THE DEVELOPMENT OF CLONE-UNSPECIFIC MICROPROPAGATION PROTOCOLS FOR THREE COMMERCIALLY IMPORTANT *EUCALYPTUS* HYBRIDS

by

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Psalm 16:8-11

" I have set the Lord always before me.

Because He is at my right hand,

I will not be shaken.

Therefore my heart is glad

And my tongue rejoices.

My body also will rest secure

Because You will not abandon me to the grave,

Nor will you let your Holy One see decay.

You have made known to me

The Path of Life;

You will fill me with joy

In Your presence,

With eternal pleasures at Your right hand."

PREFACE

The experimental work described in this thesis was carried out in the School of Life and Environmental Sciences, University of Natal, Durban, from January 1999 to December 2000, under the supervision of Prof. M.P. Watt.

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.

S. Chetty.

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ABSTRACT

Micropropagation methods are often used to supplement existing clonal programmes for *Eucalyptus* species. However, genotypic differences among clones require the implementation of clone-specific protocols, an expensive and labour-intensive exercise. Hence, this study aimed at determining high-yielding hybrid-specific rather than clone-specific, micropropagation protocols for *E. grandis* x *nitens* (GN), *E. grandis* x *nitens* (NH), and *E. grandis* x *urophylla* (GU). Different conditions for surface sterilisation, bud-break (3 protocols, 2 media), multiplication (4 media), elongation (2 protocols) and rooting (4 media) were tested. A single successful surface sterilisation approach was possible for all clones of the tested hybrids (0.0-11.8% contamination, 0.0-22.9% necrosis). It involved rinsing nodal explants in a fungicide mixture (1g/l Benlate, 1g/l boric acid, 0.5ml/l Bravo, Tween 20) for 15 minutes followed by calcium hypochlorite (10g/l with Tween 20) for three minutes. Results at each culture stage were dependent on genotypes, and results indicated here represent ranges in values among the clones of each hybrid. The highest bud-break values for GN clones (87-90%) and NH clones (17-75%) were achieved on a medium containing MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.04mg/l NAA, 0.11mg/l BAP and 0.05mg/l kinetin. In GU clones, bud-break values on this medium (84-97%) were not significantly different to those achieved directly on a multiplication medium (80-91%) (MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.2mg/l BAP, 0.01mg/l NAA). Shoot multiplication yields for GN clones (4-13 shoots/bud) and GU clones (2-6 shoots/bud) were achieved on a medium consisting of MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.2mg/l BAP and 0.01mg/l NAA. As genotypic effects were highly significant among NH clones, a single multiplication medium for all clones of this hybrid could not be determined. The best method of elongation for clones of all three hybrids involved culturing shoots on MS, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, 0.35mg/l NAA, 0.1mg/l kinetin and 0.1mg/l IBA, under photoperiod conditions, rather than total darkness, for 6 weeks. This resulted in 82.3-86.6% elongation and shoot lengths increasing by 22.9-35.2 mm for GN clones, 80.2-82.3 % elongation and an increase in length of 24.7-32.2 mm for NH clones and 70.8-78.1% elongation, and shoot elongation of 21.6-29.3 mm for GU clones from

passage 1-2. For all the above stages, media contained 20/25 g/l sucrose and 3.5g/l Gelrite, and cultures were maintained at 25°C ± 2°C day/ 21°C night with a 16 h light/ 8 h dark photoperiod (PPFD 66μmol/m^2/s). In terms of rooting, cultures on different media were initially subjected to a 72 hour period of total darkness at room temperature, then a 16 h light/8 h dark photoperiod (PPFD 37μmol/m^2/s) at 24°C day/ 21°C night for 7 days. This was followed by a 16 h light/ 8 h dark photoperiod (PPFD 66μmol/m^2/s) at 25°C ± 2°C day/ 21°C night for 21 days. Tested clones of the three hybrids were all rooted successfully (56-93% rooting in GN clones, 36-76% rooting in NH clones and 46-96% rooting in GU clones) on a medium containing ¼ MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.1mg/l IBA, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$, 15g/l sucrose and 3.5g/l Gelrite. Predicted yields from the established protocol are also presented (168-667 plants of *E. grandis* x *nitens* (GN), 35- 854 plants of *E. grandis* x *nitens* (NH) and 54-349 plants of *E. grandis* x *urophylla* from 100 initial nodal explants, depending on the clone). Hence, the established protocols can be used successfully for some of the clones, but the implementation of specific media and methods to obtain high yields may still be necessary for certain clones.

TABLE OF CONTENTS

CHAPTER 3: ESTABLISHMENT OF PROTOCOLS FOR THE MICROPROPAGATION OF *EUCALYPTUS* HYBRIDS

CHAPTER 4: CONCLUDING REMARKS AND FUTURE RESEARCH STRATEGIES

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

%	percent
µ	micro
µmol/m^2/s	micromole per metre squared per second
°C	degrees centigrade
BA/BAP	6-benzylaminopurine
CaOCl	calcium hypochlorite
cm	centimetre
g	gram
g/l	grams per litre
GN	*Eucalyptus grandis* x *nitens*
GU	*Eucalyptus grandis* x *urophylla*
h	hour
ha	hectares
$HgCl_2$	mercuric chloride
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
l	litre
M	molar (mole/l)
m^3	metres cubed
mg	milligrams
mg/l	milligrams per litre
min.	minutes
ml	millilitres
ml/l	millilitres per litre
mm	millimetres
mM	millimolar
MS	Murashige and Skoog (1962) basal nutrients
NAA	1-naphthylene-acetic acid

NH	*Eucalyptus grandis* x *nitens*
NaOCl	sodium hypochlorite
pH	hydrogen ion concentration
PPFD	photosynthetic photon flux density
R	Rands
s	second
sp/spp.	species
Tween 20	polyoxyethylene sorbitan monolaurate

Chapter 1: Introduction

1.1 The history of the forestry industry in South Africa

The forestry industry appears to have had its beginnings in the Cape, during the period of van Riebeeck and the early Cape governors, when they realised that the already heavily exploited indigenous forest would not recover from over-utilisation, and that it would be necessary to import hardier and faster-growing exotic tree species (Luckhoff, 1973). Among the first species to be imported was the common oak, which was extensively planted by Simon van der Stel and later during the seventeenth century, Cluster pine (*Pinus pinaster*) and Stone pine (*Pinus pinea*) were also introduced (Luckhoff, 1973). Legislation was introduced which compelled landowners to plant one hundred trees as well as a further two trees for every one they felled. Further to this, oak transplants were raised in large numbers and issued free for roadside and farm planting. The Newlands plantation, probably the first plantation in South Africa, was planted in 1670, by governor W.A. van der Stel, covering 34 hectares initially, but was later extended by a further 17 hectares (Luckhoff, 1973).

It was in 1876, that the Superintendant of Plantations, J. Storr-Lister, established a plantation of fast-growing eucalypts at Worcester, to provide fuel for railway locomotives. The following year, he began a regime of reclaiming the driftsands at Bellville, by spreading town refuse and sowing seed of *Acacia cyclopis* and *Acacia cyanophylla*. Furthermore, it was around this time that the Tokai and Kluitjieskraal plantations were established and these included stands of *Pinus radiata*, which has since become the major plantation species in the winter rainfall areas of the Cape. During World War I, this plantation provided an invaluable timber resource for South Africa, and about 734 000 cubic feet of *Pinus radiata* was made available due to the "timber famine" of the time (King, 1951; Luckhoff, 1973).

Sir David Hutchins undertook a study of suitable tree species that were likely to succeed in the South African climate, and made the recommendation that Mexican pines would be

ideal. Following this, seed of several species, including *Pinus patula*, were imported and propagated in South Africa (Luckhoff, 1973). The Second World War, which resulted in serious timber shortages, stimulated more liberal provision for State afforestation, including forestry enterprises by the private sector (King, 1951). Afforestation with conifers and with *Eucalyptus grandis* was undertaken in the sub-tropical areas of the then Transvaal lowveld and Zululand, to supply the need for mining timber, and later rayon pulp and saw timber (Luckhoff, 1973).

At the turn of the 19th century, the Department of Forestry, in anticipation of future demands for local timber supplies, began experimenting with exotic pine and eucalypt species selected from all over the world (Immelman, 1973). The depression of the early nineteen-thirties caused the government to establish a number of large pine plantations in the Northern and Eastern Transvaal, Natal and Southern Cape, to provide employment (Immelman, 1973).

Since its establishment, the forestry industry has expanded rapidly in this country and has supplied the local demand for wood products and is said to be one of the fastest growing sectors of the South African economy (Denison and Kietzka, 1993a). Industrial plantations, rather than natural forests or imports, supply South Africa's demand for wood, for several reasons. Natural forests do not provide enough suitable wood to supply the demand by industry for raw materials, and a domestic source of low-cost wood offers more commercial opportunities than the utilisation of natural forest or importing (Anon, 1996).

The forestry industry is dominated by large companies and, until recently, by the state-owned company SAFCOL (Anon, 1996). However, re-structuring of ownership in recent years has seen the privatisation of SAFCOL plantations (332 000 ha) and those owned by the Department of Water Affairs and Forestry (75 000 ha) (Grafton, 1999a). Currently, most of the forestry resources rest in the hands of a few large companies, a source of some concern to the present government (Anon, 1996). Commercial farmers and other private individuals involved in commercial forestry are registered with the South African

Timber Grower's Association (SATGA) and own plantations that were estimated to total to about 308 000 hectares in 1993/1994 (Anon, 1996).

1.2 The impact of the forestry industry on South Africa's economy

The South African timber industry represents a powerful economic force and a major exporter of forest products, making it a significant contributor to the earning of valuable foreign exchange (Cellier, 1993). Furthermore, the forest and forest products industry represent a major employer in South Africa, employing between 80 000 to 100 000 forestry workers in 1996, of whom nearly 80% were in Mpumalanga and Kwazulu-Natal. Further to this, about 120 000 people were employed in industries that utilised wood as a raw material (Anon, 1996). The industry appears to be completely dominated by the pulp and paper sector, which was the main application for round-wood harvested from plantations, as at 1996/1997 (Table 1.1).

Table 1.1 Sales of roundwood harvested from plantations in South Africa, as at 1996/97 to forestry products industries, by volume and value. (Anon, 1998a).

Product	**Sales By Volume** '000 m^3	**Sales by Value** R million
Sawlogs and veneer logs	6.122	415.1
Pulpwood	8.788	1187.2
Mining Timber	2.206	89.1
Poles	704	29.0
Matchwood	17	7.8
Other	804	7.7
Total	18641	1745.9

1.3 Constraints and legislation impacting on the South African forestry industry

According to Schuch (1991), the future demand for forest products may not be met due to the lack of available land on which to grow plantations, and this is certainly true for South Africa. This appears to arise partly through competition between the forestry industry and the crop and livestock farming industry, which both require prime land with good rainfall, climate and soils (Celliers, 1993; Denison and Kietzka, 1993a).

The expansion of the forestry industry is also restricted by the inherent costs associated with the industry (land use, plantation establishment and maintenance during the life of the plantation), as well as the costs of harvesting which includes felling, log removal, debarking, and loading onto trucks (Greaves *et al.*, 1997). Further constraints on the expansion of the forestry industry include the high costs associated with long haulage distances to processing mills and legislation that impacts the forestry industry in various ways, which are discussed further (Denison and Kietzka, 1993a).

The forest policy implemented in 1996 (Anon, 1996) delineated aims for several forestry sectors, including industrial. Some of these aims for industrial forestry in particular, included the fostering of international and local competition, the countering of adverse effects on water resources and biodiversity, equitable, efficient and sustainable allocation of resources through the issuing of permit allocations and catchment management, minimising barriers to the entry of small farmers and entrepreneurs into the industry and addressing all options to increase timber yields and efficiency through research, and innovation, recycling and waste minimisation and development of alternative fibre resources (Anon, 1996).

The ongoing controversy surrounding the adverse effect that forestry has on water resources began in the 1920's (Anon, 1996) and is currently reflected in legislation as the National Water Act, passed in 1998, which classifies forestry as a "stream-flow reduction activity" (Anon, 1998b). As such, foresters were expected to register with the Department of Water Affairs and Forestry, to obtain permits for the use of water. Commercial

foresters were also expected to pay for rainwater used (Anon, 1998b). However, recent amendments have been made to the act to the effect that the proposed charge for water used has been deferred, and will only be implemented in 2041 (Grafton, 1999b). Foresters are currently expected to pay a levy for "water resource management", for services rendered by the Department of Water Affairs and Forestry, a figure in the region of R6.00 per hectare of afforested area per annum (Grafton, 1999b). The forestry industry, however, believes that it is being discriminated against, since these levies are currently only being applied to tree-growers and not to any other dry-land crop growers, and further, that its activities and expansion has already been severely curtailed for the past 27 years by the permit system implemented by the government (Grafton, 1999b).

1.4 The utilisation of commercial forest tree species in South Africa

In 1996/97, South Africa was estimated to have a total plantation area of 1518 138 hectares, which equates to about 1.3% of the total area of the country. Of this area, 797 610 ha comprised various species of softwood, whilst the remainder were made up of hardwood (Anon, 1998a).

Commercial forestry plantations in South Africa are made up primarily of pine, *Eucalyptus* and wattle, with a few farmers planting poplar. According to the recent White Paper on Sustainable Forestry in South Africa, the growth of industrial forests in this country was about 1.45 million hectares in 1994; 56% of this area consisted of pine, 32% eucalypts and 11% wattle. Poplar, grown by small commercial farmers, comprised 2800 hectares of this total and is used for matches (Anon, 1996).

The distribution of forest tree species, which includes hardwood species (e.g. eucalypts) and softwood species, such as pines, in South Africa is represented in Table 1.2 below.

Table 1.2. The provincial distribution of hardwood and softwood tree plantations in South Africa as at 1996/1997 (Anon, 1998a).

