0.5 mg l^{-1} IBA was selected as the best concentration of IBA for root induction in shoots produced by indirect organogenesis.

Table 3.11: Effect of rooting pre-treatments (1 – 3) and IBA (0 - 1 mg l^{-1}) on root production in shoots obtained via indirect organogenesis. Results after 4 weeks in culture. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 15 - 30).

Shoots regenerated from calli were isolated and subjected to the following pre-treatments:

1: placed directly onto MS medium (MS salts and vitamins, 15 g l^{-1} sucrose and 4 g l^{-1} Gelrite) for 1 week and then transferred to rooting media;

2: first multiplied on semi-solid multiplication medium for 4 - 6 weeks and new shoots placed singly onto rooting media; and

3: first multiplied as in 2 and then as in 1.

Pre-treatment	IBA (mg l^{-1})	% rooting	no. roots/shoot	root length (cm)
1	0	0	0	0
	0.01	13.3[a]	1.5[a]	1[a]
	0.1	40[a]	1.5[a]	1[a]
	0.5	53.3[a]	1.5[a]	1[a]
	0.75	NT	NT	NT
	1	NT	NT	NT
2	0	13.3[a]	1.5[a]	3.17[b]
	0.01	20[a]	1[a]	1.31[a]
	0.1	46.7[a]	2.7[a]	1.84[a]
	0.5	46.7[a]	5[b]	1.43[a]
	0.75	NT	NT	NT
	1	NT	NT	NT
3	0	26.7[a]	1.3[a]	2.7[a]
	0.01	33.3[a]	1.6[a]	2.34[a]
	0.1	66.7[a]	3.4[a]	2.52[a]
	0.5	83.3[b]	5.5[b]	3.29[b]
	0.75	90[b]	13.4[c]	2.12[a]
	1	86.7[b]	11.2[c]	1.31[a]

NT: Not tested

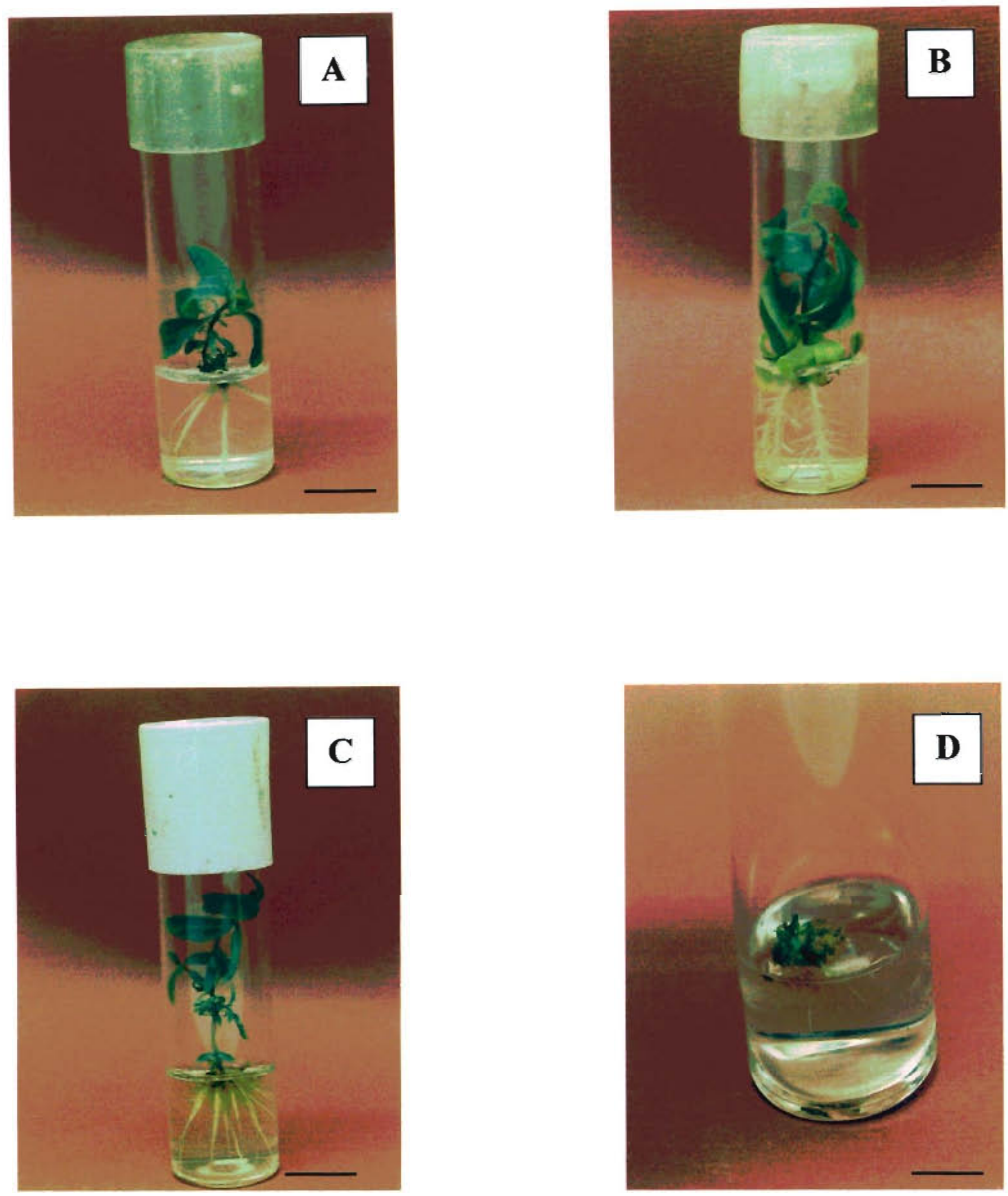

Figure 3.2: *In vitro* root induction of *E. grandis* x *E. urophylla* indirect organogenic shoots at different IBA concentrations. A) 0.1 mg l^{-1} IBA, bar = 1.03 cm; B) 0.5 mg l^{-1} IBA, bar = 1.03 cm; C) 0.75 mg l^{-1} IBA, bar = 1.03 cm; and D) 1 mg l^{-1} IBA, bar = 2.3 cm.

3.2.4 Acclimatization of regenerated plants

In a preliminary study, rooted shoots were planted in autoclaved sand in a seedling tray and enclosed in a large plastic bag. However, this resulted in 100% death of plants (results not shown). Subsequently, attempts were made to acclimatize plants individually in plastic pots (5.5cm diameter) containing either autoclaved sand or a rooting mix (2 perlite: 1 coir). Once planted in pots, plants were sealed individually in plastic bags and the humidity of the microclimate was gradually reduced by punching holes in the bags after two weeks, and by the fourth week the plastic bags were completely removed. From Table 3.12, it can be seen that there was a significant difference in percentage survival for the three treatments. Shoots that were acclimatized in the rooting mix with added nutrients produced the significantly highest percentage survival (95%). This treatment produced healthy acclimatized plants (Figure 3.3) and was therefore used for further work. Bolar *et al.* (1998) obtained similar levels of survival (70 – 100%) when they used a similar protocol for acclimatization of micropropagated apple shoots.

Table 3.12: Effect of different substrates and the presence of a nutrient source on acclimatization success. The two substrates tested were autoclaved sand and 2 perlite: 1 coir rooting mix. The nutrient source provided was 1/3 MS salts. Survival rates after 28 d of acclimatization. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 20 - 30).

Substrate	Nutrients	% survival
Autoclaved sand	-	64^{a}
Rooting mix	-	76.7^{b}
Rooting mix	+	95^{c}

Figure 3.3: Hardened-off plant of *E. grandis* x *E. urophylla* produced by indirect organogenesis, bar = 0.5 cm

3.3 COMPARISON OF DEVELOPED PROTOCOL WITH A PROTOCOL FROM PUBLISHED LITERATURE

The protocol developed was compared with a protocol published by Bandyopadhyay *et al.* (1999) that reported a plant regeneration method using seedling explants of *Eucalyptus nitens* and *Eucalyptus globulus*. The different types of media used in the developed protocol and that of Bandyopadhyay *et al.* (1999) were tested. Both protocols were tested using GU185 and TAG31 shoots multiplied on semi-solid medium. The protocol of Bandyopadhyay *et al.* (1999) produced a significantly lower percentage of explants with callus (80 – 90%) than the developed protocol (100%) for both clones tested (Table 3.13). Further, the former resulted in a significantly lower percentage of callus with shoots (3.4 – 4.9%) than the developed (Hajari) protocol (11.7 – 46.8%). However, with respect to the number of shoots/dish, only GU185 produced the significantly highest number of shoots/dish (18 shoots/dish) while, TAG31 shoots subjected to the Hajari protocol produced a similar number of shoots/dish (7 shoots/dish) to GU185 and TAG31 shoots subjected to the protocol of Bandyopadhyay *et al.* (1999) (6 and 3 shoots/dish for GU185 and TAG31 respectively). This suggested that for the *E. grandis* x *E. urophylla* hybrid (GU185) and for the pure *E. grandis* clone (TAG31), the protocol of Bandyopadhyay *et al.* (1999) was not suitable [even though there was no significant difference in the number of shoots produced per dish for TAG31 shoots subjected to the Hajari protocol and GU185 and TAG31 shoots subjected to the protocol of Bandyopadhyay *et al.* (1999)]. The differences may be attributed to Bandyopadhyay *et al.* (1999) having worked with temperate eucalypt species, whereas the clones tested in this study are semi-tropical. Further, the protocol of Bandyopadhyay *et al.* (1999) was developed using seedling explants, whereas the developed protocol used *in vitro* shoots.

Table 3.13: A comparison of the developed protocol with that of Bandyopadhyay *et al.* (1999) for callus and shoot production. Work done using GU185 and TAG31 shoots obtained from semi-solid multiplication medium. Results after 2 subcultures of 5 weeks each for the developed protocol and 3 - 4 weeks each for the protocol of Bandyopadhyay *et al.* (1999). There were 5 fragmented *in vitro* shoots/Petri dish. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 50 - 60).

Media composition:

- Hajari: MS salts and vitamins, 30 g l^{-1} sucrose, 4 g l^{-1} Gelrite, 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP; and
- Bandyopadhyay *et al.* (1999): MS salts and vitamins, 30 g l^{-1} sucrose, 4 g l^{-1} Gelrite, 1 mg l^{-1} NAA and 0.5 mg l^{-1} BAP.

Protocol	Clone	% explants with callus	% callus with shoots	no. shoots/ dish
Hajari	GU185	100[a]	46.8[a]	18[a]
	TAG31	100[a]	11.7[b]	7[b]
Bandyopadhyay *et al.* (1999)	GU185	80[b]	4.9[c]	6[b]
	TAG31	90[b]	3.4[c]	3[b]

* Data for Hajari GU185 as in Table 3.6.

3.4 STUDIES WITH OTHER CLONES

3.4.1 Testing the developed protocol on other clones

The developed protocol was tested for its applicability to other eucalypts using the hybrids GU180 and GU177 and the pure species TAG31 and ZG14. Explants from all clones tested produced 100% callus (results not shown). The lowest percentage of callus with shoots was produced by GU177 (5.3%) followed by TAG31 (11.7%) and GU180 (12.7%) with ZG14 and GU185 shoots (original experimental data) producing a significantly higher yield (31.7 and 46.8% respectively). A similar trend was observed with the number of shoots/dish i.e. 6 shoots/dish by GU177, 7 shoots/dish by TAG31 and 9 shoots/dish by GU180. However, in this instance, ZG14 produced more shoots/dish (24 shoots/dish) than GU185 (18 shoots/dish), although this difference was not significant (Table 3.14). These results suggested that the different clones were exhibiting different genotype-dependent responses to the same treatment. Laine and David (1994) also reported a genotypic effect in the regeneration of plants from leaf explants of micropropagated clonal *E. grandis*.

GU180 and GU177 shoots were not tested at the rooting and acclimatization stages as severe losses due to contamination were experienced in earlier stages and consequently there was insufficient material available for subsequent stages. For the clones tested, there was no significant difference in the percentage of rooting (83.3 – 92.5%) (Table 3.14), although TAG31 produced the highest percentage rooting (92.5%). Further, there was no significant difference in percentage survival (90.7 - 95%). The results obtained in Table 3.14 indicated that the indirect organogenesis protocol developed for the *E. grandis* x *E. urophylla* hybrid (GU185) could be applied to other *E. grandis* x *E. urophylla* hybrids (GU180 and GU177) although these clones produced a significantly lower percentage of callus with shoots and number of shoots/dish than GU185 (Table 3.14). Further, the developed protocol could also be successfully applied to *E. grandis* clones (TAG31 and ZG14). It is possible to estimate that using the developed protocol, if 100

initial explants are used, yields of 114 acclimatized TAG31, 404 acclimatized ZG14 and 286 acclimatized GU185 plants could be produced.

Table 3.14: Responses of GU180, GU177, TAG31 and ZG14 to the protocol developed for GU185. Callus induction media was supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. There were 5 fragmented *in vitro* shoots/Petri dish. Data for percentage rooting are presented using 0.5 mg l^{-1} IBA and percentage survival using 1/3 MS nutrients. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 50 - 60).

Clone	% callus with shoots	no. shoots/ dish	% rooting	% survival
GU180	12.7[a]	9[a]	NT	NT
GU177	5.3[a]	6[a]	NT	NT
TAG31	11.7[a]	7[a]	92.5[a]	90.7[a]
ZG14	31.7[b]	24[b]	90[a]	91.4[a]
GU185	46.8[b]	18[b]	83.3[a]	95[a]

NT: Not tested.

3.4.2 Studies to improve yield

3.4.2.1 Root production

Although 90 – 92.5% rooting is considered good, attempts were made to increase this percentage by testing higher concentrations of IBA. The rooting protocol reported in section 3.2.3 for the *E. grandis* x *urophylla* hybrid GU185 was tested on pure *E. grandis* species TAG31 and ZG14 to investigate the general application of the protocol. The percentage of indirect organogenic TAG31 shoots that produced roots increased with increasing IBA concentration up to 0.5 mg l^{-1} and thereafter declined at higher concentrations (Table 3.15), however this difference was not significant. There was a significant difference however, in the number of roots/shoot, as shoots placed on media containing 0.5 and 1 mg l^{-1} IBA produced the highest number of roots/shoot (6.2 and 5.6 roots/shoot respectively). It was concluded that the best concentration of IBA for root

production in TAG31 shoots was 0.1 – 0.5 mg l^{-1}, as the highest percentage rooting was obtained at this concentration (even though 0.1 mg l^{-1} IBA yielded significantly fewer roots per shoot than 1 mg l^{-1} IBA). Further, roots of the desired morphology i.e. long, thick roots with a few well developed lateral roots were obtained on media containing 0.1 – 0.5 mg l^{-1} IBA. This is a similar concentration of IBA that was suggested for root production in GU185 shoots (Table 3.11), and as in Table 3.14.

Table 3.15: Effect of IBA (0 - 1 mg l^{-1}) on root production in TAG31 shoots produced by indirect organogenesis. Results after four weeks in culture. Rooting conditions were the same as those described in Table 3.10. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, $n = 40$).

IBA (mg l^{-1})	% rooting	no. roots/shoot	root length (cm)
0	70^a	1.4^a	3.46^a
0.01	85^a	1.7^a	1.57^b
0.1	93.8^a	3.2^a	2.51^a
0.5	92.5^a	6.2^b	1.77^b
0.75	75^a	3^a	1.74^b
1	70^a	5.6^b	1.03^b

With ZG14, there was a significant difference in the percentage of rooting and number roots/shoot for the different IBA concentrations (Table 3.16), as 0.5 and 0.75 mg l^{-1} IBA produced a significantly higher percentage of rooting (90%) and number of roots/shoot (7.2 and 7.5 roots/shoot respectively) than the other tested concentrations. From the results, it can be seen that the best concentration of IBA for root induction of ZG14 shoots was 0.5 and 0.75 mg l^{-1}. Since there was no significant difference in percentage rooting (Table 3.16) and the number of roots/shoot and root length for these two concentrations, the recommended concentration of IBA for root induction of ZG14 was 0.5 mg l^{-1}.

Table 3.16: Effect of IBA (0 - 0.75 mg l^{-1}) on root production in ZG14 shoots produced by indirect organogenesis. Results after four weeks in culture. Rooting conditions were the same as those described in Table 3.10. a – b = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30).

IBA (mg l^{-1})	% rooting	no. roots/shoot	root length (cm)
0	20^a	1.2^a	2.16^a
0.1	33.2^a	1.5^a	4.18^b
0.5	90^b	7.2^b	2.13^a
0.75	90^b	7.5^b	1.87^a

3.4.2.2 Acclimatization of regenerated plants

Similarly to the rooting, an investigation was performed to attempt to further increase percentage survival of explants during the acclimatization process. Towards this end, the rooting mix used for the acclimatization of regenerated plants was overlaid with a 1 – 2 cm layer of river sand and a source of water was provided in Petri dishes for plants during the acclimatization process. The results obtained (Table 3.17) indicated that high survival rates were obtained for both treatments (93.3 – 96.7%), and that the use of river sand was not justified.