	Plantation area (ha)	
Province	**Softwood**	**Hardwood**
Northern	29 068	31 519
Mpumalanga	333 674	292 351
Northwest	0	0
Gauteng	0	0
Free-state	0	108
Kwazulu-Natal	211 030	366 758
Eastern Cape	145 522	24 574
Northern Cape	0	0
Western Cape	78 316	5 218
Total	797 610	720 528

The wattle industry in South Africa is based primarily on the cultivation of the Black Wattle (*Acacia mearnsii*), a species of *Acacia* native to Australia, and introduced to South Africa in 1864 (Dunlop *et al.*, 2000). The lucrative local market dealing in wattle is driven mainly by the high quality vegetable tannin in the bark of *A. mearnsii*, however, *Acacia* also lends itself to several other applications, such as firewood, fodder, poles for fencing and construction, wood for carving and furniture, medicines and gum resins and brushwood for fencing, and high quality fibre for pulp and paper (Barnes, 2000; Roux *et al.*, 2000). Most of 130 000 ha planted during the 1990's, was in Kwazulu-Natal and in the south-eastern parts of Mpumalanga (Roux *et al.*, 2000).

Pine species have been grown in South Africa to provide a cheap local source of sawlogs and, until the construction of the Richard's Bay pulp and liner board mill in 1983, pulpwood (Denison and Quaile, 1987; Denison and Kietzka, 1993b). Several pine species are currently cultivated in different parts of South Africa, *e.g.* Mpumalanga, Northern Province and Kwazulu-Natal, the commercially important species being *Pinus patula*, *P. elliotti*, *P. taeda* and *P. greggii*, for use as pine sawtimber (Anon, 1999).

Mondi Forests, a division of the Mondi Paper Milling Company Ltd. has been committed to tree research and improvement since 1968 (Denison and Quaile, 1987), with the main objective of manipulating the vast genetic variation inherent in the various forest species grown locally, so that improved quality and productivity can be achieved. With the construction of the Mondi Richards Bay Pulp and Liner Board Mill in 1983, the emphasis shifted from pine sawlogs and pulpwood to hardwood fibre which resulted in the implementation of the clonal eucalypt programme in the same year (Denison and Quaile, 1987; Denison and Kietzka, 1993b). The main species used for making pulp at the Richards Bay mill is *Eucalyptus grandis* from clonal plantations (Meadows, 1999).

1.5 The role of *Eucalyptus* in the South African Forestry Industry

The genus *Eucalyptus* (Family Myrtaceae) includes 450-700 species, which can occur in one of three sub-genera: Symphomyrtus, Monocalyptus and Corymbia (Turnbull, 1991). Species in the sub-genus Symphomyrtus usually occur in areas of high soil fertility, whilst species in the other two sub-genera are associated with soils of a lower nutrient status (Mueller-Dombois, 1992). In a short time, many species of *Eucalyptus* can yield abundant biomass, which can be used as sawn timber, mining timber, pulp for paper, poles, firewood, charcoal, essential oils, honey, tannin, shade and shelter (Lakshmi Sita, 1986; Gupta and Mascarenhas, 1987; Turnbull, 1991; Patil and Kuruvinashetti, 1998; Bandyyopadhyay *et al.*, 1999).

Eucalyptus globulus was originally used for paper and pulp in Portugal in 1906, and since then eucalypt pulp production has attained global status (Turnbull, 1991). There are distinct differences in pulp quality and yield between species, such that wood can be tailored to produce pulp with a specific end-use. *Eucalyptus* has found application in the pulp and paper industry due to the shift in emphasis from long to short fibre pulp. Plantation-grown wood also gives uniform material, with high brightness, good opacity and bulk, making the pulp suitable for the production of printing, writing, and tissue papers. Because the majority of eucalypts grow fast, coppice easily and burn well when

air-dried, leaving little ash, they are also widely utilised as fuelwood. Since some *Eucalyptus* species have timber with good colour and appearance, they have a high potential for conversion to high value sawn products (Turnbull, 1991).

Besides the major industrial uses of wood and pulp, eucalypts also have found application in the production of "non-wood products" such as tannin, honey, ectomycorrhizal fungi and essential oils. Essential oils, for example, are obtained by steam distillation of eucalypt leaves and are used in medicine, industrial products and perfumery. Industrial products include disinfectants from phellandrene and synthetic menthol from piperitone. Perfumery oils are based mainly on citronellal extracted from *E. citriodora*. In South Africa, essential oils are extracted primarily from *E. dives, E. radiata* and *E. smithii* (McComb and Bennet, 1986; Gupta and Mascarenhas, 1987; Turnbull, 1991).

In South Africa, the most important and widely planted eucalypt is *E. grandis*, and is estimated to cover approximately 72.6% of the total commercial forestry area planted with eucalypts (van Wyk, 1990; Sneddon *et al.*, 2000). However, several other species are also screened and planted commercially (van Wyk, 1990; Anon, 1999) (Table 1.3). *Eucalyptus grandis* is prominent as a pure species, but more importantly, as a hybrid with other *Eucalyptus* species, especially *E. camaldulensis, E. tereticornis, E. urophylla* and *E. nitens* (Anon, 1999). Hybridisation, the crossing of diverse species, has allowed the manipulation of natural genetic variation to result in increased flexibility of pure species. Further, hybrid vigour increases forest productivity and allows the extension of growing sites to include drier and hotter areas as well as cooler and more frost susceptible locations. In addition, greater disease-resistance can be obtained, wood properties for specific end-products can be improved and rotation age can be reduced (Denison and Kietzka, 1993b; Malan, 1993; Anon, 1999). In South Africa, the most suitable areas have already been planted with the fastest-growing *E. grandis*. Thus, hybrids are required for the remaining marginal areas (Jones and van Staden, 1994). In this country, sub-tropical areas are planted with *E. grandis* x *camaldulensis*, *E. grandis* x *urophylla* and *E. grandis* x *tereticornis,* whilst *E. grandis* x *nitens*, and *E. grandis* x *macarthurii* are grown in the more temperate locations (Denison and Kietzka, 1993b).

Table 1.3 List of *Eucalyptus* species currently being planted and tested in South Africa by Mondi Forests (Anon, 1999).

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

1.6 Traditional propagation of forest tree species

Generally, plants can be propagated via their two developmental life-styles, *viz.* the sexual phase, which involves the fusion of gametes and subsequent production of zygotic embryos contained within seeds, and the asexual phase (George, 1993). Traditionally, forest trees, including *Eucalyptus* species have been regenerated from seedlings derived from bulked seed collected in nature or, more recently, from seeds collected from randomly pollinated superior trees (Ahuja, 1993). In areas of natural forest, where trees are removed by selective felling, seeds are used to regenerate trees. Where clear-felling of trees for wood-chips occurs, some stands of good quality trees are left to re-seed the area that has been cleared (McComb and Bennet, 1986).

In most of the forests arising from propagation via seeds, there is a large variation in growth, form and vigour, which can be attributed to the genetic diversity among offspring and between parents and offspring. This is undesirable where a uniform and selected or engineered stand of superior trees needs to be established (Lakshmi Sita, 1986; Ahuja, 1993). Some important forest tree species are characterised by poor or irregular seed set, *e.g. Eucalyptus dunnii* (Lakshmi Sita, 1986). In addition, in some cases, the seed may be prone to genetic damage or rapid loss of viability (Ahuja, 1993), and some tree species, such as certain species of *Eucalyptus* take a long time to flower, precluding this method

of propagation in a commercial environment, where quick establishment of plants has to occur (Cresswell and de Fossard, 1974; Lakshmi Sita, 1986). For example, Jones *et al* (2000) reported that problems of biennial flower production, or irregular flowering as well as high capsule abortion rates are characteristic of many eucalypts, and limit seed supply. However, seed production still plays a major role in commercial forestry propagation and breeding programmes, in that it forms the basis for maintaining a wide genetic base from which to select and develop superior trees. Selected plus-trees are pollinated and the seed arising from this procedure, is collected and planted out in provenance trials (Watt *et al.*, 1995).

Grafting is another approach to propagating trees that can be used to establish seedling nurseries for several cultivars (McComb and Bennett, 1986). At the Institute for Commercial Forestry Research (ICFR), Pietermaritzburg, grafted plants are used to establish clonal arboreta, which act as 'gene conservation blocks' for further tree improvement research (Gardner, 1995). However, McComb and Bennett (1986) argued that this technique is labour intensive and expensive and that care must be taken to select the appropriate kind of graft and ensure the condition of the stock. Furthermore, observations with *E. nitens* and *E. macarthurii* showed that the size of rootstocks plays an important role in the procedure and those that have not reached a suitable size, can delay grafting for up a month (Gardner, 1995). This author further reported that the problem of rootstock/scion incompatibility in *E. macarthurii*, both in the field and the nursery, highlighted the need to move away from grafting as a means of producing ramets for this species (Gardner, 1996). Air-layering is another method of propagation but the expense associated with this technique, and the high failure rate makes its implementation feasible only in special purpose plantations (Cresswell and de Fossard, 1974; McComb and Bennet, 1986).

To avoid the shortcomings associated with the previously discussed vegetative propagation techniques, propagation via stem cuttings has found application in the commercial propagation of several forestry species (McComb and Bennet, 1986; Zobel, 1993; Maile and Nieuwenhuis, 1996; Wilson, 1994, 1996, 1999; Elster and Perdomo,

1999). The offspring are identical to the parent plant and one another, so desirable traits are retained as there is genetic and phenotypic uniformity among the plants. However, there are certain disadvantages associated with this technique. For example, selection of elite mother trees can only occur after they have reached the adult stage and have been genetically proven to display the desired superior qualities (Mott, 1981; McComb and Bennet, 1986). However, in many forestry species, such as *Quercus*, *Fagus*, *Eucalyptus* and most conifers, woody cuttings taken from mature trees are difficult to root, or the rooting frequency is rather low (Mott, 1981; Ahuja, 1993). For *Eucalyptus*, the problem of maturity of shoots has been circumvented by felling superior trees and rooting the juvenile coppice shoots that arise from the stump (McComb and Bennet, 1986). However it is not always possible to fell superior trees or the stump might not coppice easily, *e.g.* *E. regnans* does not coppice at all (Blomstedt *et al.*, 1991). Further to this, Wilson (1998) pointed out that cuttings are considered more expensive and difficult to produce than seeds, and are also quite fragile prior to root development which itself, is prone to variability. In addition to these shortfalls, many hybrids and clones of *Eucalyptus* produce only a limited number of coppice shoots per plant that are deemed suitable for cutting propagation, thus rendering the technique inefficient for the testing of progeny (Watt *et al.*, 1995).

Many workers recognised that there is a need to explore new technologies, including *in vitro* tissue culture techniques such as micropropagation, which could potentially improve the speed and efficiency of tree propagation (Biondi and Thorpe, 1981; McComb and Bennet, 1986). Those authors pointed out that while a rooted cutting can produce a single plant from which, several years later, further cuttings may be taken, tissue culture systems can produce several shoots from a single explant. Furze and Cresswell (1985) alluded that tissue culture techniques could eliminate seed production and that commercial production of genotypes could be obtained 1-2 years after selection, as opposed to 4-10 years using traditional breeding methods. However, conventional breeding practises would always be important, especially for creating new genotypes that could subsequently be mass-propagated asexually.

1.7 Micropropagation of forest tree species

Traditional vegetative propagation methods such as propagation by cuttings are termed "macropropagation" (George, 1993). In comparison, the term "micropropagation" which refers to *in vitro* methods of propagation, stems from the use of very small pieces of plant tissue (explants) excised from the parent plant. These explants are then manipulated using various *in vitro* tissue culture techniques (George, 1993).

Propagation *in vitro* can proceed via one of two major routes, *viz.* somatic embryogenesis or organogenesis (Hussey, 1978). Somatic embryogenesis refers to the asexual induction of embryos from somatic cells. Embryo development proceeds through stages similar to that observed for zygotic embryo development, i.e. globular, heart, torpedo and cotyledonary stages. Embryos can develop either directly on the explant surface or they may differentiate from a mass of proliferating cells (callus) that develops from the explant (Tulecke, 1987; Wann, 1988; Watt *et al.*, 1999). The present discussion will deal only with organogenesis.

Organogenesis encompasses *in vitro* methods of tissue culture where organ primordia, e.g. buds, are initiated on an explant in response to the application of exogenous plant growth hormones (Ammirato, 1986). The development of these primordia into organs is essentially the same as in the intact plant and, in most cases, shoot primordia followed by leafy vegetative shoots are formed and these are then rooted (Thorpe, 1980, 1983; Ammirato, 1986; Nashar, 1989; Cheliak and Rogers, 1990). Propagation via organogenesis can occur via several different paths, one of which, adventitious shoot development, involves the production of multiple shoots either directly on the explant, or indirectly via callus, followed by rooting. Indirect organogenesis involves developmental changes, which include the formation of callus from explant tissue and subsequent reorganisation into plantlets (Hu and Wang, 1983). Axillary bud proliferation, another technique that involves the organogenic route of regeneration, refers to the *in vitro* multiplication, elongation and rooting of axillary buds initiated from nodal explants. Production of plantlets via axillary bud proliferation involves multiplication of a shoot

that has already been differentiated *in vivo*, and all that is required is elongation and rooting (Hu and Wang, 1983). Each technique of *in vitro* propagation holds certain advantages and altogether, the spectrum of tissue culture methods collectively termed 'micropropagation techniques' offer several advantages over conventional propagation methods. These as well as certain limitations associated with the application of tissue culture methods to forest tree species, are discussed in Chapter 3. Of note, is the problem of clonal specificity in terms of the requirements of each genotype for optimum growth and development *in vitro*, and the fact that media and methods have to be specific for each clone that is propagated. This will be discussed further in Chapter 3. At present, micropropagation techniques are used to supplement existing clonal forestry programmes, which employ traditional and vegetative methods of mass-propagation.