Table 3.17: Effect of application of a 1 – 2 cm layer of river sand over the rooting mix on acclimatization of regenerated shoots of TAG31. Results after 28 d of acclimatization. Means with the same letter are not significantly different (Mann-Whitney U Test, $p \geq 0.05$, n = 40 – 60).

River sand	% survival
-	96.7^a
+	93.3^a

3.4.2.3 Choice of explant for callus and shoot production

a) Testing the effect of the system (semi-solid and RITA) to generate explants for callus and shoot production

The effect of the system to generate explants for callus and shoot production was also tested on another *E. grandis* x *E. urophylla* hybrid clone (GU180) as well as a pure *E. grandis* clone (ZG14) to test if the conclusions from that result had a broader application. *In vitro* shoots from both systems induced 100% callus (Table 3.18). For GU180, shoots generated in the RITA system produced a significantly higher percentage of callus with shoots (14.21%) than shoots generated in the semi-solid system (12.7%). However, the number of shoots/dish (9 shoots/dish) was identical for the two systems. Conversely, for ZG14, shoots generated in the semi-solid system produced a significantly higher percentage of callus with shoots (31.7%) than those generated in the RITA system (25.8%). However, shoots generated in the RITA system produced a significantly greater number of shoots/dish (38 shoots/dish) than those from the semi-solid system (24 shoots/dish). For GU185 (Table 3.18), shoots generated in the semi-solid system consistently produced a significantly higher percentage of callus with shoots (46.8%) and number of shoots/dish (18 shoots/dish) than shoots generated in the RITA system (7.9% and 9 shoots/dish respectively). These results indicated that in contrast to clone GU185, for clone GU180 of the same hybrid both the semi-solid and RITA systems provided suitable explants for callus and shoot organogenesis. *E. grandis* (ZG14) responded similarly to GU180.

Table 3.18: Effect of system to generate explants for callus and shoot production using GU180, ZG14 and GU185 shoots. Results after 2 subcultures of 5 weeks each on callus induction media supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. There were 5 fragmented *in vitro* shoots/Petri dish. The two systems used to generate explants were the semi-solid and RITA systems. a – d = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 30 – 60).

Clone	System to generate explants	% explants with callus	% callus with shoots	no. shoots/ dish
GU180	Semi-solid	100	12.7^{a}	9^{a}
	RITA	100	14.2^{b}	9^{a}
ZG14	Semi-solid	100	31.7^{c}	24^{c}
	RITA	100	25.8^{d}	38^{d}
GU185	Semi-solid	100	46.8^{c}	18^{ac}
	RITA	100	7.9^{a}	9^{a}

* Data for GU185, GU180 and ZG14 semi-solid as in Table 3.14 and GU185 RITA as in Table 3.7.

b) Testing the effect of the elongation status of shoots (with different internodal distances) as explants for callus and shoot production

An important factor to consider for callus and shoot regeneration is the morphology of the plated explants used. As discussed previously (section 3.2.2), work with GU185 indicated that explants with a short internode (0.3 cm) produced more callus with shoots and indirect organogenic shoots/dish than shoots with longer internodes (1.7 cm). Similarly, this effect was tested with other clones available *viz.* GU177 and TAG31. However, only semi-solid medium was used and two internode distances were tested. This study considered the effect of using shoots with different degrees of elongation (internode distances) as explants for callus and shoot organogenesis. Type I (internode distance of 0.3 – 0.5 cm) and Type II (internode distance of 1.5 – 1.7 cm).

There was no significant difference in the percentage of callus with shoots (5.3 and 4.6%) and number of shoots/dish (6 and 4 shoots/dish) between Type I and Type II explants of GU177 (Table 3.19). For TAG31, Type II explants produced a higher (but not significant) percentage of callus with shoots than Type I explants (15.7 vs. 11.7%), and a significantly greater number of shoots/dish (17 shoots/dish vs. 7 shoots/dish). For GU185, Type I explants consistently produced a significantly higher percentage of callus with shoots (46.8%) and number of shoots/dish (18 shoots/dish) than Type II explants (15.4% and 8 shoots/dish). The results suggested that for GU177, Type I explants produced a higher percentage of callus with shoots and number of shoots/dish than Type II explants although this difference was not significant (Table 3.19). The same conclusion was reached for GU185 (Table 3.8). For TAG31, the reverse was true.

Table 3.19: Effect of elongation status (internode distance) of *in vitro* shoots used as explants for callus and shoot production using GU177, TAG31 and GU185 shoots. Results after 2 subcultures of 5 weeks each on callus induction media supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. There were 5 fragmented *in vitro* shoots/Petri dish. Different internodal distances were generated by incubating shoots on semi-solid multiplication or elongation medium for 4 – 6 weeks. Explant type: Type I = 0.3 – 0.5 cm internodal distance and Type II = 1.5 – 1.7 cm internodal distance. a – c = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 50 – 60).

Clone	Explant type	% explants with callus	% callus with shoots	no. shoots/ dish
GU177	Type I	100	5.3^{a}	6^{a}
	Type II	100	4.6^{a}	4^{a}
TAG31	Type I	100	11.7^{a}	7^{a}
	Type II	100	15.7^{a}	17^{b}
GU185	Type I	100	46.8^{b}	18^{b}
	Type II	100	15.4^{a}	8^{c}

* Data for explant Type I GU177, TAG31 and GU185 as in Table 3.14.

c) Testing the effect of hyperhydric shoots as explants for callus and shoot production

The use of liquid media for micropropagation often results in the production of hyperhydric shoots (Ziv, 1991; Sandal *et al.*, 2001). These shoots are usually discarded. An attempt was made to determine if such shoots could be 'salvaged' and used for callus and shoot organogenesis (Table 3.20). For both clones tested (GU185 and TAG31), 100% of explants produced callus (results not shown). For GU185, non-hyperhydric shoots produced a significantly higher percentage of callus with shoots (46.8%) and number of shoots/dish (18 shoots/dish) than hyperhydric shoots (4.1% and 4 shoots/dish respectively). Conversely, for TAG31, hyperhydric shoots produced the significantly highest percentage of callus with shoots (15.8 vs. 11.7%) and number of shoots/dish (21 vs. 7 shoots/dish). This result indicated that hyperhydric shoots are capable of producing calli and shoots through indirect organogenesis. In addition, these results implied varying sensitivities of the different clones to the physiological state of the explant used for callus and shoot organogenesis and further that hyperhydric shoots of *E. grandis* clones need not be discarded as they have potential use as a source of explants for indirect organogenesis.

Table 3.20: Effect of hyperhydric shoots as explants for callus and shoot production for GU185 and TAG31 shoots. Results after 2 subcultures of 5 weeks each on callus induction media supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP. There were 5 fragmented *in vitro* shoots/Petri dish. a – d = mean separation within columns, Scheffe's multiple range test ($p \leq 0.05$, n = 50 – 115).

Clone	Physiological state of explant	% callus with shoots	no. shoots/ dish
GU185	Hyperhydric	4.1^{a}	4^{a}
	Non-hyperhydric	46.8^{b}	18^{b}
TAG31	Hyperhydric	15.8^{c}	21^{c}
	Non-hyperhydric	11.7^{d}	7^{d}

* Data for non-hyperhydric explants as in Table 3.14.

4. DISCUSSION

4.1 The need for an indirect organogenesis protocol for *Eucalyptus* species and hybrids

A protocol for the regeneration of plants via indirect organogenesis is of perceived value to the forestry industry for: 1) the potentially high multiplication rates that may be achieved (Sha Valli Khan *et al.*, 2002); 2) the production of new genotypes through somaclonal variation and mutagenesis (Karp, 1995) and; 3) most importantly, the application of genetic engineering technology to yield transgenic trees with altered genotypes (Altman, 2003). However, the commercial planting of transgenic *Eucalyptus* trees will not become a reality overnight, as much research and field testing remains to be completed before such trees can be deployed for operational use. If the underpinning methods, such as the development of indirect organogenesis protocols that would allow for the regeneration of transformed *Eucalyptus* cells, could be established, this would ensure that when other aspects of the technology such as gene transfer methods, have been adequately researched, it can be rapidly applied to existing clonal programmes.

The ideal situation would be to establish a protocol for indirect organogenesis using clonal material as these genotypes have been selected and identified as possessing desirable traits (Schween and Schwenkel, 2002) with predictable growth characteristics. This is in contrast to populations of plants raised from seed whose genotypes cannot be predicted with any consistency due to heterozygosity (Tibok *et al.*, 1995). Only a small amount of literature is available that reports on indirect organogenesis protocols in eucalypts, and most of that work has been done using explants derived from seeds (section 1.4.3.2).

In the present study, the first attempt to establish an indirect organogenesis protocol for clones of *Eucalyptus*, involved testing the protocol of Bandyopadhyay *et al.* (1999) for seedling material. The results for the *E. grandis* x *E. urophylla* hybrid (GU185) and a pure *E. grandis* clone (TAG31) presented in Table 3.13 indicated that callus induction

was successful as 80 – 90% of explants produced callus. However, a low percentage of induced callus regenerated shoots (3.4 – 4.9%). Further, the number of shoots produced per dish was also low (3 – 6 shoots/dish), which was deemed to be an inadequate yield. This highlighted the need for the establishment of a high-yielding indirect organogenesis protocol specific for non-seedling material and for the genotypes of interest to the South African forestry industry.

4.2 Establishment of protocol with one clone (GU185)

A number of parameters are known to influence callus induction and shoot regeneration such as the type and ratio of plant growth regulators (Gamborg and Shyluk, 1981; Tao *et al.*, 2002; Faisal and Anis, 2003), explant type (Evans *et* al., 1981; Rani *et al.*, 2003), genotype (Schween and Schwenkel, 2003), environmental factors such as light (Yepes and Aldwinckle, 1994; Sha Valli Khan *et al.*, 2002), type of culture vessel (Choi *et al.*, 2001) and the gaseous environment therein (Ammirato, 1986), chemical factors such as gelling agents (Tanimoto and Ishioka, 1991; Wilson and James, 2003) and carbon source (El-Bakry, 2002). In the present study, only the following parameters were considered: the effect of different culture vessels (tubes and Petri dishes), plant growth regulator combinations (5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP vs. 5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin), age of parent plants (3 and 5 months) and the system to generate *in vitro* shoots (semi-solid and RITA systems) (Tables 3.5, 3.6 and 3.7).

For all parameters tested, 100% of explants produced callus. Therefore, the strategy employed in this study to fragment *in vitro* shoots for use as explants for callus induction (Das *et al.*, 2002) proved to be a good one. Further, the careful destruction of the axillary buds, as well as discarding premature shoots after the first subculture, ensured that shoot regeneration occurred from callus cells only. When different culture vessels were used, even though explants incubated in Petri dishes produced a lower percentage of callus with shoots (51.8 – 78.9%) than those incubated in tubes (14.6 – 100%), the former were selected to be used for subsequent work as they were technically easier to handle than tubes, and seemed to produce more consistent results. The observed difference in the

percentage of callus with shoots for the two vessel types may be attributed to the different microenvironments within the vessels (McClelland and Smith, 1990). Further, the two vessels used in this study had different types of closures, (culture tubes were capped while the Petri dishes were sealed with Parafilm) due to the need to prevent microbial contamination and desiccation of plant tissues and the culture medium (Jackson *et al.*, 1991; Buddendorf-Joosten and Woltering, 1994). The two vessel types used also had different volumes of gases present. This, together with the different types of closures, has effects on the ventilation of cultures and subsequent composition of the gaseous environment (Zobayed *et al.*, 1999). According to those workers, the environment of the headspace within a culture vessel had a strong effect on the growth and development of cauliflower callus cultures; in a tightly sealed system, high levels of ethylene and CO_2 accumulated which inhibited callus and shoot growth. Jackson *et al.* (1991) found that in vessels that were loosely sealed, very little ethylene accumulated. Similarly, Choi *et al.* (2001) found that shoot regeneration in persimmon was significantly influenced by the type of culture vessel, as shoot regeneration levels were higher in the comparatively larger Erlenmeyer flasks than in Petri dishes.

In the present study, it appears that when explants were incubated in Petri dishes that were sealed with Parafilm, the tightly sealed vessel allowed for the accumulation of gases in the headspace that were detrimental to the growth and morphogenesis of induced callus. Similarly, presumably better ventilation was afforded in culture tubes that were not sealed with Parafilm (Table 3.5). The increased vessel volume of tubes relative to the amount of cultured material may also have had an impact on callus growth and morphogenesis. These factors could collectively account for the levels of regeneration of indirect organogenic shoots observed in tubes. Nevertheless, for the reasons mentioned above, Petri dishes were found to be suitable for the present study.

It has been well established that auxins and cytokinins are the most important plant growth regulators in inducing morphogenesis (e.g. Horgan 1987; George, 1993). The commonly used auxins include indoleacetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 4-chlorophenoxyacetic acid (CPA),

napthaleneacetic acid (NAA), indole-3-butyric acid (IBA) and 4-amino-3,5,6-trichloropicolonic acid (Picloram or TCP) (Collins *et al.*, 1978; Zaerr and Mapes, 1982; Hughes, 1981). Auxins are usually required for the induction and maintenance of callus cultures (Zaerr and Mapes, 1982; Minocha, 1987). In some plants, relatively high concentrations of auxins induce regeneration of roots, for example, Zaerr and Mapes (1982) reported that roots were induced from callus when NAA was used. Indeed, NAA and IBA are the auxins commonly used for root induction in plants (Minocha, 1987). This may explain the formation of roots from callus in the present study when NAA was used as the source of auxin (Table 3.5). Similarly, Tao *et al.* (2002) also reported that when NAA was used for callus and shoot organogenesis in *Citrus grandis*, roots were produced from calli.

The commonly used cytokinins are 6-benzylaminopurine (BAP), isopentyladenine (2iP), kinetin, 6-(4-hydroxy-3-methyl-trans-2-butenylamino) purine (zeatin) and thidiazuron (TDZ) (Zaerr and Mapes, 1982; Hughes, 1981; Huetteman and Preece, 1993; Barciszewski *et al.*, 1999). Cytokinins are usually used in combination with auxins for the initiation and maintenance of callus cultures. The ratio of auxins: cytokinins determine whether roots or shoots will be induced *in vitro* (Minocha, 1987).

The present study focused on two combinations of plant growth regulators (IAA and BAP vs. NAA and kinetin) and their levels (5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP vs. 5 mg l^{-1} NAA and 0.5 mg l^{-1} kinetin) because these combinations and ratios were identified as ideal for callus and shoot production using clonal *E. grandis* x *E. urophylla in vitro* shoots by another worker in our laboratory (Gaffoor, 2002). Of these, 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP proved to be the best combination, as it produced the significantly highest percentage of callus with shoots (78.9 – 100%) (Table 3.5).

Factors relating to the nature of the parent plant have been reported to influence the efficiency of shoot regeneration (George and Tripepi, 1994). In the present study, the effect of the age of parent plants that were used to generate a source of *in vitro* shoots for indirect organogenesis was considered in a small preliminary study (Table 3.6). The few

results obtained indicated that *in vitro* shoots from 3-month-old parent plants produced more indirect organogenic shoots (33 shoots/dish) than *in vitro* shoots from 5-month-old parent plants (18 shoots/dish). This finding is in general agreement with other reports that juvenile material is more responsive in culture (Warrag *et al.*, 1991) and in specific cases is capable of regenerating more shoots by indirect organogenesis than older material (Bandyopadhyay *et al.*, 1999; Tao *et al.*, 2002). Unfortunately, such young material was not available for an extended, in depth study, so 5-month-old material that was readily available and proved to be adequate for the purpose of this study (Table 3.6), were used in all subsequent work.

Progress in this study (and in any other that depends on the availability of *in vitro* shoots), required the bulking up of a large number of explants. Shoots can be multiplied *in vitro* using the semi-solid or RITA systems. RITA bioreactors are capable of multiplying *Eucalyptus in vitro* shoots at a much faster rate, and producing greater yields (McAlister *et al.*, 2002) than the semi-solid system. However, it was necessary to determine if *in vitro* shoots produced in the RITA system would provide suitable explants for callus and shoot organogenesis. The results presented in Table 3.7 indicated that *in vitro* shoots multiplied in the semi-solid system yielded significantly more indirect organogenic shoots (18 shoots/dish) than those multiplied in the RITA system (9 shoots/dish). Therefore, although the RITA system offers great advantages in terms of quick and high shoot multiplication rates, at present the best system to generate *in vitro* shoots for use as explants for callus and shoot production is the semi-solid system. Nevertheless, better yields than those obtained in this study are predicted for when a protocol for the specific use of *in vitro* shoots from RITA vessels is optimised. Parameters that could be targeted towards this end include different levels, ratios and types of plant growth regulators (Ziv *et al.*, 2003), immersion regimes, etc. Other studies that tested the elongation status of shoots obtained from semi-solid and RITA systems gave an indication of possible future studies in this regard. This will be discussed further in section 4.3.2.

As previously mentioned in the results section, all calli produced were very small regardless of the system used to generate *in vitro* shoots and all other callus induction

parameters tested. This observation was important as this factor had an effect on the subsequent yield of shoots. A similar result was reported by Watt *et al.* (1991) for somatic embryogenesis in calli of *E. grandis* and by Tibok *et al.* (1995) for indirect organogenesis in calli of *E. urophylla*. In the present study, attempts were made to increase callus proliferation by subculturing the induced callus onto fresh medium but this was unsuccessful, as no size increase was observed. This represents a critical issue in terms of potential plant yields that needs to be resolved.