1.8 Aim

Eucalyptus plays a pivotal role in the forestry industry in South Africa, lending itself to several wood and non-wood applications. Expected shortfalls in the supply of wood to meet increasing demands in the near future requires the development of technologies that can ensure a constant reliable supply of this resource. In this regard, micropropagation has the potential to increase yields of superior genotypes of commercial importance and is being used to supplement clonal programmes of *Eucalyptus* hybrids. However, genotypic differences within commercially-important genotypes of *Eucalyptus* hybrids require clone-specific sterilisation and micropropagation protocols, which are costly and labour-intensive, therefore impacting negatively on the commercial application of this technique. Hence, the aim of this study was to establish hybrid-specific, rather than clone-specific protocols for (a) surface sterilisation and (b) *in vitro* axillary bud proliferation of clones of three commercially important *Eucalyptus* hybrids, *viz. E. grandis x nitens* (GN), *E. grandis x urophylla* (GU) and *E. grandis x nitens* (NH). The approach involved testing various surface sterilisation procedures (Chapter 2) and methods for the subsequent stages of the culture process (Chapter 3) to establish a single, high-yielding hybrid-specific protocol for each stage of micropropagation.

Chapter 2: Establishment of Aseptic Explant Material

2.1 Introduction and Literature Review

2.1.1 Sources of microbial contamination in culture systems

2.1.1.1 Common microbial contaminants in culture systems

According to Constantine (1986), *in vitro* plant cultures are susceptible to three forms of contamination. Acute contamination is sudden, severe and occurs during the establishment of cultures, and is caused mostly by ineffective surface sterilisation. Contamination which occurs after establishment of cultures is introduced during subculture or caused by micro-organisms concealed within the explant. Another type of contamination is chronic contamination, which is deep-seated, long-lived and usually results in the simultaneous contamination of a batch of cultures after a long period of apparent sterility.

Organisms that can be construed as contaminants in culture systems include viruses, bacteria, fungi, yeasts, arthropods, rickettsias and mollicutes (Leifert and Waites, 1990; George, 1993). Although bacterial contamination is considered by those authors to be the most serious, usually fungal contamination, especially in woody species from sub-tropical regions, often present a major hazard in tissue culture, resulting in significant losses in the laboratory (Watt *et al.*, 1996). Furthermore, Leifert and Waites (1994) also contend that fungi, viruses and yeast contaminants are as hazardous as their bacterial counterparts because of their equally deleterious effects on *in vitro* plants and the economic losses they can incur.

Bacterial contamination of plant cultures has been dealt with extensively in the literature on the subject. Various bacterial genera have been isolated from cultures and nearly all are described as plant pathogens, opportunistic plant pathogens, or saprophytic bacteria of aerial plant surfaces or the rhizosphere (Young *et al.*, 1984; Leifert and Waites, 1990;

Danby *et al.*, 1994; Gunson and Spencer-Phillips, 1994; Reed *et al.*, 1995; Reed *et al.*, 1998; Tanprasert and Reed, 1998).

Higher plants provide a vast source of nutrients for the myriad species of bacteria in the environment (*e.g.* Alfano and Collmer, 1996). Bacteria, as with other forms of microbial contamination, can occur on the surface of the plant tissues. Also, bacteria are small enough to pass through stomata and other natural openings into the apoplast of the host plant, where they establish themselves up against the cell wall of the plant cells, in the intercellular spaces of plant organs or in the xylem, in which case they are known as latent or endogenous contaminants (Leifert and Waites, 1990, 1994; George, 1993; Alfano and Collmer, 1996). This form of contamination is not eliminated by standard surface sterilisation techniques and does not immediately manifest its presence in cultures (Leifert and Waites, 1990, 1994; George, 1993).

Constantine (1986) stated that the slow growth of bacteria allows them to remain undetected during visual screening of *in vitro* cultures for contamination. Furthermore, bacteria tend to appear as haloes around the base of the explant, growing in fractures in the gel medium, rather than forming distinct colonies, which allows them to escape immediate detection. The identification of bacterial contaminants was usually done by the observation of a wide range of phenotypic characters. More recently, however, serological and genetic identification methods and fatty acid profiling methods are being used to identify bacteria down to species level (Leifert and Waites, 1990).

Common phytopathogenic bacteria that have led to devastating agricultural losses generally tend to be gram-negative, rod-shaped apoplastic colonising bacteria (Alfano and Collmer, 1996; Jackson and Taylor, 1996). These are usually of the genera *Erwinia, Pseudomonas* and *Xanthomonas*, and observations of *in vitro* bacterial contamination appear to confirm this (Gunson and Spencer-Phillips, 1994; Reed *et al.*, 1995; Reed *et al.*, 1998; Tanprasert and Reed, 1998).

Gunson and Spencer-Phillips (1994) isolated several bacterial genera from *in vitro* cultures of *Ficus benjamina, Dieffenbachia, Syngonium* and *Zantedeschia*. These included *Agrobacterium, Erwinia, Pseudomonas, Streptomyces* and various coryneform bacteria. Tanprasert and Reed (1998) identified most of the bacteria contaminating cultures of strawberry runner explants as being gram-negative, rod-shaped, non-spore forming species, such as *Pseudomonas fluorescens, Xanthomonas spp., Enterobacter cloacae A* and other *Pseudomonas* species. Moreover, *in vitro* cultures of hazelnut showed contamination by *Pseudomonas fluorescens, Xanthomonas spp. Agrobacterium radiobacter B, Enterobacter asburiae Flavobacterium spp.* and *Alcaligenes spp.* (Reed *et al.*, 1998).

Yeasts such as *Candida*, and *Rhodotorula* are adapted to conditions of high sugar and salt concentrations and thus find the prevailing conditions in plant tissue culture media ideal to support growth (Leifert and Waites, 1990), even in the absence of explant material. Yeasts also exist on the external surfaces of plants (George, 1993), and can thus be introduced with the explant, but Leifert and Waites (1990) believe that the main route of introduction into culture is during media preparation. This view is in accordance with the findings of a study conducted by Danby *et al.* (1994), whereby at least five different species of yeast were isolated from the laboratory air, i.e. the media preparation room, the subculturing area, storage areas and growth rooms. These included *Rhodotorula rubra, Rhodotorula glutinis, Cryptococcus albidis, Candida spp.* and other non-identifiable samples. In the same study, other species were isolated from the tissue cultures themselves, *viz. Candida spp., Debaryomyces polymorphus, Trichosporum cutaneum* and *Candida humicola*.

The presence of yeast infections in cultures is distinguishable by the characteristic fermentation smell when the culture vessel is opened (George, 1993). Yeast contaminants are usually identified on the basis of morphological tests detailing colony form, mycelium, fruiting bodies and sexual/asexual spore formation. In addition to this, yeasts can also be identified by testing for phenotypic characteristics such as growth on different sugars and other substrates, and metabolic capabilities (Leifert and Waites, 1990). Test

strips have also been successfully used to identify different strains of yeast infecting plant tissue cultures. In this regard, Danby *et al.* (1994) were able to identify the different strains of yeast contaminating their cultures and immediate environment, through the use of test strips.

According to Jackson and Taylor (1996), numerous species of fungi from each of the major phylogenetic groups cause serious plant diseases, and these include lower fungi such as Plasmodiophoromycetes, Chitridomycetes and Oomycetes, and higher fungi like Ascomycetes, Basidiomycetes and Deuteromycetes. Like bacteria and viruses, many opportunistic fungi enter their hosts through natural openings or wounds, however, true phytopathogenic fungi secrete a cocktail of hydrolytic enzymes that digest away the cuticle and cell wall of the epidermis (Knogge, 1996). As common plant pathogens and saprophytic soil inhabitants, many genera come to be associated with plants in tissue culture, through introduction with the explant material. Fungal contamination usually becomes apparent shortly after establishment of a culture (George, 1993), and presents an especially serious hazard in tissue culture in tropical and sub-tropical regions (Watt, *et al.*, 1996). Fungi can be introduced into cultures together with the explant, should sterilisation procedures be inadequate to eliminate them completely. However, like other ubiquitous contaminants, fungi occur in the general environment around the workers, and can thus be inadvertently introduced during media preparation or through inefficient sterile technique.

As with yeasts, Danby *et al.* (1994) found that, not only were several genera of fungi isolated from their *in vitro* cultures of *Coffea, Iris, Musa* and *Primula*, but there was also a direct correlation between fungi isolated from the laboratory air and from *in vitro* cultures that had been maintained for a minimum of a year. Such genera included *Penicillium, Cladosporium, Aspergillus, Botrytis, Alternaria, Fusarium* and *Phoma*. Furthermore, those authors found that certain fungal genera appeared to be host-specific *in vitro*, for example, only *Penicillium* spp. were isolated from *Iris* and *Musa* cultures, whilst *Coffea* was found to be contaminated by *Botrytis* and *Cladosporium*. Besides the

two genera infecting *Coffea*, they found that *Primula* was also infected with *Aspergillus* and *Phoma*.

Many fungal pathogens can originate from seedling nurseries and field-grown sources of explant material. For example, Viljoen *et al.* (1992), found that nurseries of *Eucalyptus* and *Pinus* seedlings were infected by a variety of fungal microflora which produced disease symptoms such as damping-off, rot, mould, blight, mildew and leaf spot. The fungal genera isolated from these nursery seedlings included *Pythium, Rhizoctonia, Cylindrocladium, Fusarium, Phytophthora, Botrytis, Sphaeropsis, Colletrichum, Coniella, Hainesia, Harknessia, Phaeoseptoria, Pseudocercospora and Sphaerotheca.* Linde *et al.* (1994) supported this assertion that South African nurseries of both juvenile and mature pine and eucalypts are under threat and have been infected by fungal species, especially *Pythium* and *Phytophthora* species.

In a separate study, by Crous and Swart (1995), those authors isolated several fungal pathogens from the leaves of eucalypt trees. These included known foliar pathogens such as *Aulographina eucalypti, Cylindrocladium quinquiseptatum, Kirramyces epicoccoides, Pseudocercospora eucalyptorum, Harknessia hawaiiensis, Codinea eucalypti, Codinea septata, Arnaudiella eucalyptorum, Mycotribulus mirabilis, Clypeophysalaspora latitans, Glomerella cingulata, Cylindrocladium candelebrum* and *Propolis emarginata.* All of these fungal genera have been found in South African stands of eucalypts, with the exception of *C. quinquiseptatum* and *M. heimii* (Crous and Swart, 1995). It is conceivable that introduction of inadequately sterilised explants from such stock plants would result in the gross contamination of cultures.

A recent survey of forest plantations in Mpumalanga and Kwazulu-Natal, South Africa, showed that die-back of eucalypt species, clones and hybrids was occurring as a result of *Botryosphaeria dothidea* and *Colletrichum gloeosporiodes* (Smith *et al.*, 1998). It seemed that clones of *Eucalyptus grandis* and its hybrids with *Eucalyptus camaldulensis* were especially susceptible to infection from *B. dothidea.* Significantly, *Colletrichum* was found to display asymptomatic infection of *Eucalyptus nitens* and *Eucalyptus grandis*

(Smith *et al.*, 1998), which could lead to the inadvertent introduction of this pathogen into *in vitro* cultures of field-grown material, and subsequent culture loss or dissemination of infected plants.

Rickettsias are small prokaryotic bacteria-like parasites with a well-defined cell wall, the outer margin of which is usually scalloped (Roberts and Boothroyd, 1984). Most of the rickettsias that cause plant diseases are obligate parasites and some are surface contaminants of plants *in vivo*, but may also occur within the vascular tissues, infecting the xylem and phloem. They are usually transmitted to plants by insect vectors such as aphids (George, 1993). Mollicutes are a group of prokaryotic organisms, including mycoplasmas and spiroplasmas. They lack a true cell wall and are instead surrounded only by an external lipoprotein membrane (Roberts and Boothroyd, 1984; George, 1993). These organisms are sub-microscopic, vary in shape and usually have a small genome size. Originally known as pleuropneumonia-like organisms, mollicutes are generally classed under six genera – *Mycoplasmas, Acholeplasma, Ureaplasma, Spiroplasma, Thermoplasma* and *Anaeroplasma* (Roberts and Boothroyd, 1984). Like the larger rickettsias, they can occur as surface or endogenous (in the phloem) contaminants (George, 1993).

Insect infestations present a year-round problem in tropical and sub-tropical climes, whereas in temperate regions this problem only becomes serious during the summer months (Blake, 1988, George, 1993). Due to their very small size, certain insects and arachnids are able to enter culture vessels where they can multiply rapidly without immediate detection. Ants, especially the small ubiquitous Pharoah's ant (*Monomorium pharaonis*) can be a serious problem since they can enter culture vessels, and are difficult to exclude from laboratories. The larger cockroaches, although unable to enter culture vessels, are able to introduce and spread contaminants in otherwise clean rooms. Thrips are common pests on glasshouse grown plants, and could thus be introduced into culture with the explant, although it is more likely that these insects enter the laboratory with the plant material, and subsequently contaminate cultures by moving from vessel to vessel (Blake, 1988). Infection of cultures by mites or other small arthropod pests is usually

recognisable by a characteristic "trail" of fungal and/or bacterial growth over the plant growth medium (Blake, 1988; George, 1993).

The term "viroids" is used to denote a group of sub-viral pathogens and are the smallest known agents of infectious disease. They usually consist of a short strand of RNA with a low molecular weight (75,000 – 125,000 daltons) which replicates in the host, leading to manifestation of the disease symptoms (Roberts and Boothroyd, 1984). Viroids have been responsible for diseases such as potato spindle tuber disease, citrus exocortis, chrysanthemum stunt, cucumber pale fruit, and chrysanthemum chlorotic mottle. Of significance in *in vitro* culture of plant tissues is the fact that the causative agents for all these diseases are mechanically transmissable, meaning that the viroid could be inadvertently spread from infected plants to uninfected propagules (Roberts and Boothroyd, 1984).

Plant viruses, another class of phytopathogens of major economic significance, are classified according to their genome type and organisation, and other physiochemical, biochemical and biological criteria (Jackson and Taylor, 1996), with the result that over 40 families of plant viruses have been defined. All plant viruses replicate in the initially infected cells and usually rely to some extent on host components to supplement their own replication processes. Following this, they are then able to move from cell to cell and through the vascular system, over long distances by exploiting and modifying pre-existing pathways for macro-molecular movement (Carrington *et al.*, 1996). According to Leifert and Waites (1994), viroids and viruses present a severe hazard in plant tissue culture because they are not readily detected and symptoms sometimes only manifest themselves after several subcultures or even after the plants have been weaned. This not only leads to serious economic losses, but could also contribute to the spread of the viruses via the infected plants.

George (1993) stated that although infected explant material could be used for establishment of cultures, the viral infection appeared to disappear from some or all the cells after a period of time in culture. However, more recent studies indicate that this is an

erroneous assumption. Following long-term culture of three peach cultivars at 4°C, the presence of *Prunus* necrotic ringspot virus was still detected, using a cRNA probe (Heuss *et al.*, 1999). Furthermore, Cohen *et al.* (1999) were able to detect the presence of tobacco and tomato mosaic virus in deliberately inoculated leaves of *Petunia* cultivars, by using the enzyme-linked immuno-sorbent assay (ELISA). Moreover, they found that up to 95% infection by tobacco mosaic virus occurred when a sterile knife was passed through an infected shoot prior to its being used to remove cuttings from healthy *Petunia* plants. This suggests that mechanical transmission of the viral particle can occur during culture manipulations, due to inefficient sterile technique.

2.1.1.2 Effect of source and type of explant on microbial contamination

Researchers working with *in vitro* cultures have used different types of explants from various sources in an effort to establish viable axenic cultures of plants *in vitro*. These explants include stems, leaves, petioles, lignotubers, roots, axillary and apical buds, floral organs, zygotic embryos, seeds and *in vitro* germinated seedlings (Le Roux and van Staden, 1991a, George, 1993, Watt *et al.*, 1996). The source and type of explant material chosen plays a significant role in controlling the severity of contamination experienced *in vitro* at culture establishment.

In this regard, de Fossard and de Fossard (1988) and later, Leifert and Waites (1990, 1994) reported that explant material growing close to soil or roots and other underground organs of soil-grown plants are generally highly contaminated and are therefore not ideal for initiating cultures. It is further contended by de Fossard and de Fossard (1988) that plants grown in tropical climates and exposed to overhead irrigation display an increased prevalence of contamination, through introduction of aerial contaminants with the overhead spray. In this regard, Grattapaglia *et al.* (1990) found that the use of drip irrigation of stock plants drastically reduced the prevalence of contamination in tissues. Also, older tissues or explants with rough surfaces are usually more heavily infected with superficial contaminants than younger, smoother explant material. Watt *et al.* (1996) further suggested that the long life-cycle of many woody trees gives them a longer

exposure to soil micro-organisms and hence introduces a higher probability of latent contamination.

Explants harvested from etiolated shoots also appear to be less contaminated than normal, possibly due to the fact that the contaminants do not have suitable conditions in which to multiply rapidly during elongation, especially if this occurs under relatively dry conditions (George, 1993). Furthermore, shoots of plants are less contaminated when they are actively growing than at other times. Seedling explants derived from seeds germinated under aseptic conditions would be ideal and have been used by many authors as explant material to initiate cultures (Blomstedt *et al.*, 1991; Watt *et al.*, 1991; Tibok *et al.*, 1995; Termignoni *et al.*, 1996; Lux *et al.*, 1998; Nepovim and Vanek, 1998; Watt *et al.*, 1998; Bandyopadhyay *et al.*,1999; Mederos Molina and Trujillo, 1999a; Zobayed *et al.*, 1999).

Although viruses have been known to occur in various concentrations in the cells of an intact plant, many are not transmitted through seeds. From this, it can be inferred that viruses may not infect the reproductive organs of plants, suggesting that anthers, ovaries, stamens etc. would be an attractive option in the culture of heavily contaminated plants. Furthermore, apical shoot and root meristems are usually virus-free (George, 1993).

2.1.1.3 Effects of microbial contaminants on *in vitro* cultures

To survive and grow properly, *in vitro* plant cultures need to be free of pests, fungi and bacterial infection. Arthropod pests, such as mites and thrips do not usually damage the *in vitro* plants directly, but act as vectors, introducing and encouraging the spread of other contaminants like fungi, bacteria and yeast in aseptic cultures. However, Carter and Hummer (1999) observed that gooseberry mite (*Cecidphyopsis grossulariae* Collinge) infestations in dormant black currant flower buds actually decreased the cold-hardiness of the tissues *in vivo*.

On the other hand, yeast contaminants in cultures metabolise the carbohydrates in the medium, producing phytotoxic fermentation products such as ethanol and acetic acid, thus dropping the pH of the medium to below three. The attack is thus two-pronged: an extremely acidic pH as well as phytotoxic compounds that affect the plant directly. This unfavourable environment has been known to result in plant death within one to three subcultures after the introduction of the contaminant (Leifert and Waites, 1990).

Besides the pathogenic and saprophytic genera of bacteria found in tissue cultures, there are non-pathogenic genera which are found on explant material and are adapted to metabolise dead or stressed tissue as well as the nutrients of the growth medium, and thus rapidly overgrow the explant (George, 1993). However, it would seem that unlike yeasts, endogenous bacteria require the presence of explant material on Murashige and Skoog medium (1962) to persist and grow (Leifert and Waites, 1990). Although those authors contend that competition between latent bacteria and plants for nutrient in the growth medium is not the main reason for poor plant growth, George (1993) states that even normally harmless bacterial species can interfere with growth *in vitro* and lead to death in plant cultures.

Jackson and Taylor (1996) stated that the gram-negative bacteria that are largely responsible for crop loss and appear so frequently in contaminated cultures appear to attack host plants by one of two mechanisms. They are either biotrophic pathogens, which kill their host slowly, thus allowing a maximum time for replication, or necrotrophic pathogens, which employ a "brute force strategy", resulting in rapid tissue death. It is likely that the former mechanism allows the pathogen to become established as a cryptic contaminant and thus escape visual detection. Toxins produced by gram negative necrotrophic bacteria are highly diffusable and can produce symptoms beyond the site of the original infection. These toxins can produce pores in the plasma membrane, degrading its integrity, inhibit enzymes in the chloroplast or even mimic certain key molecules in the plant, resulting in various symptoms such as necrosis or chlorosis (Alfano and Collmer, 1996).

Furthermore, bacteria can affect *in vitro* plant cultures by producing phytotoxic metabolites such as lactic acid, and other secondary metabolites such as organic acids, antibiotics, plant growth regulators and/or cyanides, which have been found to affect growth and development of plants *in vivo* (Leifert and Waites, 1990). George (1993) confirms this through his assertion that even if contaminants in plant tissue culture did not cause growth retardation and/or death, their presence is not desirable. Some bacterial genera are able to supply plant cultures with utilisable organic substances, which then leads to non-reproducible anomalous results between batches of cultures or in critical physiology experiments, as was the case in *in vitro* cultures of *Pinus radiata* buds (Horgan, 1987). An unidentified white bacterial growth manifested its presence at the base of the explant in the medium, or where a pine needle had broken and touched the surface of the medium. It was found that cultures contaminated with this bacteria tended to yield conflicting and variable results. Furthermore, it was found that contaminated cultures grew faster on agar-gelled medium than in liquid medium, whilst the reverse was true of sterile cultures. Similarly, de Fossard *et al.* (1974) stated that the microbial flora from field-grown plants multiply rapidly on nutritive growth media and are able to modify the composition of the medium and produce conditions that cannot be repeated. Furthermore, these microbes are also likely to over-run the explant tissue or kill it by their toxins.

Like yeast and bacterial contaminants, fungi are considered serious hazards because they grow well on nutrient media and can kill plant cultures by reducing the pH, producing phytotoxic metabolites and by competing for nutrients from the growth medium (Leifert and Waites, 1994). Although fungal infections generally result in the death of the explant, according to George (1993), it is possible to maintain dual cultures of the fungus and plant. Latent bacteria and viruses, which do not produce symptoms or manifest their presence *in vitro* present a different and perhaps more serious threat than contaminants that appear more immediately in cultures (Leifert and Waites, 1994). These cryptic contaminants can reduce multiplication and rooting rates, and thus the productivity of the plant cultures, or suddenly manifest their presence after long periods of apparent sterility, resulting in unexpected and disastrous losses (Leifert and Waites, 1994; George, 1993).

Mollicutes and rickettsias are responsible for leaf yellowing, wilt-inducing and stunting diseases of major economic importance and it is thus imperative that they, like all other contaminants, be eliminated from *in vitro* cultures (Roberts and Boothroyd, 1984; George, 1993).

2.1.2 Strategies for eliminating *in vitro* microbial contamination

Microbial contamination is responsible for the high losses of *in vitro plants* experienced in tissue culture laboratories. Furthermore, propagation of *in vitro* plants which harbour endogenous contaminants may lead to the dissemination and further spread of these contaminants in the greenhouse and field. It is thus desirous if not imperative that these contaminants be eliminated from tissues before any effort can be made to culture the explant. In this regard, several methods have been employed over the years to rid explant material of contaminants, both exogenous and endogenous. The use of chemical sterilants, ultraviolet radiation, and heat treatments has been documented in the literature, with varying degrees of success accorded to each method. In general, it could be said that the best method of sterilising explant material would involve the elimination or at least the reduction of contamination in cultures without leading to the irreversible senescence of the tissues as a result of the procedure employed. Sterilisation is effected on many levels, including the pre-treatment of stock plants to reduce overall contamination, the surface sterilisation of explant material prior to initiation in cultures, the elimination of endogenous contaminants that are unaffected by surface sterilisation techniques and the maintenance of aseptic plant cultures *in vitro*.

2.1.2.1 Pre-treatment of stock plants

It has been reported by several authors that even glasshouse-grown plants are infected by a range of fungal, bacterial, viral and arthropod contaminants. In this regard, it is often recommended that regular treatment of stock plants prior to sterilisation be carried out, to

reduce overall surface contamination. Treatment of stock plants would involve the regular application of anti-microbials such as fungicides, insecticides and bactericides.

Pre-treatment of stock plants has been done by several workers (Hu and Wang, 1983; Cohen, 1986; Ikemori, 1987; de Fossard and de Fossard, 1988; Donald and Newton, 1991; Le Roux and van Staden, 1991b; Jones and van Staden, 1994; Leifert and Waites, 1994; Beck *et al.*, 1998; Meszaros *et al.*, 1999). Jones and van Staden (1994) treated their stock plants of *Eucalyptus* hybrids with a combination of Benlate (active ingredient benomyl) and antibiotics, whereas Ikemori *et al.* (1987) applied Benlate to epicormic branches of *Eucalyptus grandis*. Boulay (1987) found that by keeping three-year old stock plants of *Auracaria excelsa* under very dry conditions for a minimum of six months prior to use, microbial infection was reduced and a milder sterilisation protocol could be successfully used.

2.1.2.2 Surface sterilisation of explant material

Before any attempt can be made to successfully micropropagate plants *in vitro*, the explant material harvested from the stock plants has to be adequately sterilised so that it is free of contaminants that could subsequently overrun the cultures and/or kill the explants. Surface sterilisation of explant material is thus aimed at reducing or completely eliminating the contaminants that are on the surface of stock plants. Commonly used sterilants include hypochlorites, antiseptics, antibiotics, fungicides, and heavy metal ions (George, 1993). Of these, the hypochlorite sterilants are the most widely used to disinfest plant tissue, and have been used as sodium hypochlorite from commercial bleach, or calcium hypochlorite and freshly prepared chlorinated lime. However, hypochlorites have been known to destroy only superficial contaminants and do not eliminate endogenous ones. The efficacy of hypochlorite solutions in the elimination of contaminants relates to their oxidising capacity, and the hypochlorous acid (HOCl) and OCl^+ ions (George, 1993). According to that author, some workers have suggested that calcium hypochlorite is less effective than its sodium counterpart, but other authors agree that it is less likely

than sodium hypochlorite to result in senescence and the production of browning exudates from sterilised tissues.

Ethanol is the most widely used alcohol in disinfecting plant tissue, although it is rarely used singly. This is because alcohols not only disinfectant tissues directly, but also remove surface waxes from plant tissue, facilitating the action of subsequent sterilant solutions (George, 1993).

The next most common sterilant is mercuric chloride, despite its associated toxicity and environmental hazards (George, 1993). Heavy metal ions have been known to be toxic to plants in tissue culture so due precautions have to be observed to remove all traces of the sterilant prior to introduction of the explant into culture (George, 1993).

Examples of sterilants used in plant tissue culture, their concentrations and exposure times are presented in Table 2.1 below. As part of the initial sterilisation step, antiseptics have been successful in eliminating microbials from explant material, although, like alcohols, these are rarely used singly (George, 1993).

Fungicides have also found application in the sterilisation of explant material besides the general maintenance of stock plants. Several different fungicides have been tested over the years, with varying success and deleterious effects on explant material. These include benomyl, propamocarb hydrochloride, chlorothalonil, imazalil, captafol, carbendazim and propiconazole (Shields *et al.*, 1984; Hannweg, 1995; Watt *et al.*, 1996; Camargo *et al.*, 2000).

Recently, the use of biological treatments to control *in vitro* and *ex vitro* contaminants has come under discussion. In this regard, the use of anti-fungal and antibacterial extracts derived from various plant sources has been detailed extensively in the literature and is currently being studied by other students in our laboratory. An example of "bio-control" of contaminants is the use of quaternery benzophenanthridine alkaloids (QBAs) derived

Table 2.1 Common sterilants used in *in vitro* tissue culture, their concentrations (conc.), exposure times (exp. time), the initial explant material used, and whether the sterilant used was part of a sequence of sterilisation steps (seq.). A * indicates that pre-treatment of stock plants was carried out prior to sterilisation.