Once shoots were obtained via indirect organogenesis, the next step was to establish optimal conditions for root induction. When this was initially attempted on medium devoid of plant growth regulators, root induction was low (21%). Blomstedt *et al.* (1991) also reported sporadic rooting of *Eucalyptus regnans* shoots on medium without plant growth regulators. Auxins are commonly used for the induction of roots (Nemeth, 1986; Gaspar and Coumans, 1987; Riov, 1993) and IBA is the most widely used auxin for root induction of *Eucalyptus* shoots produced by axillary bud multiplication. It has been applied as a pulse treatment, where a high concentration is provided for a short period of time (Pelosi *et al.*, 1995; Willyams *et al.*, 1998; Mokotedi *et al.*, 2000; Fett-Neto *et al.*, 2001) and as a chronic treatment, where a lower concentration is provided for the duration of the culture period (Curir *et al.*, 1990; Blomstedt *et al.*, 1991; Bennett *et al.*, 1994; Jones and van Staden, 1994; Yang *et al.*, 1995).

In the present study, increasing concentrations of IBA up to 0.1 mg l^{-1} promoted root induction in regenerated shoots (53.2%) (Table 3.10). Attempts were made to increase observed levels of root induction by subjecting shoots to specific pre-treatments prior to transfer to root induction medium (Table 3.11). In this respect, pre-treatment 3 *viz.* multiplying shoots produced by the developed protocol for indirect organogenesis, and then placing new shoots on medium without plant growth regulators for one week (conditioning), before transferring them to rooting medium, was selected as the best strategy for root induction using the *E. grandis* x *E. urophylla* hybrid GU185, as a high percentage of rooting (up to 90%) as well as roots of superior morphology (long, thick roots with a few well developed lateral roots) were produced. Bennett *et al.* (1994)

reported on the beneficial effects of a 'conditioning' medium prior to root induction in *Eucalyptus globulus*.

When high concentrations of IBA were used (1 mg l^{-1}) to induce rooting, extensive callusing was observed at the base of shoots (Figure 3.2D). Similar results were reported with *E. regnans* (Blomstedt *et al.*, 1991) and *E. grandis* x *E. nitens* (Mokotedi *et al.*, 2000). This is not ideal, as the proliferation of callus at the base of shoots during root induction may interrupt vascular connections between roots and the stem, thereby interfering with the transport of water and nutrients to the shoot (Martin, 1985). Therefore, it was concluded that the best concentration of IBA for root induction of GU185 shoots was 0.5 mg l^{-1}.

In vitro propagation protocols are often limited by poor survival of plantlets when transferred to *ex vitro* conditions (e.g. Rohr *et al.*, 2003). This may be attributed to the fact that plantlets need to adapt to a number of changes during the acclimatization process including those that relate to the control of water loss and autotrophic growth (Marin, 2003). One hundred percent mortality was obtained when plantlets were acclimatized using autoclaved sand with plantlets enclosed in a large plastic bag in a seedling tray. Therefore, in order to minimise possible stressful conditions during acclimatization, plantlets were acclimatized individually in small plastic pots enclosed in plastic bags and the humidity of the microclimate was gradually reduced over a period of four weeks. Further, different substrates for acclimatization (autoclaved sand and 2 perlite: 1 coir rooting mix) were tested, and nutrients ($^1/_3$ MS salts) were provided to attempt to increase survival levels (Table 3.12). These conditions resulted in good survival of regenerated plants (up to 95%). It may be suggested that the relative differences in plant volume: air volume in the different size plastic bags presumably had an effect on acclimatization success, as it is known that the gaseous environment has an effect on the growth and development of plants (Jackson *et al.*, 1991).

In conclusion, the protocol developed for indirect organogenesis in the *E. grandis* x *E. urophylla* hybrid clone GU185 involves fragmenting *in vitro* shoots obtained via axillary

bud multiplication of greenhouse-established inserts and after destruction and removal of buds using a scalpel blade, placing them onto media supplemented with 5 mg l^{-1} IAA and 0.25 mg l^{-1} BAP for two subcultures of five weeks each in the dark. Shoots regenerated from calli are then isolated and rooted on media supplemented with 0.5 mg l^{-1} IBA. Rooted plants were acclimatized individually in small plastic pots containing a rooting mix (2 perlite: 1 coir), provided with nutrients ($^1/_3$ MS salts) and sealed in plastic bags. The humidity of the microclimate was reduced by punching holes in the bags and by the fourth week, the bags were removed.

4.3 Evaluation of strategies developed to overcome practical constraints

A number of practical constraints were encountered during the establishment of the indirect organogenesis protocol. One was the availability of parent plants. The material for this work was supplied by Mondi Forests, which is located at Hilton, Pietermaritzburg. When parent plants were needed, and if they were available, they had to be transported to Durban, which is a 150 km return trip. In order to deal with these constraints, the strategy for the present study was to use greenhouse-established inserts as parent plants, which were used to initiate *in vitro* shoot multiplication cultures. These were used to induce callus, as research in our laboratory (Chetty, 2001), as well as published literature (Yasodha *et al.*, 1997), has suggested that *in vitro* shoot cultures are generally more responsive to culture manipulations than explants initiated directly into culture from *ex vitro* material. This phenomenon has been attributed to a proposed reversion to the juvenile phase (Wilkins, 1991; Raghava Swamy *et al.*, 1992).

4.3.1 Dealing with microbial contamination

A problem often encountered in the use of *in vitro* culture systems is that of contamination (Kunneman and Faaij-Groenen, 1988). Contamination results in the loss of cultures when microorganisms proliferate and overgrow the plant material (Reed *et al.*, 1998) and/or reduced multiplication and rooting rates due to the production of phytotoxic metabolites (Leifert *et al.*, 1989; 1992; Thomas, 2004). Any of these translate into

economic losses (Reed *et al.*, 1995) that can have serious implications in a commercial environment. Contaminants of plant tissue cultures include bacteria, fungi, yeasts, viruses and micro-arthropods such as mites and thrips (Shields *et al.*, 1984; Young *et al.*, 1984; Long and Cassells, 1986; Blake, 1988; Niedz, 1998). It is often difficult to determine the exact source of contamination of plant cultures however some identified sources include ineffective decontamination, poor aseptic technique, normal airborne and human associated microorganisms and those that originate from contaminated stock plants when pre-treatment of such plants with chemicals prove to be ineffective (Gunson and Spencer-Phillips, 1994; Leifert and Waites, 1994; Reed and Tanprasert, 1995; Leifert and Cassells, 2001).

In order to minimise contamination problems, there are two standard practices: 1) maintenance and treatment of parent plants and; 2) surface decontamination of explants prior to culture. The former involves adequate watering and fertilization regimes as well as treatments involving (usually) the application of fungicides (section 2.1) on a regular basis in an attempt to maintain parent plants free of contaminants. In the present study, minimal efforts were made to try to eliminate endogenous contamination in the parent plants. This is because experience gained at the University of KwaZulu-Natal and at Mondi Forests has indicated that young plants and particularly inserts are very sensitive to fungicide treatments. Therefore, in this study, only a mild fungicide treatment was applied to the inserts. However, in view of the results obtained (Tables 3.1, 3.2, 3.3 and 3.4), it is suggested that this strategy should be investigated further.

A range of decontamination methods were therefore tested in order to obtain explants for the development of the indirect organogenesis protocol, since the decontamination method that is routinely used in our laboratory for *Eucalyptus* explants proved to be ineffective, as up to 100% of explants were contaminated at the bud break stage (Table 3.1). Ideally, a decontamination regime is used that is sufficiently toxic to microorganisms but that has a minimal phytotoxic effect on plant material (de Fossard and de Fossard, 1988; Gordon, 1991). The method employed depends on the plant

species, type of explant, phytotoxicity, nature of contaminant and cost (Niedz and Bausher, 2002).