Sterilant	Conc. (%)	Time (min.)	Seq.	Explant Material	Reference
NaOCl	0.10	5	Yes	*Eucalyptus* – nodal segments	Yasodha *et al.*, 1997
	0.12	15	No	*E. dunnii* – seeds	Termignoni *et al.*, 1996
	0.50	5	No	*Karwinskia parvifolia* – seeds	Lux *et al.*, 1997-1998
	0.50	10	No	*E. sideroxylon* – nodal segments	Burger, 1987
	1	20	No	*Stevia rebaudiana* – seeds	Nepovim & Vanek, 1998
	1	15	No	*Cucumis sativus* – seeds	Lou *et al.*, 1996
	1	15	Yes	*E. grandis* – nodal segments	Lakshmi Sita & Shobha Rani, 1985
	1	15	No	*E. dunnii x Eucalyptus* spp. – nodal segments	Fantini Jr. & Cortezzi-Graça, 1989
	1	20	No	*Eucalyptus* hybrids – nodal segments	Watt, *et al.*, 1995
	1	20	Yes	*E. grandis* – seeds	Watt *et al.*, 1991
	1	30	Yes	*Pinus patula* - seeds	Watt *et al.*, 1998
	1.05	20	No	*Chionanthus virginicus* – fruit	Chan and Marquard, 1999
	1.2	12	No	*Simmondsia chinensis* – nodal segments	Roussos *et al.*, 1999
	1.35	25	Yes	*Azadirachta excelsa* – shoots	Liew & Teo, 1998
	1-2	15	Yes	*Alloxylon flammeum* - seedlings	Donovan *et al.*, 1999
	2	5	No	*Brassica oleracea* – seeds	Zobayed, *et al.*, 1999
	2	20	Yes	*E. marginata*	
				E. calophylla	
				E. citriodora	
				E. diversicolor - unopened flower buds	McComb & Bennet, 1982
	2	15	Yes	*Anthurium parvispathum* – seeds	Atta-Alla *et al.*, 1998
	5	10	Yes	*Corylus avellana* – shoots	Yu and Reed, 1993
	5	30		*E. globulus*	
			No	*E. nitens* - seeds	Bandhyopadhyay, *et al.*, 1999
	7	7	Yes	*Pinus brutia* – seedlings	Abdullah, *et al.*, 1986
	10	10		*E. grandis* and	
			No	*E. nitens* - nodal segments	Furze & Cresswell, 1985
	10	5	Yes	*Ficus religiosa* – nodal segments	Deshpande *et al.*, 1998
	16.60	2	Yes*	*E. grandis* – epicormic shoots	Ikemori, 1987
	20	30	No	*E. urophylla* – seeds	Tibok *et al.*, 1995
	20	30	Yes	*E. tereticornis* – shoots	Das & Mitra, 1990
	20	15-20	Yes	*Eucalyptus* – nodal segments	Warrag *et al.*, 1990
	30	30	Yes	*E. regnans* – seeds	Blomstedt *et al.*, 1991
$Ca(OCl)_2$	1.20	10	No	*E. grandis x urophylla* - shoots	Yang *et al.*, 1995
	4	30	Yes	*Salvia canariensis* - branches	Mederos Molina, *et al.*, 1997
	5	5-10	No	*E. globulus* - epicormic shoots	Trindade *et al.*, 1990
	5	15	No	*Vitis vinifera* - shoot tips	Mederos Molina, *et al.*, 1998
	7	15	No*	*E. radiata* - epicormic shoots	Donald and Newton, 1991
	9	30	No	*Malus* x *domestica* - shoots	Piccioni, 1997
	10	8		*E dunnii*	
	10	8	No	*E. dalrympleana* - shoots	Franclet and Boulay, 1982
	10			*E. dalrympleana*	
	10		No	*E. delegatensis* - shoots	Boulay, 1983
$HgCl_2$	0.02	15	No*	All explants	Le Roux and Van Staden, 1991
	0.02	3-4	No	*Pinus patula* - juvenile shoots	Watt, *et al.*, 1998
	0.05	15	Yes	*E. tereticornis* - nodal explants	Patil and Kuruvinashetti, 1998
	0.05	15	No	*E. citriodora* - vegetative buds	Gupta *et al.*, 1981
	0.05	10	Yes	*Plantago major* - shoot tips	Mederos *et al*, 1997-98
	0.05	20	Yes	*Syzygium alternifolium* - stem cuttings	Sha Valli Khan *et al*, 1999
	0.05	15	Yes	*Saussurea lappa* - buds	Sudhakar Johnson *et al.*, 1997
	0.05	10		*E. camaldulensis, E. globulus,*	
	0.05	10	Yes	*E. tereticornis & E. citriodora* - nodal explants	Gupta and Mascarenhas, 1987
	0.10	20	Yes	*Pistachia* spp. - seeds	Mederos Molina *et al.*, 1999a
	0.10	15	Yes	*E. grandis* - nodal explants	Lakshmi Sita and Shobha Rani, 1985
	0.10	not given	No	*E. grandis* - shoots	Rao and Venkateswara, 1985
	0.10	15	No	*E. grandis* - shoots	Lakshmi Sita, 1986
	0.10	10-15	Yes	*Dalbergia latifolia* - nodal explants	Raghava Swamy *et al.*, 1992
	0.10	15	No	*Morus indica* - nodal explants	Vijaya Chitra and Padmaja., 1999
	0.20	10	Yes*	*Melissa officinalis* - shoots	Meszaros *et al.*, 1999
	0.20	15	Yes*	*Eucalyptus* hybrids - nodal segments	Jones and van Staden, 1994
	0.50	30	No	*Populus tremula* - axillary buds	Mandal, 1989

from plants from the family Papaveraceae. These alkaloids are potentially effective in the control of some fungal diseases caused by *Sphaerotheca pannosa* var. *rosae*, the cause of powdery mildew in roses. Glasshouse-grown roses were treated with a 150mg/l spray of the extract from *Macleaya cordata*, a plant that is said to be rich in QBAs (Newman, *et al.*, 1999). Rabe and van Staden (1998) screened *Plectranthus* for *in vitro* antibacterial activity, and found that methanolic extracts were effective against at least two of four Gram-positive bacteria, but ineffective against Gram-negative genera.

2.1.2.3 Elimination of endogenous contamination from tissues

Endogenous contaminants have proven to be a source of great frustration to researchers due to their unpredictability and the unexpected losses that can be incurred, should the plants produced be distributed. Several different types of antibiotics have been used to reduce bacterial contamination of explant material following establishment of cultures, as well as in the initial surface sterilisation treatments (Table 2.2). In addition, antibiotics sprayed on stock plants, can also be effective in reducing the level of contamination (George, 1993), and several different antibiotics have been used to this end. However, the application of this method for controlling bacterial contamination is not generally advocated due to the danger of antibiotic-resistant strains of bacteria arising, that could subsequently be released into the environment, with serious agricultural and medical ramifications (Falkner, 1990). Furthermore, more than one type of bacterium could be present in plant tissues, which may require the use of two or more antibiotics. In this regard, the possibility of incompatibility of antibiotics needs to be considered. The potential toxicity of the antibiotic to humans is another factor that needs to be taken into account prior to the use of antibiotics in tissue culture media (Barrett and Cassells, 1994). The effect of antibiotics on *in vitro* cultures appears to vary, with the antibiotic being used, the concentration, the explant and the species being propagated. Some authors have reported that the use of antibiotics has resulted in the inhibition and or necrosis of *in vitro* plants (Pollock *et al*, 1983; Young *et al.*, 1984; Tsang *et al.*, 1989; Leifert *et al.*, 1992; Yepes and Aldwinckle, 1994), whereas other workers report a stimulation of *in vitro*

plant development (Barrett and Cassell, 1994; Humara and Ordas, 1999; Nauerby *et al*, 1997).

Of the various classes of antibiotics tested, several authors have observed that aminoglycoside antibiotics seem to exhibit the highest toxicity (Pollock *et al.*, 1983; Tsang *et al.*, 1989; Leifert *et al.*, 1992; Yepes and Aldwinckle, 1994; Kapaun and Cheng, 1999). Tested antibiotics sometimes resulted in the necrosis of the explant (Young *et al.*, 1984; Tsang *et al.*, 1989; Humara and Ordas, 1999) or more commonly, inhibited *in vitro* plant growth and development (Tsang *et al.*, 1989; Leifert *et al.*, 1992; Yepes and Aldwinckle, 1994; Reed *et al.*, 1995; Kapaun and Cheng, 1999). Even at very low concentrations (5-20mg/l), kanamycin inhibited shoot regeneration and further growth in leaf explants of apple (Yepes and Aldwinckle, 1994), and strongly inhibited shoot regeneration, and caused necrosis within ten days of whole *Pinus pinea* cotyledons even at very low doses of 2.5 μg/ml (Humara and Ordas, 1999). Although streptomycin in combination with timentin was very effective in eliminating bacteria from micropropagated hazelnut, the treatment resulted in culture losses due to phytotoxicity (Reed *et al.*, 1995). Leifert *et al.* (1992) found that aminoglycoside antibiotics, either singly or in combination, had a tendency to reduce multiplication rates in *Clematis, Delphinium, Iris, Hosta* and *Photinia*. Streptomycin and gentamicin also inhibited root formation in *Photinia*. Kapaun and Cheng (1999) provided evidence for the toxicity of aminoglycoside antibiotics to leaf explants of Siberian Elm. Geneticin was highly toxic and killed explants at concentrations as low as 4mg/l and inhibited shoot regeneration at 1mg/l after a week's exposure. Inhibition of shoot regeneration by neomycin also occurred at 450 mg/l and by kanamycin at 225mg/l.

Other antibiotics besides aminoglycosides have also been reported to have a phytotoxic effect on plant cultures. Carbenicillin (1000mg/l) reduced shoot formation in leaf discs of *Nicotiana tabacum* by 44% and in cotyledons by 49% after two months in culture and 500mg/l cefotaxime inhibited shoot regeneration from cotyledons, as well as rooting of shoots from leaf discs (Nauerby *et al.*, 1997). Methotrexate partially inhibited bud formation in *in vitro* cultures of zygotic embryo explants of *Picea glauca* at

Table 2.2 Examples of the antibiotics used in plant tissue culture protocols, as a component of growth media.

Antibiotic	Mode of Action	Effect	References
Aminoglycosides Streptomycin Kanamycin Neomycin Gentamycin Tobramycin Amikacin Spectinomycin Geneticin Paromomycin G418 Hygomycin B	Inhibit protein synthesis by interacting with 30S or 50S ribosome	Bactericidal	Pollock *et al.*, 1983 Young *et al.*, 1984 Tsang *et al.*, 1989 Falkner, 1990 Kneifel and Leonhardt, 1992 Leifert *et al.*, 1992 Barrett and Cassells, 1994 Yepes and Aldwinckle, 1994 Reed *et al.*, 1995 Reed *et al.*, 1998 Humara and Ordas, 1999 Kapaun and Cheng, 1999
Quinolones Naladixic Acid Ofloxacin Norfloxacin Enoxicin Ciprofloxocin	Interfere with DNA replication by inhibition of DNA gyrase	Bactericidal	Falkner, 1990 Kneifel and Leonhardt, 1992
ß-Lactams Penicillin Ampicillin Carbenicillin Cephradine Cephamandole Cefuroxime Ceftazidime Sulbactam Imipenem Aztreonam Cefataxime Ticarcillin Cephalothin Cefoxiur Piperacillin	Inhibits cell wall synthesis	Bactericidal	Pollock *et al*, 1983 Young *et al.*, 1984 Tsang *et al.*, 1989 Santos and Salema, 1989 Falkner, 1990 Kneifel and Leonhardt, 1992 Leifert *et al.*, 1992 Barrett and Cassells, 1994 Yepes and Aldwinckle, 1994 Nauerby, *et al.*, 1997 Humara and Ordas, 1999
Macrolides and Lincosamides Erythromycin Lincomycin	Inhibit protein synthesis by acting on 50S ribosome	Bacteriostatic	Pollock *et al*, 1983 Falkner, 1990 Kneifel and Leonhardt, 1992 Barrett and Cassells, 1994
Glycopeptides Vancomycin	Interfere with bacterial cell wall synthesis	Bactericidal for gram + bacteria only	Pollock *et al*, 1983 Falkner, 1990 Kneifel and Leonhardt, 1992 Humara and Ordas, 1999
Polymixins Polymixin B Polymixin E	Attach to cell membrane and modify ion flux, resulting in cell lysis	Bactericidal for Gram -ve bacteria, especially *Pseudomonas*	Pollock *et al.*, 1983 Young *et al.*, 1984
Tetracyclines Chlortetracycline Aureomycin Biomycin Tetracycline	Inhibit protein synthesis by acting on 30S ribosome	Bacteriostatic	Pollock *et al.*, 1983 Young *et al.*, 1984 Falkner, 1990 Kneifel and Leonhardt, 1992 Barrett and Cassell, 1994
Trimethoprim Sulphonamides	Inhibit synthesis of tetrahydrofolate	Bacteriostatic	Pollock *et al.*, 1983 Kneifel and Leonhardt, 1992
Chloramphenicol	Inhibits protein synthesis by acting on 50S ribosome	Bacteriostatic	Pollock *et al.*, 1983 Kneifel and Leonhardt, 1992 Barrett and Cassell, 1994
Rifampicin	Interferes with mRNA formation by binding to RNA polymerase	Resistance emerges readily	Pollock, *et al.*, 1983 Young *et al.*, 1984 Kneifel and Leonhardt, 1992 Reed *et al.*, 1995

concentrations as low as 2.5μg/ml, whilst complete inhibition occurred at 5μg/ml (Tsang *et al.*, 1989).

Although β-lactam antibiotics are said to be less toxic than other antibiotics generally (Pollock *et al.*, 1983), evidence of phytotoxicity has been observed with carbenicillin (Leifert *et al.* 1992; Yepes and Aldwinckle, 1994; Nauerby *et al,* 1997), imipenem and Kathon (Kneifel and Leonhardt, 1992), and cephalothin (Leifert *et al.*, 1992). Contrary to these observations, however, cefotaxime has been found to be non-toxic to *in vitro* cultures and even promotes growth (Young *et al.*, 1984; Tsang *et al.*, 1989; Barrett and Cassell, 1994; Yepes and Aldwinckle, 1994; Humara and Ordas, 1999). Furthermore, in a study by Santos and Salema (1989), penicillins were able to promote synthesis of various enzymes, including nitrate reductase, glutamate dehydrogenase, and glutamine synthetase, by acting like cytokinins. Thus, it seems that certain antibiotics are able to stimulate growth in *in vitro* plant cultures by acting like growth hormones. Pollock *et al.*, (1983) found ampicillin and carbenicillin to be the least toxic of the antibiotics they tested, and further maintained a wide spectrum of bactericidal activity. Ticarcillin, another β-lactam antibiotic, was tested by Humara and Ordas (1999), on *Pinus pinea* cotyledons, and was found to have a stimulatory effect on shoot regeneration.

In the same way as antibiotics are incorporated into media to inhibit the proliferation of bacteria, fungicides are often added to prevent the growth of fungi *in vitro*. It has been observed that the incorporation of antibacterial agents into media suppressed bacterial growth, but allowed for the proliferation of *in vitro* fungal contaminants (Falkner, 1990). Under those circumstances, the addition of fungicides into the media was necessary to prevent fungi overrunning cultures.

Several different fungicides have been used over the years to retard fungal growth in *in vitro* cultures with varying degrees of success in disinfestation and different effects on the growth and development. In contrast to literature regarding the effect of anti-bacterial compounds *in vitro*, fewer reports are available on the effect of commercial fungicides on *in vitro* cultures (Watt *et al.*, 1996). Criteria for the selection of an appropriate fungicide

are very similar to those for antibiotic selection. In this regard, Shields *et al.* (1984) stated that the ideal fungicide should be fungicidal in plant tissue culture media with a broad spectrum of activity, whilst remaining non-toxic to the explant.

It has generally been observed by several workers that benomyl (as Benlate) a benzimidazole fungicide, exhibits a high degree of phytotoxicity both to *in vitro* and *in vivo* plant tissue (Shields *et al.*, 1984; Hannweg, 1995; Watt *et al.*, 1996; Reicher and Throssell, 1997). Benomyl, whilst showing good antifungal activity, also exhibited marked toxicity to protoplast cells at all concentrations tested, and further, tended to stimulate the development of callus (Shields *et al.*, 1984). A study by Watt *et al.* (1996) on the effect of different fungicides on *in vitro* cultures of *Eucalyptus* again provided evidence for the phytotoxicity of benomyl through observations of stunted growth in micropropagated *in vitro* shoots, as well as inhibition of multiplication and poor shoot survival. Furthermore, after hardening-off, a carry-over effect of the fungicide was observed, resulting in plants with reduced fresh mass. Reicher and Throssell (1997) observed that the treatment of bentgrass turf with benomyl not only tended to reduce the fresh weight of clippings, suggesting an inhibitory effect on shoot growth, but also increased the incidence of yellow tuft, and reduced the water-soluble carbohydrate content of grass clippings.

Other fungicides that have been shown to have deleterious effects on *in vitro* cultures include N-substituted imidazoles, such as miconazole and clotrimazole (Shields *et al.*, 1984). These tended to kill protoplast cells at fungicidal concentrations, the latter proving severely detrimental to root and callus cultures although it was safely used to eliminated yeast contaminants from coconut embryos (Shields *et al.*, 1984). Imizalil was found to have little toxicity, but did tend to slow germination and induced some deformation of lateral roots in culture.

In the study discussed above, Shields *et al.* (1984) observed that Amphotericin B, a polyene fungicide, was observed to be more toxic to protoplasts than nystatin, although both antimicrobials inhibited root and callus cultures to varying degrees. However, Watt

et al.(1996) showed that, despite inhibiting the number of explants that multiplied, this fungicide increased the number of shoots per explant and had a stimulatory effect on rooting.

Chlorothalonil, a chlorine substituted aromatic compound, was also observed to inhibit shoot survival and multiplication in *Eucalyptus* and further stunted shoot growth and inhibited rooting at low concentrations of the fungicide (Watt *et al.*, 1996). Chlorothalonil, applied weekly and alternated with other fungicides, increased rooting of bentgrass turf (Reicher and Throssell, 1997) but, like benomyl and propiconazole treatments, also reduced the water-soluble carbohydrate concentration of clipping; when alternated with propiconazole, it increased the incidence of pink snow mould. Propamocarb hydrochloride is a carbamate fungicide and has been proven to stunt shoot growth and stimulate rooting in *Eucalyptus* cultures *in vitro* but a carry-over effect of treatment with this fungicide resulted in a significant drop in the fresh weight of hardened-off plants (Watt *et al.*, 1996).

Latent contaminants, which also include fungi, bacteria and viruses present great hazards in tissue culture (Leifert and Waites, 1994). This category of contaminants poses problems in tissue culture because of the cryptic nature of the pathogen. Whilst some authors advocate meristem culture as a preventative measure when culturing plants infected with viruses (Hu and Wang, 1983; George, 1993, Cohen *et al.*, 1999), eradicating viruses from *in vitro* plants is also an issue that needs to be considered. In this regard, George (1993) stated that the use of heat therapy applied to whole plants and the culture of meristems at elevated temperatures could both be used to eliminate viruses from tissues. Stein and Spiegel (1990) successfully eliminated *Prunus* necrotic ringspot virus from micropropagated peach cultivars, by exposing the infected plantlets to alternating temperatures (38°C, 16 hours light and 28°C, 8 hours dark) for 16 days. This resulted in 70% of the plantlets surviving, all of which were virus-free. These were subsequently regenerated to intact plants, which remained free from viral infection.

Chemical treatment of virus-infected plants would be simpler than thermotherapy techniques, which are difficult and time-consuming. Certain compounds have been successfully used to eliminate viruses from tissues, *viz.* 2-thiouracil, malachite green, amantidine, ribavirin and adenine arabinoside (George, 1993). Cohen *et al.* (1999) were able to prevent the transmission of tobamovirus infection in *Petunia* by heat sterilisation of knives and treatment with sodium triclosene (2.8g/l) for 15 seconds. They further contended that disinfectants that have been successfully used against human viruses cannot be used against plant viruses, and have usually failed because plant viruses are more stable and thus much more difficult to de-activate than human viruses. Those authors suggested a system of precaution and prevention, entailing the observance of strict hygiene and sterilisation protocols and the maintenance and propagation of virus-indexed and screened stock plants, to control the spread and subsequent dissemination of the virus among propagated plants.

2.1.3 Aim

The maintenance and success of tissue culture protocols depends, in part on the elimination of contaminants and the maintenance of sterility *in vitro*. Furthermore, in order to survive and grow properly, plants generally need to be free of microbial pathogens. The *in vitro* culture environment presents ideal conditions for contaminants to proliferate rapidly and overrun cultures, killing the explant or altering the immediate chemical environment. It is thus imperative that explants are sterile prior to any manipulations *in vitro*, which requires the implementation of appropriate surface sterilisation methods. As mentioned in chapter 1, variability among different clones requires that specific methods be implemented in their micropropagation, and this includes surface sterilisation protocol. This is time-consuming and costly, thus, the aim of this experiment was to establish non-clone specific sterilisation protocols that would successfully eliminate microbial contaminants without resulting in the necrosis of explant material, nor affect subsequent growth and development of the plants *in vitro*.

2.2 Materials and Methods

2.2.1 Plant material

Cutting-derived potted plants of clones from three hybrids were obtained from Mountain Home Laboratory, Mondi Forests, Hilton (Kwazulu-Natal, South Africa): *E. grandis* x *nitens*, produced by a controlled cross between a female *E. grandis* and a male *E. nitens* (clones GN1, GN9, GN15, GN108 and GN121), *E. grandis* x *nitens*, a "natural hybrid" produced by open pollination between female *E. nitens* and a male *E. grandis* (clones NH0, NH58, NH69 and NH70) and *E. grandis* x *urophylla*, produced by crossing a female *E. grandis* and male *E. urophylla* (clones GU21, GU151, GU244 and GU297).

2.2.2 Maintenance of stock plants

Stock plants of the hybrid clones were maintained in the greenhouse at the University of Natal, Durban (29°52'S, 30°59'E; 25°C day/18°C night) and sprayed with fungicides and fertilizers on a weekly basis for the duration of this study. The fungicides included a mixture of 2g/l Dithane® (mancozeb; Efekto, South Africa) and 1ml/l Bravo® (chlorothalonil; Shell, South Africa), applied as a foliar spray once a week, and a mixture of 1g/l Sporgon® (prochloraz manganese chloride; Hoechst Schering AgrErvo, South Africa) and 1.25ml/l Folicur® (tebuconazole; Bayer, South Africa) applied as a soil spray, also once a week. The fertilizers were 2.5ml/l Trelmix® trace element solution (18g/l Fe, 4g/l Cu, 2g/l Zn, 1g/l B and 0.4g/l Mo; Hubers, South Africa) as a foliar spray, and 1g/l Mondi Orange® 1N-2P-1K (Harvest Chemicals, South Africa), applied as a soil spray, applied alternately once a week. These stock plants were cut-back every three to four weeks to stimulate coppice growth (Figure 2.1). Nodal explants were harvested and subjected to the tested sterilisation protocols outlined below in 2.2.3.

Figure 2.1 **Stimulation of coppice growth (right) in a clone of *E. grandis* x *nitens* (NH 69) by cutting-back stock plants every three to four weeks (left).** Bar= 5cm

2.2.3 Sterilisation of nodal explant material

A summary of the various protocols tested on nodal explants of GN 1 is presented in Table 2.3. Following treatment with each sterilant, nodal explants were rinsed thoroughly with sterile distilled water. The most suitable sterilisation method for this clone of *E. grandis* x *nitens* (GN) was subsequently tested on other genotypes of this and other hybrids.

Table 2.3. Sterilisation protocols employed in the eradication of microbial contaminants in nodal explants of *E. grandis* x *nitens* (GN 1). The autoclaved fungicide rinse consisted of 1g/l Benlate® (benomyl; Effekto SA), 1g/l boric acid and 0.5ml/l Bravo® (Shell SA), with a drop of Tween 20. Fungicides incorporated into the bud-break medium were 1g/l Benlate® (Effekto SA), 1ml/l Previcur® (propamocarb hydrochloride; FBC Holdings).

Protocol	Fungicide Rinse (min.)	Mercuric chloride (0.2g/l) (min.)	Calcium hypochlorite (10g/l) (min.)	Fungicides in medium
1	-	10	10	-
2	30	10	10	-
3	30	10	10	+
4	30	3	3	-
5	15	10	10	-

2.2.4 Analysis of data

The mean and standard error was determined from an average of three replicates, each with a sample size of 45-64 nodal explants. Data were analysed using a one way analysis of variance (ANOVA) and means were contrasted using Scheffe's multiple range test, at the 95% confidence interval.

2.3 Results and Discussion

One of the main criteria for successful micropropagation is the prevention or avoidance of microbial contaminants in *in vitro* cultures by disinfection of plant material taken from the field or greenhouse (George, 1993; Danby *et al.*, 1994; Reed and Tanprasert, 1995). As detailed earlier in this chapter, authors have pre-treated stock plants with fungicides to reduce overall contamination (section 2.1.2.1). In the present study, stock plants that were maintained in the greenhouse at the University of Natal, Durban, were similarly treated with fungicides on a weekly basis (section 2.2.2) to reduce systemic and surface microbial contamination. Nevertheless, suitable surface sterilisation methods were still required to disinfect explant material adequately. Hence, studies were conducted into establishing a protocol for the elimination of microbial contaminants from such parent stock plants. Initial studies were conducted on a single clone of *E. grandis* x *nitens* (GN 1), and the protocol optimised for this clone was subsequently tested on other clones of that and other hybrids, *viz. E. grandis* x *nitens* (NH) and *E. grandis* x *urophylla* (GU).

2.3.1 Establishment of surface sterilisation protocols using GN 1

The initial tested protocol to sterilise nodal explants involved 0.2g/l mercuric chloride and 10g/l calcium hypochlorite (10 minutes each). This was unsuccessful, in that contamination levels were unacceptably high (77.7 ± 10.01 %), although no necrosis of explants was observed (Table 2.4).

Since the initial protocol was ineffective in reducing contamination, a more stringent sterilisation method was tested (Table 2.3). This method (protocol 2) involved a 30 minute wash in a fungicide solution (1g/l Benlate, 1g/l boric acid, 0.5ml/l Bravo) followed by sterilisation as per protocol 1 (see above), and yielded 0 % contamination, but resulted in very high levels of necrosis (61.1 ± 3.20 %) (Table 2.4). A concurrent investigation (Protocol 3) into the effects of including fungicides in the bud-break medium (1g/l Benlate, 1ml/l Previcur; surface sterilisation as per protocol 2) resulted in a

slightly higher but not significantly different contamination level (3.4 ± 1.51 %) and than that experienced using protocol 2 alone (0.0 ± 0.00 %) (Table 2.4). Furthermore, necrosis levels from protocol 2 (61.1 ± 3.20 %) were not significantly different to those from protocol 3 (56.2 ± 9.37 %) (Table 2.4).

Protocol 4 was devised to attempt to inhibit necrosis by reducing the exposure time of explants to mercuric chloride (0.2g/l) and calcium hypochlorite (10g/l) to three minutes each. However, this proved unsuccessful as necrosis was 31.9 ± 7.35% and contamination was 16.7 ± 6.37% (Table 2.4). Protocol 5 was based on reducing the exposure time of explants to the fungicide rinse, from 30 minutes to 15 minutes, followed by sterilisation as per protocol 1. Contamination and necrosis levels using this method were 0% and 21.6 ± 3.53% respectively, which was deemed to be within acceptable limits (Table 2.4).

In summary, extremely harsh sterilisation methods (protocols 2, 3, 4) resulted in necrosis of the nodal explant material. This was manifested as browning of the tissues and the exudation of phenolics into the nutrient medium from the base of the nodal cutting, accompanied by the release of strong, distinctive-smelling volatile oils when the culture vessel was opened. George (1993) attributes this reaction to a wound response by the explant to excision and sterilisation procedures, which leads to the damage and senescence of cells. This phenomenon has also been documented by other authors working with woody tree species (Chang *et al.*, 1992: *Eucalyptus radiata*; Mederos Molina *et al.*, 1999b: *Pistachia* spp.). However, other authors using mercuric chloride and calcium hypochlorite at similar or higher concentrations to those used in this study have not reported high levels of necrosis in their explants following culture establishment (Boulay, 1983; Franclet and Boulay, 1982; Mandal, 1989; Jones and van Staden, 1994; Meszaros *et al.*, 1999) (Table 2.1). With reference to protocol 5, reducing the exposure time of the explants to the fungicide rinse had a positive effect on necrosis and contamination level, suggesting that long exposure to the fungicides in the rinse was one of the key contributors to the high necrosis levels experienced for this clone.