Fungal contamination proved to be a problem, not surprisingly since in our laboratory and at Mondi Forests, fungal contamination of *Eucalyptus* plants has been identified as one of the most important causes of losses during micropropagation (Watt *et al.*, 1996). In an attempt to deal with fungal contamination, explants were immersed in a fungicide cocktail comprising 1g l^{-1} Benlate, 1g l^{-1} boric acid, 0.5 ml l^{-1} Bravo and two drops of Tween 20 for 10 – 30 minutes (Table 3.2). Benlate and Bravo fungicides were used, as they are easily available in South Africa, are widely used in agriculture and forestry and a combination of these fungicides exhibits a broad spectrum of activity (Watt *et al.*, 1996). It has been reported that Benlate acts by interfering with fungal microtubules (Shields *et al.*, 1984) and the active ingredient in Bravo reacts with the thiol groups of cell constituents (Hassall, 1990). Various immersion times of the selected fungicides were tested, as the phytotoxicity of fungicides such as Benlate has been reported (Shields *et al.*, 1984; Watt *et al.*, 1996). The results presented in Table 3.2 indicated that the time of immersion in the fungicide cocktail did have an effect on explant health, as the longer immersion times were detrimental to explants. Therefore, 10-minute immersion of explants in the fungicide cocktail was selected as the best decontamination method for *Eucalyptus* shoots as only 15% of explants were observed to be contaminated by bacteria and 20% were contaminated by fungi using this method (Table 3.2).

Fungal contamination levels were controlled with the use of fungicides (Table 3.2) but in such cases, bacterial contamination proliferated (15 – 62%). The addition of antibiotics to culture media is a widely used strategy to deal with bacterial contamination (Pollock *et al.*, 1983; Falkiner, 1990). There are dangers associated with the repeated use of antibiotics such as the development of resistance by bacteria to the antibiotics used (Falkiner, 1990; Kneifel and Leonhardt, 1992). Therefore, it has been suggested that if antibiotics are to be used, they must be used to a very limited extent and only for a short period. The ideal antibiotic should have a broad antibacterial spectrum and be applied at a concentration that is sufficient to inhibit bacteria but does not harm the plant material

(Falkiner, 1990; Kneifel and Leonhardt, 1992). In the present study, the antibiotics kanamycin (100 μg ml^{-1}) and rifampicin (20 μg ml^{-1}) were used to reduce bacterial contamination (Table 3.3). The antibiotics were applied sequentially for two days each, with a two-day recovery period for plants on antibiotic-free medium between applications. Kanamycin and rifampicin were combined, and as such, they supplied a broad spectrum of activity. Kanamycin is an aminoglycoside antibiotic that inhibits bacterial protein synthesis and translocation by binding to the 70S ribosomal subunit thereby eliciting miscoding. Kanamycin exhibits an antimicrobial spectrum that includes Gram negative and positive bacteria and mycoplasma. Rifampicin inhibits the initiation of bacterial RNA synthesis by binding to the β-subunit of RNA polymerase (Young *et al.*, 1984; Falkiner, 1990; Sigma catalogue, 2003). The treatments with the antibiotics (Table 3.3) reduced but did not eliminate bacterial contamination levels, as 22% of explants were still contaminated by bacteria. Further, fungal contamination still proved to be a problem as 32% of explants were contaminated by fungi. Therefore the use of antibiotics for decontamination of *Eucalyptus* shoots was not justified.

It has been reported that often a single surface decontamination step is insufficient in controlling both fungal and bacterial contamination (de Fossard and de Fossard, 1988; Leifert and Waites, 1994). Therefore, combinations of previously attempted methods for disinfection of explants were tried to reduce observed contamination levels (Table 3.4). The combinations of treatments tested either yielded unacceptably high levels of contamination, for example 60% bacterial contamination and 20% fungal contamination when explants were immersed for 10 minutes in the fungicide cocktail followed by incubation on antibiotic-containing media (Protocol H, Table 3.4) or very low numbers of shoots/shoot explant for example 1 – 1.2 shoots/shoot explant when explants were sprayed with 70% (v/v) ethanol and then immersed for 10 minutes in the fungicide cocktail (Protocol G, Table 3.4) or when explants were sprayed with 70% (v/v) ethanol and then placed on antibiotic-containing media (Protocol I, Table 3.4). Therefore, it was decided that protocol B (10-minute immersion of explants in fungicide cocktail followed by standard decontamination protocol) (Table 3.2) was the best decontamination method. The levels of contamination encountered using this method (15% bacterial contamination

and 20% fungal contamination, Table 3.2) were deemed acceptable for the purposes of this study which was to provide a supply of *in vitro* shoots for development of the indirect organogenesis protocol. Similarly, Wilson and James (2003) first optimised *in vitro* shoot proliferation conditions in order to generate a supply of decontaminated *in vitro* apple shoots to be used as explants for regeneration and transformation work.

4.3.2 The potential use of RITA bioreactors

In vitro shoots are conventionally multiplied using semi-solid multiplication medium. This process is laborious and time consuming as it takes time to multiply a suitable amount of shoots that can be used as explants for callus and shoot production. As discussed previously (section 4.2), an alternative to this constraint was thought to be the use of the temporary immersion bioreactor RITA that uses liquid multiplication medium to rapidly multiply *in vitro* shoots (Teisson and Alvard, 1995; see section 2.2 of Materials and Methods).

There are a number of advantages in the use of liquid media for micropropagation including that media can be easily renewed or replaced, media can be sterilised by microfiltration, generally more uniform culture conditions are created with the use of liquid media, much larger containers can be used for culturing, transfer times can be reduced and the cleaning of containers after an incubation period is much easier (Etienne *et al.*, 1999; Etienne and Berthouly, 2002). A number of workers have reported improved multiplication levels using temporary immersion bioreactors rather than conventional semi-solid medium. For example, McAlister *et al.* (2002) reported a four-to-six-fold increase in the yield of *Eucalyptus* clones in half the time using RITA bioreactors. Similar conclusions were obtained by Escalona *et al.* (1999) for pineapple, by Lorenzo *et al.* (1998) for sugarcane and by (Alvard *et al.*, 1993) for banana. Temporary immersion systems have also been used to enhance somatic embryogenesis in cotton (Gawel and Robacker, 1990), rubber (Etienne *et al.*, 1997), tea (Akula *et al.*, 2000) and *Citrus* (Cabasson *et al.*, 1997).

According to Martre *et al.* (2001) and Etienne and Berthouly (2002), the high success rate of temporary immersion systems may be attributed to the fact that these systems allow for contact between all parts of the plant tissue and the nutrient medium and also enables complete renewal of the atmosphere inside the vessel by forced ventilation; this combination of characteristics does not usually occur in other culture systems. Another factor that contributes to the success achieved with RITA systems is that the desiccation of plant material is prevented by the presence of a capillary film of medium over the explants. Also, the short immersion times ensures that there is minimal disruption of the gas exchanges between the plant and atmosphere (Etienne *et al.*, 1999; Teisson and Alvard, 1995). There are however a few disadvantages associated with the use of temporary immersion systems, such as the phenomenon of hyperhydricity (Paek *et al.*, 2001; Sandal *et al.*, 2001; Etienne and Berthouly, 2002) but opportunities exist to minimise such problems in RITA bioreactors by, for example, manipulating the frequency and duration of immersion times (Etienne and Berthouly, 2002).

It has been reported that liquid medium often results in better growth and multiplication levels than semi-solid medium (Paek *et al.*, 2001). Therefore, it can be suggested that the high multiplication levels in RITA vessels may result in plants that are physiologically different from those multiplied more slowly in the semi-solid system (Teisson and Alvard, 1995). A manifestation of these fast growth rates could be the longer shoots that are routinely produced in RITA vessels (Murch *et al.*, 2004). Other effects may be manifested at the cellular level in terms of ability of cells to de-differentiate to produce callus, morphogenetic potential of cells, etc. For these reasons, it was necessary to investigate some parameters relating to the unique nature of shoots produced in RITA bioreactors to assess the usefulness of these *in vitro* shoots as explants for indirect organogenesis. When the elongation status of shoots multiplied in the semi-solid and RITA systems were investigated as explants for indirect organogenesis (Table 3.8), it was discovered that elongated shoots from either system generally did not provide suitable explants as these shoots produced a lower number of shoots/dish (3 – 8 shoots/dish) than shorter explants (9 – 18 shoots/dish). Further, when 1st generation shoots (produced by axillary bud multiplication of greenhouse-established inserts) and 2nd generation shoots

(produced by indirect organogenesis) were used as explants for callus and shoot production, shoots from the semi-solid system provided better explants for indirect organogenesis than those from the RITA system (Table 3.9) as the former yielded a greater number of shoots/dish (18 shoots/dish) than the latter (4 – 9 shoots/dish).