Table 2.4. The effect of different sterilisation protocols on contamination and necrosis of GN 1, a clone of *E. grandis* x *nitens*. Results were obtained after one week in culture.

Protocol 1 = 10 min. $HgCl_2$ (0.2g/l), 10 min. CaOCl (10g/l);

Protocol 2 = 30 min. fungicides (1g/l Benlate, 1g/l boric acid, 0.5ml/l Bravo), 10 min. $HgCl_2$ (0.2g/l), 10 min. CaOCl (10g/l);

Protocol 3 = Protocol 2 with 1g/l Benlate, 1ml/l Previcur incorporated in bud-break medium;

Protocol 4 = 30 min. fungicides (1g/l Benlate, 1g/l boric acid, 0.5ml/l Bravo), 3 min. $HgCl_2$ (0.2g/l), 3 min. CaOCl (10g/l);

Protocol 5 = 15 min. fungicides (1g/l Benlate, 1g/l boric acid, 0.5ml/l Bravo), 10 min. $HgCl_2$ (0.2g/l), 10 min. CaOCl (10g/l);

Protocol	% Contamination	% Necrosis
1	77.7 ± 10.01^{b}	0.0 ± 0.00^{a}
2	0.0 ± 0.00^{a}	61.1 ± 3.20^{c}
3	3.4 ± 1.51^{a}	56.2 ± 9.37^{c}
4	16.7 ± 6.37^{a}	31.9 ± 7.35^{bc}
5	0.0 ± 0.0^{a}	21.6 ± 3.53^{ab}

a-c= mean separation within columns, Scheffe's multiple range test, n=3, $p \leq 0.05$.

Based on the results presented on Table 2.4, it was concluded that the cause of this necrosis was the long exposure time of the explants to components of the fungicide wash (Benlate, in particular), as well as to the mercuric chloride. As mentioned in section 2.1.2.3, Benlate has phytotoxic effects, ranging from stunted growth and leaf drop to the development of small twisted leaves in ornamental and vegetable crops (Kelly, 1993; Watt *et* al., 1996; Camargo *et al.*, 2000). However, the successful non-phytotoxic use of Benlate as a sterilisation agent has been reported. Cortezzi-Graça and Mendes (1989) employed a stringent disinfection protocol, involving immersion of *Eucalyptus dunnii* nodal explants in 0.5g/l Benlate for 30 minutes followed by successive treatments with commercial detergents and sodium hypochlorite, but did not report any deleterious effects arising from the use of this systemic fungicide. Mederos Molina *et al* (1997) used 1g/l

Benlate to soak nodal explants of *Salvia canariensis* for 10 minutes and this protocol, followed by 30 minutes in 40g/l calcium hypochlorite, resulted in no observed necrosis and 0% contamination of cultures for the duration of that study. Beck *et al.* (1998) successfully disinfected coppice material of *Acacia* by employing a 10 minute soak in a mixture of Benlate (0.2%) and boric acid (0.1%), followed by 15 minutes in mercuric chloride (0.1%), with no apparent necrosis.

The phytotoxicity associated with the use of Benlate in culture media (protocol 3) has also been observed by several authors and has been discussed earlier in this chapter (section 2.1.2.3) (Shields *et al.*, 1984; Watt *et al.*, 1996; Camargo *et al.*, 2000). Benlate has been shown to have phytotoxic effects at concentrations as low as 0.5g/l and 1g/l (Watt *et al.*, 1996) and even lower concentrations of 0.05g/l and 0.1g/l proved to be lethal to axillary shoot cultures, and completely inhibited rooting, compared to cetoconazole and nystatin (Camargo *et al.*, 2000). Furthermore, in this study, the addition of fungicides to the bud-break medium following surface sterilisation (protocol 2, 30 minutes fungicides, 10 minutes mercuric chloride and calcium hypochlorite each) resulted in a slight but not significantly different increase in explant contamination, compared to the use of protocol 2 alone (Table 2.4). A similar effect was observed by Camargo *et al.* (2000), when Benlate, incorporated into the growth medium did not eliminate contamination in cultures of *Eucalyptus*. Hannweg (1995) also documented an increase in contamination when both Benlate and Previcur N were incorporated into the growth medium for *Haworthia limifolia*, suggetsing that the fungicides, in combination, acted antagonistically, resulting in enhanced fungal growth. Several authors have discussed the antagonistic action of antibiotics as a criterion for their selection and use *in vitro* (Pollock *et al*, 1983; Young *et al.*, 1984; Tsang *et al.*, 1989; Falkner, 1990; Leifert *et al.*, 1992; Yepes and Aldwinckle, 1994), and it appears that this *caveat* extends to the use of fungicides.

2.3.2 Testing of established protocol on other genotypes

Based on the positive results obtained with protocol 5 on GN 1, the remaining four clones of this hybrid, as well as those of the other two hybrids, were subjected to this sterilisation treatment.

Clones of *E. grandis* x *nitens* (GN) generally responded well to the protocol, with contamination levels ranging between 0%-1.7% among clones (Table 2.5). Necrosis levels were within acceptable levels (6.5%-23.2%), with the exception of clone GN 121, which exhibited a marked sensitivity to this protocol (51.7 ± 4.4 % necrosis) (Table 2.5).

Table 2.5. The effect of protocol 5 on contamination and necrosis of four other clones of *E. grandis* x *nitens* (GN). Results were obtained after one week in culture. Details as in Table 2.4.

Clones	% Contamination	% Necrosis
GN 9	0.0 ± 0.0[a]	6.6 ± 4.43[a]
GN 15	0.0 ± 0.0[a]	16.7 ± 1.67[a]
GN 108	0.0 ± 0.0[a]	23.2 ± 6.83[a]
GN 121	1.7 ± 1.67[a]	51.7 ± 4.41[b]

a-b = mean separation within columns, Scheffe's multiple range test, n=3, p≤0.05.

Contamination levels among clones of *E. grandis* x *nitens* (NH) were also low (0%-4.1%) and were not significantly different among clones (Table 2.6). However, with reference to necrosis, the application of protocol 5 proved suitable only for NH 0 (25.5 ± 1.80 %) and NH 58 (4.7 ± 1.33 %), since the remaining two clones exhibited a highly sensitive response (60.7 ± 5.48% for NH 69; 83.6 ± 2.85 % for NH 70) (Table 2.6).

Table 2.6. The effect of protocol 5 on contamination and necrosis of four clones of *E. grandis* x *nitens* (NH). Results were obtained after one week. Details as in Table 2.4.

Clones	% Contamination	% Necrosis
NH 0	4.1 ± 2.67^{a}	25.5 ± 1.80^{b}
NH 58	0.7 ± 0.65^{a}	4.7 ± 1.33^{a}
NH 69	0.0 ± 0.0^{a}	60.7 ± 5.48^{c}
NH 70	3.6 ± 1.86^{a}	83.6 ± 2.85^{d}

a-d= mean separation within columns, Scheffe's multiple range test, n=3, $p \leq 0.05$.

The application of protocol 5 to the surface sterilisation of nodal explants of *E. grandis* x *urophylla* clones was successful in terms of achieving low levels of contamination (1.4%-5.5%) among clones (Table 2.7). However, all proved sensitive to this disinfection method, as evidenced by the high necrosis values among them (30.6%-46.0%) (Table 2.7).

Table 2.7. The effect of protocol 5 on contamination and necrosis of four clones of *E. grandis* x *urophylla* (GU). Results were obtained after one week. Differences between means within a column are indicated by different letters, Scheffe's multiple range test, n=3, $p \leq 0.05$. Details as for Table 2.4

Clone	% Contamination	% Necrosis
GU 21	5.5 ± 1.45^{a}	35.2 ± 3.62^{a}
GU 151	3.8 ± 2.98^{a}	38.9 ± 5.01^{a}
GU 244	3.9 ± 0.20^{a}	46.0 ± 2.41^{a}
GU 297	1.4 ± 1.40^{a}	30.6 ± 7.35^{a}

In conclusion, although protocol 5 was employed successfully in the disinfection of all clones of the three tested hybrids, the high levels of necrosis experienced in some clones (GN 121, NH 69, NH 70, all GU clones) still posed a problem. Therefore, further study was necessary to achieve a suitable sterilisation method that would achieve lower necrosis levels without compromising asepsis.

2.3.3 Development of a common optimised sterilisation protocol to all tested genotypes

Protocol 6 was devised and tested on all clones of the three *Eucalyptus grandis* hybrids. It involved a 15-minute fungicide wash, followed by agitation in 10g/l calcium hypochlorite for three minutes only, thus eliminating the mercuric chloride step. Generally, this method proved successful for all clones of the three hybrids (Table 2.8). Although contamination levels were usually higher than those experienced using protocol 5, they still fell within an acceptable and manageable range (GN clones: 2.8-6.9%; NH: 1.4-11.8%; GU: 0.0-11.8%) (Table 2.8). Further, Scheffes multiple range test indicated that these values were not significantly different from clone to clone ($p \geq 0.05$; Table 2.8). The elimination of the mercuric chloride step had a drastic effect in terms of reducing necrosis levels for all clones of all three hybrids to levels below those experienced using protocol 5 (GN clones: 0.0%-4.2%; NH: 4.2%-22.9%; GU: 4.2%-9.9%) (Table 2.8).

As mentioned earlier in this section (section 2.3.2), the high levels of necrosis experienced in several clones was attributed to mercuric chloride. Although George (1993) asserted that the calcium ions in calcium hypochlorite provide a protective buffer for the explant to the sterilant, when formulating protocol 6, it was decided that a reduction in the exposure time of this sterilant from 10 minutes to 3 minutes was also advisable. These steps evidently yielded the desired results, with contamination levels maintained at a low level for all tested clones and necrosis levels reduced (Table 2.8), compared to explants exposed to protocol 5 (Tables 2.5, 2.6, 2.7).

Table 2.8 The effect of protocol 6 on contamination and necrosis of nodal explants from five clones of *E. grandis* x *nitens* (GN), four clones of *E. grandis* x *nitens* (NH) and four clones of *E. grandis* x *urophylla* (GU). Results were recorded after one week.

Protocol 6 = 15 min. fungicides (1g/l Benlate, 1g/l boric acid, 0.5ml/l Bravo), 3 min. 10g/l CaOCl.

Clone	% Contam.	% Necrosis
GN 1	5.6 ± 3.68 [a]	1.5 ± 1.45 [a]
GN 9	5.6 ± 1.39 [a]	4.2 ± 0.00 [ab]
GN 15	2.8 ± 1.39 [a]	0.0 ± 0.00 [a]
GN 108	6.9 ± 2.78 [a]	0.0 ± 0.00 [a]
GN 121	5.6 ± 1.36 [a]	2.8 ± 2.78 [ab]
NH 0	11.8 ± 4.67 [a]	7.7 ± 3.85 [ab]
NH 58	6.9 ± 2.78 [a]	4.2 ± 2.41 [ab]
NH 69	1.4 ± 1.39 [a]	14.3 ± 2.38 [ab]
NH 70	9.4 ± 7.86 [a]	22.9 ± 2.08 [b]
GU 21	6.7 ± 6.67 [a]	6.7 ± 6.67 [ab]
GU 244	0.0 ± 0.0 [a]	4.2 ± 0.0 [ab]
GU 297	11.8 ± 1.10 [a]	8.1 ± 0.21 [ab]
GU 151	0.0 ± 0.0 [a]	9.9 ± 5.10 [ab]

a-b= mean separation within columns, Scheffe's multiple range test, n=3, $p \leq 0.05$.

The establishment of a common sterilisation method (protocol 6) is a significant contribution to the commercial application of micropropagation protocols, in that it saves time, money and labour associated with the employment of different disinfection methods for each clone of a hybrid. Further, in this study, the elimination of toxic sterilants such as mercuric chloride from the sterilisation protocol allowed for the aseptic production of axillary buds that were healthy and unaffected by the sterilisation protocol, as has been documented by other authors (Shields *et al.*, 1986; Watt *et al.*, 1996). In addition, the protocol is safe, since mercuric chloride has been shown to have deleterious effects on the environment and to be toxic to humans and animals (George, 1993).

2.3.4 Conclusion

The established protocol (protocol 6) involved washing the nodal explants in a fungicide solution (1g/l Benlate, 1g/l boric acid, 0.5ml/l Bravo, Tween 20) for 15 minutes, followed by agitation in calcium hypochlorite (10g/l, Tween 20) for 3 minutes, with thorough rinsing with sterile distilled water following each sterilant application. It was found to be successful in maintaining contamination levels for all clones below 15%, and necrosis levels below 25%.

It was also noted that genotypic differences between clones existed with reference to their responses to a given protocol. Moreover, it would appear that GN clones are less susceptible to necrosis resulting from harsh sterilisation procedures than NH and GU clones. Despite this, a single common sterilisation method, applicable to all tested clones, was devised.

Chapter 3: Establishment of protocols for the micropropagation of *Eucalyptus* hybrids

3.1 Introduction and Literature Review

3.1.1 Applications and limitations of micropropagation in forest species

Although conventional methods of propagation such as vegetative propagation by cuttings have a vital role to play in many commercial forestry breeding programmes, new techniques like those offered in tissue culture may be of great importance towards achieving progress in the propagation and breeding of commercial forest tree species (Konar and Nagmani, 1973; Bonga, 1977; McComb and Bennet, 1986; Ahuja, 1993). As discussed in Chapter 1, micropropagation methods are techniques that involve the *in vitro* manipulation (via somatic embryogenesis or organogenesis) of cells or very small pieces of organs excised from the parent plant, that results in the multiplication of regenerated plants (Thorpe, 1980; Ammirato, 1986; Nashar, 1989; George, 1993).