The use of liquid media for micropropagation facilitates greater nutrient uptake and therefore promotes growth, but the phenomenon of hyperhydricity is common (Paek *et al.*, 2001; Etienne and Berthouly, 2002) in *Eucalyptus* and other species (Whitehouse *et al.*, 2002). Hyperhydric (or vitrified) plants are identified by a glassy, water-soaked appearance and anomalous growth (Ziv, 1991). These plants are characterised by having different patterns of development compared with non-hyperhydric plants, poor survival *ex vitro* as well as reported losses with regard to the regenerative ability of tissue (Gribble, 1999; Gaspar *et al.*, 2002; Franck *et al.*, 2004; Kevers *et al.*, 2004). In the present study, a number of plants obtained from RITA bioreactors were observed to be hyperhydric. These plants are usually discarded. In any laboratory but particularly in a commercial environment, this wastage can translate into significant losses of time and other costs. Therefore, these hyperhydric shoots were used for callus and shoot production to evaluate their use as explants (Table 3.20). Hyperhydric shoots were capable of producing callus and regenerating shoots. For the *E. grandis* x *E. urophylla* hybrid clone GU185, non-hyperhydric shoots produced significantly more indirect organogenic shoots (18 shoots/dish) than hyperhydric shoots (4 shoots/dish). The reverse was true for the pure *E. grandis* clone TAG31. Hence, in a commercial laboratory, hyperhydric shoots need not be discarded and can be used as explants for callus and shoot production.

4.4 Suitability of developed protocol for other clones

In a commercial laboratory, the ideal situation would be for any protocol that is developed to have a broad general application i.e. suitable for a wide variety of clones. However, experience in our laboratory as well as reports in published literature (e.g. Thorpe *et al.*, 1991; Rodriguez and Vendrame, 2003) indicate that this situation is seldom

true. Usually a protocol that is developed using one clone must be adjusted and optimised in order to yield similar or better results in another clone. This optimisation process is costly and time consuming and is not ideal in a commercial environment where propagation is carried out on a large scale and research activities are kept to a minimum. Therefore, a clone-unspecific protocol for indirect organogenesis in *Eucalyptus* species and hybrids is of perceived value to the commercial forestry industry. For these reasons, the indirect organogenesis protocol that was developed for the *E. grandis* x *E. urophylla* hybrid GU185 was tested on other hybrid clones (GU177 and GU180), as well as on pure *E. grandis* clones (TAG31 and ZG14) (Table 3.14). As expected (Laine and David 1994), the results indicated a genotype-dependent response. Nevertheless, in general, the developed protocol up to the shoot development stage, exhibited a broad general applicability to the clones tested with GU177, GU180 and TAG31 producing lower yields of shoots (6, 9 and 7 shoots/dish) than ZG14 and GU185 (24 and 18 shoots/dish).

The system used to generate explants for callus and shoot production (semi-solid and RITA) was also tested using GU180 and ZG14 clones (Table 3.18). For the *E. grandis* x *E. urophylla* hybrid GU180 a similar yield of shoots was produced for both systems (9 shoots/dish). For the pure *E. grandis* clone ZG14, a significantly higher percentage of indirect organogenic calli with shoots was produced using shoots from the semi-solid system, but shoots from the RITA system regenerated a greater number of shoots/dish (38 shoots/dish) than those from the semi-solid system (24 shoots/dish). These results indicated that the pure *E. grandis* clone ZG14 responded in a similar manner to the hybrid clone GU180 i.e. both semi-solid and RITA systems provided suitable explants for callus and shoot organogenesis. This is in contrast to GU185, where shoots from the semi-solid system provided the best explants for callus and shoot production.

When the elongation status of shoots were compared among hybrid clones (GU177 and GU185) and pure species (TAG31) (Table 3.19), it was concluded that for both hybrid clones, Type I explants (0.3 – 0.5 cm internodal distance) provided better explants for callus and shoot production than Type II explants (1.5 – 1.7 cm internodal distance) as the former yielded more indirect organogenic shoots (6 – 18 shoots/dish) than the latter (4

- 8 shoots/dish). Conversely, for TAG31, Type II explants yielded a greater number of shoots/dish (17 shoots/dish) than Type I explants (7 shoots/dish). This result indicated that for TAG31, the longer shoots yielded more indirect organogenic shoots than the shorter shoots, in contrast to the situation in the hybrid clones tested.

Both the *E. grandis* x *E. urophylla* hybrid GU185 and the pure *E. grandis* species TAG31 and ZG14 illustrated good rooting responses (83.3 – 92.5%) and survival after acclimatization (90.7 – 95%). With respect to the root induction stage, a similar concentration of IBA (0.5 mg l^{-1}) is suggested for the production of a high percentage of rooting (≥90%), with roots of the desired morphology for the hybrid clone GU185 and the pure species TAG31 and ZG14 (Tables 3.15 and 3.16). For the acclimatization stage, similarly high survival (in excess of 90%) was recorded for both the hybrid and pure species (Table 3.14). Attempts were made to increase survival levels of TAG31 and ZG14 by application of a layer of river sand over the rooting mix during the acclimatization process (Table 3.17). High survival percentages were recorded using this treatment, but the extra work associated with the use of the river sand treatment does not seem to justify the use of this method in future work. Hence, the strategy devised for the acclimatization of GU185 regenerated shoots was also considered to be suitable for TAG31 and ZG14.

In conclusion, the protocol developed for the hybrid clone GU185 (Figure 4.1), can be applied to other hybrid clones and pure species, as all clones tested were capable of producing shoots via indirect organogenesis. However, the yield of shoots regenerated was clone-dependent, as has been reported in published literature (Das and Mitra, 1990; Laine and David, 1994; Yasodha *et al.*, 1997).

From the data obtained for all the clones, it is possible to estimate final total yields. If 100 initial explants are used, then 286 acclimatized plants of the *E. grandis* x *E. urophylla* hybrid GU185 can potentially be produced while for the pure *E. grandis* clones, 114 acclimatized TAG31 plants and 404 acclimatized ZG14 plants can be produced. Optimisation of specific areas of the developed protocol can potentially further increase

observed yields. Unfortunately these results could not be compared with those from published protocols, as this information was not provided in the literature.

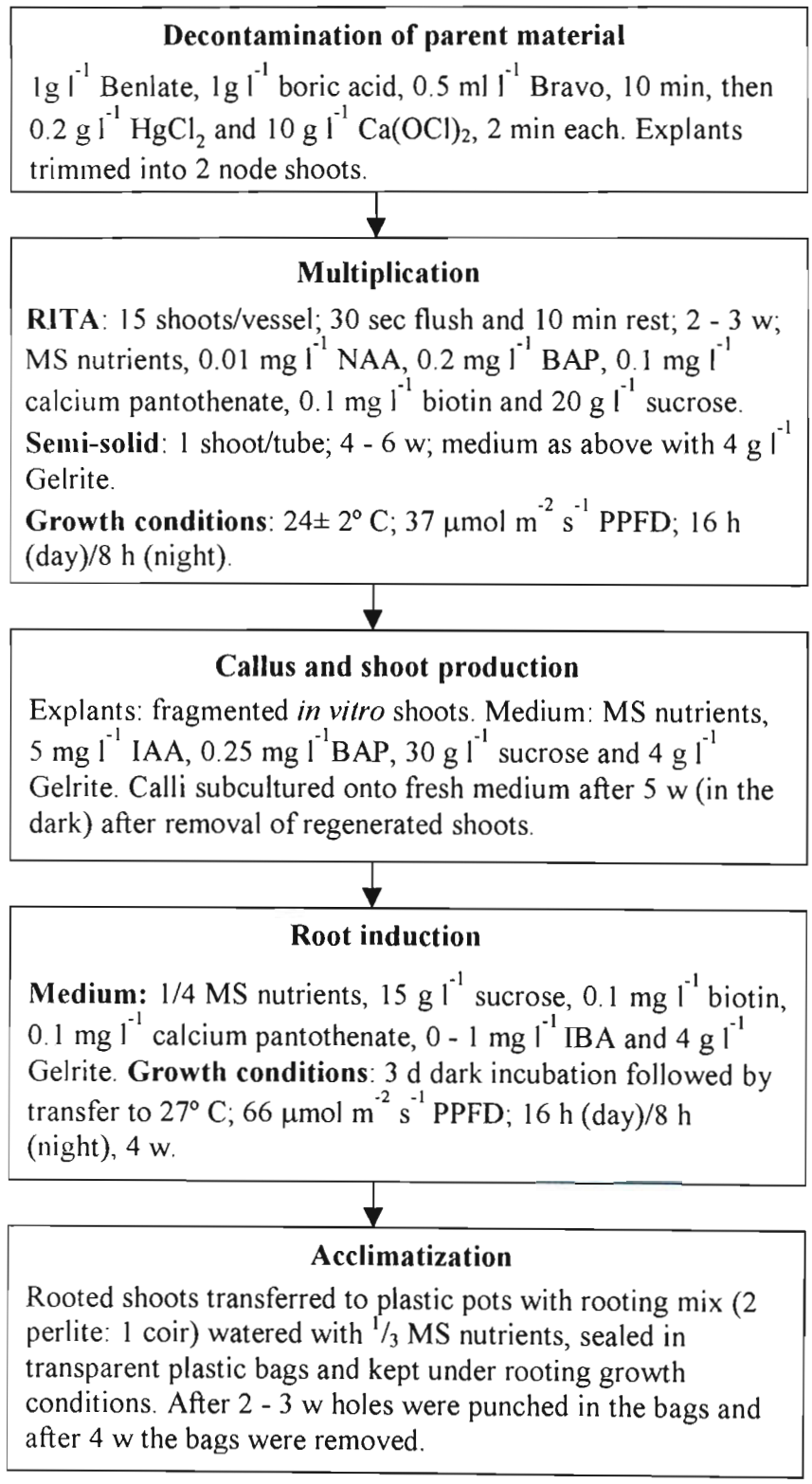

Figure 4.1: Schematic representation of indirect organogenesis protocol developed for *Eucalyptus*.

4.5 Applications of indirect organogenesis

Some of the advantages of the applications of indirect organogenesis in *Eucalyptus* species and hybrids have been mentioned in previous sections. One such advantage relates to the use of callus cultures to generate a source of variation needed in breeding programmes through the use of the phenomenon of somaclonal variation (Karp, 1995). Another advantage relates to the potentially high multiplication rates that are possible with the use of callus cultures. However, with the presently available protocol for indirect organogenesis, the high potential yield has not yet been achieved; with GU185, for example, plantlet production via multiplication of axillary buds (direct organogenesis) was 2.4 shoots/initial shoot explant and the indirect organogenesis method yielded 3.6 – 6.6 shoots/initial shoot explant. Even though the indirect method yielded more shoots than the direct method, even greater yields can potentially be obtained with callus cultures.

By far the greatest application of indirect organogenesis relates to the use of genetic engineering technology, hence the need for a protocol for indirect morphogenesis. Genetic engineering technology is of value to the forestry industry as this technology allows for the insertion of specific genes into selected elite trees. This is useful for the continued production of new genotypes of interest by making use of genes not in the gene pool of the species or hybrids. Obvious practical application is the planting of such improved trees on marginal sites. A few reports have been published on the regeneration and transformation of some *Eucalyptus* species and hybrids. Methods of transformation that have been reported include particle bombardment or biolistics of *E. grandis* x *E. urophylla* (Sartoretto *et al.*, 2002) and *E. grandis* (Walter *et al.*, 1995), *Agrobacterium tumefaciens* mediated transformation of *E. camaldulensis* (Mullins *et al.*, 1997; Ho *et al.*, 1998; Chen *et al.*, 2001) and *E. grandis* x *E. urophylla* (Tournier *et al.*, 2003) and sonication-assisted *Agrobacterium* mediated transformation of *E. grandis* x *E. urophylla* (Gonzalez *et al.*, 2002). There appears to be very few reports in the scientific literature on successful cases and field trials; it is possible that this work is being done 'in-house' and therefore remains confidential.

Specific areas of application of genetic engineering technology to clonal eucalypt programmes include the production of herbicide-tolerant genotypes that will enable transgenic trees to be resistant to specific herbicides. This would allow for the use of environmentally friendly herbicides (Peacock, 1995). Another avenue for investigation is the generation of insect-resistant genotypes i.e. genotypes that are resistant to specific insect pest species. Specific gene constructs are available which when expressed provide resistance against specific Coleopteran and Lepidopteran pests. One such example is the biological insecticide produced by *Bacillus thuringiensis* (*Bt*). *B. thuringiensis* is a bacterium that produces an insoluble crystal protein that causes starvation or death of target insects when ingested. An advantage in the use of *Bt* technology is that only target insects are killed. In contrast, conventional methods of spraying chemical insecticides are usually non-specific and could result in the death of a number of beneficial insects (Harcourt *et al.*, 1995).

The use of transgenic trees alleviates some of the problems associated with conventional spraying of herbicides and insecticides, for example high costs of chemicals, coordinating the timing of application with the correct stage of the insect life cycle, favourable weather conditions and inaccessibility of many plantations (Harcourt *et al.*, 1995). Another area where genetic engineering technology is of use to commercial forestry is with respect to the generation of trees tolerant to abiotic stresses such as cold climates, drought, etc.

Of particular interest to commercial eucalypt forestry, is the ability to increase the productivity of trees by altering trees for specific end uses. For example, the modification of lignin biosynthesis pathways to allow for easier and cheaper pulping of *Eucalyptus* trees (Burdon, 2003). In this regard, Chen *et al.* (2001) have reported on the production of the first transgenic *E. camaldulensis* trees carrying the cinnamate 4-hydroxylase gene that is involved in the process of lignin biosynthesis. Once these workers produced transgenic plants, they were vegetatively propagated for field testing by the rooting of cuttings. This provides an example of the integration of new technologies with existing conventional programmes. In this manner, the true value of genetic engineering can be realised, as it

provides a mechanism for improving economically important traits that cannot be modified by conventional methods in a reasonable period (Chen *et al.*, 2001).

The great advantages that can be achieved with the use of genetic engineering technology are evident but there are risks associated with the use of this technology. These include gene flow through the spread of transgenes through pollen diffusion and hybridisation with closely related or wild species, horizontal gene transfer, negative effects on non-target organisms, creation of 'superweeds', ethical issues, etc (Giovannetti, 2003). Strategies have been developed to attempt to minimise these risks. One such strategy is the engineering of sexual sterility in trees (Brunner *et al.*, 1998) by, for example, the production of trees that do not develop floral structures or that produce non-reproductive floral structures (Brunner *et al.*, 1998). Other reports have advocated the creation of refuges to create a buffer effect (Burdon, 2003).

Currently there exists strict regulation regarding the release of genetically modified organisms into the environment. An overview of the current status and regulations regarding the release of genetically modified organisms is presented by Nap *et al.* (2003). The great potential benefits of genetic engineering technology are evident, but the controversy surrounding this issue is unlikely to abate, as public perception of genetically engineered organisms will continue to be a volatile issue. Only after extensive research and field testing should genetically modified organisms be released and even then, it is not possible to predict all the ecological implications. It is apparent, however, that at the present time, genetic engineering technology cannot be disregarded in the face of predicted timber and food shortages and increased population growth.

5. CONCLUDING REMARKS

The protocol developed for indirect organogenesis was detailed in Figure 4.1. As discussed, it was found to be suitable for the clones tested (GU185, GU180, GU177, TAG31 and ZG14), with potential yields of 286 acclimatized GU185 plants, 114 acclimatized TAG31 and 404 acclimatized ZG14 plants, if 100 initial explants are used. A summary of the investigations performed during this study as well as future research opportunities is presented in Table 6.1.

Table 6.1: Summary of success achieved during this study and areas of proposed future research.

Investigation	Result
Production of decontaminated explants	Achieved (section 3.1)
Establishment of indirect organogenesis protocol: work with GU185	
Stages:	
a) Callus induction and shoot initiation	Achieved (section 3.2.1)
- studies relating to choice of explant	Preliminary investigations completed. Optimisation required (section 3.2.2)
b) Root production	Achieved (section 3.2.3)
c) Acclimatization of regenerated plants	Achieved (section 3.2.4)
Studies with other clones	
Testing developed protocol (stage a)	Achieved with GU177, GU180, TAG31 and ZG14 (section 3.4.1)
Stages b and c	Achieved with TAG31 and ZG14 (section 3.4.2)
- studies relating to choice of explant	Preliminary investigations completed. Optimisation required (section 3.4.2.3)
Comparison of developed protocol with a protocol from published literature	Section 3.3.

It must be emphasised that the high potential multiplication levels generally associated with the use of callus cultures was not achieved in this study (section 4.5). One reason for this was the small size of calli produced in all studies. This represents an area that needs to be further investigated as this parameter has an effect on the subsequent regeneration of shoots. Hence, different media and culture conditions need to be researched to attempt to increase callus production, while retaining its morphogenetic capacity.

Another aspect that requires consideration relates to the potential use of RITA bioreactors to provide a method for the rapid multiplication of explants for callus induction. In the present study, inadequate yields of shoots were obtained when *in vitro* shoots from RITA vessels were used as explants for callus and shoot production. If conditions are optimised for the use of such shoots as explants, then there exists the possibility for savings in time, labour and other costs. This is of particular importance in a commercial environment where propagation is carried out on a large scale. Parameters that could be investigated towards this end include different types and levels of plant growth regulators, immersion regimes, etc.

The protocol developed also needs to be tested on a wider range of *Eucalyptus* clones, particularly those genotypes that are cold-tolerant. This is important to determine the general application of the protocol.

In conclusion, the results presented indicate that the developed indirect organogenesis protocol is effective and future research opportunities exist to improve yields of plantlet regeneration further.

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