The first attempts at *in vitro* plant propagation involved the maintenance of isolated live cells, and was carried out by Haberland in 1902 (Nashar, 1989). This was followed by Gautheret's work on tissue cultures of carrot, some forty years later, and in the same year, Nobecourt and White conducted similar work on carrot and tobacco (Nashar, 1989). The belated discovery of plant hormones delayed further investigations into plant tissue culture, but following this, workers were able to deduce the influence of auxin:cytokinin ratios on *in vitro* morphogenesis, and subsequently manipulate this. Industrial micropropagation only truly became established in the 1970s, when Morel was able to mass-produce *in vitro* tropical orchids, in France (Nashar, 1989). Since then, *in vitro* techniques of propagation have been successfully applied to a number of plant species, including ornamentals, crops, horticultural plants species, and forest tree species of commercial importance.

Micropropagation offers several advantages that render it an attractive alternative to conventional methods of propagation. These include very high multiplication rates of genotypes (Franclet and Boulay, 1982; Ammirato, 1986; Constantine, 1986; McComb and Bennet, 1986; Le Roux and van Staden, 1991a; George, 1993; Muralidharan and Mascarenhas, 1995), an important consideration when selected superior genotypes have to be mass-produced over the shortest period of time possible (Nashar, 1989). In addition, *in vitro* techniques are faster than conventional propagation techniques and also enable propagation of species that are resistant to vegetative propagation through cuttings, making it an appealing prospect in forestry (Bonga, 1977; Ahuja, 1993; Zobel, 1993; Yang *et* al., 1995; Watt *et al.,* 1999). Less energy and space are required for propagation and maintenance of stock plants, and the plant material requires little attention between subcultures (George, 1993). Furthermore, *in vitro* rooted material can also be readily shipped overseas, since the total volume and weight is reduced (McComb and Bennet, 1986; Nashar, 1989; George, 1993).

A quick scale-up in production can be achieved via liquid cultures through growth of somatic embryos in bio-reactors (Jain and Ishii, 1997; Rival *et al.*, 1998). Liquid cultures, making use of temporary immersion systems in bio-reactors, have also resulted in high multiplication rates of different tree species via the organogenic route of propagation (Alvard *et al*, 1993; Teisson and Alvard, 1995; Nepovim and Vanek, 1998; Nixon *et al.*, 2000). Added advantages include the production of 'somatic seeds' which are somatic embryos encapsulated in beads of gel, and the long-term storage of genetic material and dormancy induction and cryopreservation of these somatic seeds (Jain and Ishii, 1997; Watt *et al.*, 1999). Apical and axillary buds have also been encapsulated, preserved in gel beads, and subsequently regenerated (Piccioni, 1997; Capuano *et al.*, 1998; Micheli *et al.*, 1998; Standardi and Piccioni, 1998; Gardi *et al.*, 1999).

However, micropropagation does have its limitations. Wilson (1998) asserted that the advocacy of micropropagation as a commercial propagation system for forest trees is not fully justified and perceived advantages appear speculative and idealistic, with the actual application of *in vitro* techniques in practice falling prey to several pitfalls. These could

include the high cost of plant production due to the high labour intensity and technicality involved. A specialised and expensive production facility is needed and fairly specific methods may be necessary to obtain optimum results from each species, variety, and explant type (George, 1993). Sterilisation of material from field-grown trees, especially forest tree species has also proven problematic (Constantine, 1986; Ikemori, 1987; McComb and Bennet, 1986, Warrag *et al.*,1990). Further, authors have experienced difficulties regenerating plantlets from material harvested from mature trees (Ikemori, 1987; Le Roux and van Staden, 1991a; George, 1993; Patil and Kuruvinashetti, 1998). Although studies have been carried out using mature material, most successful regeneration has been reported usually only when seedling or embryonic material has been used (Bonga, 1987; Le Roux and van Staden, 1991a; Merkle, 1995). This constitutes a problem since trees can be selected for desirable character traits only when they are mature, at which point they become recalcitrant to *in vitro* manipulations. Also, *in vitro* plants initially, are usually incapable of autotrophic growth, and have to go through a transition period before they are capable of self-sufficiency (Hussey, 1978; George, 1993). Hyperhydricity, a disorder where shoots take on a glassy water-soaked appearance is also a problem, leading to difficulties experienced in rooting microshoots (Constantine, 1986; Ziv, 1991).

Despite this, tissue culture has become a valuable tool for rapid clonal propagation of several important forest tree species which have been propagated successfully in this way (Mascarenhas *et al.*, 1981). Somatic embryogenesis has been achieved less frequently than organogenesis with forest tree species, and Haissig (1989) suggests that the reason for this is that the development of embryoids is physiologically more intricate than organogenesis and more difficult to control. *In vitro* propagation via somatic embryogenesis has been carried out mostly using seedling and embryonic tissues. Furthermore, reports on somatic embryogenesis show that usually only a single population of embryos is produced, of which only some may mature and germinate to form plants (Merkle, 1995). Thus, although this technique has been applied to some tree species (Table 3.1), barriers to the application of this technique for propagating highly selected forest trees of commercial importance remain (Merkle, 1995; Watt *et al.*, 1999).

Table 3.1 Examples of reported studies of recent work conducted on somatic embryogenesis in some forest tree species. Unless otherwise indicated, all studies involved indirect embryogenesis.

Species	Explant	Reference
Eucalyptus dunnii	Seeds	Termignoni *et al.*, 1996
Eucalyptus grandis	Zygotic embryos, hypocotyls	Major *et al.*, 1997
Eucalyptus grandis	*In vitro* shoots	Watt *et al.*, 1991
Eucalyptus globulus Labill.	Zygotic embryos, floral tissue	Nugent *et al.*, 1997
Eucalyptus species	Zygotic embryos, cambium	Ikemori *et al.*, 1994
Salvia officinalis, S. fruticosa	Leaf explants	Kintzios *et al.*, 1999
Dendranthema glandiflora	Leaf explants	May and Trigiano, 1991
Pinus taeda	Female gametophyte	Gupta *et al.*, 1987
Phleum pratense L.	Anther	Guo *et al.*, 1999
Picea glauca, Picea engelmani	Immature zygotic embryos	Roberts *et al.*, 1990
Acer palmatum	Immature zygotic embryos	Vlasinova and Havel, 1999
Persea spp.	Petioles, axillary buds, embryo	Raviv *et al.*, 1998
Cyclamen persicum	Ovules	Schwenkel and Winkelman, 1998
Santalum album	Internodal segments	Lakshmi Sita *et al.*, 1998
Pinus patula	Seedling material	McKellar *et al.*, 1994

On the other hand, organogenic methods of *in vitro* propagation have proven successful in the tissue culture of several species (Table 3.2). Of the techniques in organogenesis, axillary bud proliferation and culture of individual nodes are the techniques most widely used in commercial micropropagation. This method is preferred due to the ease of implementation, the greater degree of control over morphogenesis and the high yields obtained. Also, because there is no intervening callus stage, the risk of genotypic differences among propagules, arising as a result of somaclonal variation, is less than that experienced with such techniques as somatic embryogenesis and organogenesis via callus production. This is an overriding concern for commercial forestry companies that require both genotypic and phenotypic uniformity for the establishment of plantations of highly selected or engineered superior trees (Bonga, 1977; Constantine, 1986; Lakshmi Sita, 1986; McComb and Bennet, 1986; Haissig, 1989; Cheliak and Rogers, 1990; Ahuja, 1993; Watt *et al.*, 1997).

Table 3.2. Examples of reported studies of recent work conducted on organogenesis in some forest tree species. *In vitro* propagation via organogenesis was accomplished through several methods, *viz.* axillary bud proliferation, regeneration from callus produced from various explants (*) or adventitious shoot production directly from explants.

Species	Explant	Reference
Eucalyptus globulus	Lateral buds	Trindade *et al.* 1990
Simmondsia chinensis	Seedling explants	Roussos *et al,* 1999
Corylus avellana	Shoot tips, Nodal segments	Yu and Reed, 1993
Populus tremula	Axillary buds	Mandal, 1989
Salvia canariensis	Axillary nodes	Mederos Molina *et al.* 1997
Syzygium alternifolium	Nodal explant	Sha Valli Khan *et al.*, 1999
Morus indica	Nodal explants	Vijaya Chitra and Padmaja, 1999
Eucalyptus radiata	Nodal explants	Donald and Newton, 1991
Plantago major	Shoot tips	Mederos *et al.*, 1997/98
Eucalyptus botryoides	Shoot tips *	Ito *et al.*, 1996
Alloxylon flammeum	Seedling shoot tips	Donovan *et al.*, 1999
Eucalyptus urohylla	Seedling hypocotyls *	Tibok *et al.*, 1995
Olea europa	Nodal, apical buds	Micheli *et al.* 1998
Azadirachta excelsa	Axillary buds	Liew and Teo, 1998
Pinus brutia	Shoot explants	Abdullah *et al.* 1986
Malus x *domestica*	Axillary buds	Piccioni, 1997
Betula pendula	Axillary buds	Jokinen and Tormala, 1991
Paulownia elongata	Shoot tips, intenodes, leaves – *in vitro* seedlings	Chang and Donald, 1992
Pinus sylvestris	Cotyledons – germinated embryos	Haggman *et al.*, 1996
Saussurea lappa	Shoot tips	Sudhakar Johnson *et al.*, 1997
Acacia mearnsii	Nodal explant – coppice shoots	Beck *et al.*, 1998
Pinus elliotti x *caribea*	Mature embryos	Meyer, 1998
Eucalyptus grandis	Leaf explants *	Laine and David, 1994
Karwinskia parvifolia	Zygotic embryos	Lux *et al.*, 1997/98
Mondia whitei	Nodal explants – *in vitro* seedlings	McCartan and Crouch, 1998
Cajanus cajan	Mature embryonal axes	Franklin *et al.*, 2000
Cryptanthus sinuousus	Stolons, leaves, stems	Carneiro *et al.*, 1998
Polianthes tuberosa	Leaves*	Sanyal *et al.*, 1998
Pinus contorta	Zygotic embryos	Flygh *et al.*, 1993
Acer pseudoplatanus	Plumule, hypocotyl	Wilhelm, 1999
Rubus spp.	*In vitro* internodal segments, leaves	Mendoza and Graham, 1999
Ficus religiosa	Axillary nodal explants	Deshpande *et al.*, 1998
Pinus patula	Seedlings, juvenile shoots	Watt *et al.*, 1998

3.1.2 Micropropagation of *Eucalyptus* species

Several species of eucalypts have been micropropagated and there are extensive reviews detailing the various media and methods used (McComb and Bennett, 1986; Le Roux and van Staden, 1991a; George, 1993; Muralidharan and Mascarenhas, 1995) (Table 3.3).

As detailed earlier, micropropagation can proceed via somatic embryogenesis, the sequential subculture of axillary buds or adventitious budding (either directly on the explant or via callus). As for most tree species, micropropagation via axillary bud proliferation has been the method of choice for the multiplication of eucalypts (Gupta *et al.*, 1981; Muralidharan and Mascarenhas, 1987; Le Roux and van Staden, 1991a). In their comprehensive review on the tissue culture of eucalpts, Le Roux and van Staden (1991a) listed some 28 species for which complete protocols for axillary bud proliferation exist, whilst regeneration via other organogenic methods was at less than half of that number of species, and approximately only five species of eucalypts were listed for which embryogenic callus had been successfully produced (Le Roux and van Staden, 1991a, Watt *et al.*, 1999). The reason for the dearth in protocols for somatic embryogenesis of eucalypts is due to the problems experienced with mature, genetically proven tissues, as outlined earlier and successful studies in somatic embryogenesis carried out on *Eucalyptus* have mostly involved embryo or seedling material (Table 3.3), which is genetically unproven (Le Roux and van Staden, 1991a; Watt *et al.*, 1991; Termignoni *et al.*, 1996; Jain and Ishii, 1997). The use of axillary buds is the preferred route of micropropagation, for the maintenance of clonal fidelity, and the greatest success has been achieved when explants have been induced to form primordia without an intervening callus stage (Biondi and Thorpe, 1981; Constantine, 1986; Le Roux and van Staden, 1991a). As discussed earlier (section 3.1.1), the use of axillary bud proliferation is fast, ensures a high plantlet yield and maintains genotypic and phenotypic uniformity amongst the micropropagated *Eucalypptus* plants. The technique of axillary bud proliferation will be discussed further (section 3.1.3).

Table 3.3 Reported studies of micropropagation carried out on *Eucalyptus* species, through organogenesis or via somatic embryogenesis.

Species	Explant	Propagation System	Reference
E. regnans	*In vitro* nodal explants	Axillary bud proliferation	Blomstedt *et al.*, 1991
E. sideroxylon	Nodal explant	Axillary bud proliferation	Burger, 1987
E. marginata	Shoots – crown of tree	Axillary shoot proliferation	McComb and Bennett, 1982
E. radiata	Nodal explant	Shoot multiplication	Donald and Newton, 1991
E. tereticornis	Nodal explant – mature field	Axillary bud proliferation	Das and Mitra, 1990
E. citriodora	Terminal, axillary buds, Seedling explants	Shoot multiplication	Gupta *et al.*, 1981
E. dunii x E. spp.	Nodal explants	Axillary bud proliferation	Fantini Jr. and Cortezzi-Graça, 1989
E. dunii	Nodal explants	Axillary bud proliferation	Cortezzi-Graça and Mendes, 1989
E. botryoides	Shoot tips – *in vitro* seedlings	Indirect organogenesis	Ito *et al.*, 1996
E. urophylla	Seedling hypocotyls	Indirect organogenesis	Tibok *et al.*, 1995
E. grandis hybrids	Branches and sprouts – mature	Axillary bud proliferation	Warrag *et al.*, 1990
E. grandis	Nodal explants – 5 yr old trees	Axillary bud proliferation	Lakshmi Sita and Shobha Rani, 1985
E. grandis	Nodal explants	Axillary bud proliferation	Lakshmi Sita, 1986
E. grandis	Nodal explant	Axillary bud proliferation	Sankara Rao and Venkateswara, 1985
E. globulus	Nodal explants – coppice, and epicormic shoots	Axillary bud proliferation	Trindade *et al*, 1990
E. tereticornis,	Nodal explants	Axillary bud proliferation	Yasodha *et al.* 1997
E. camaldulensis	Nodal explants	Axillary bud proliferation	Yasodha *et al.*1997
E. gunnii	Leaves, internodes, nodes	Axillary bud proliferation	Herve *et al.*, in press
E. tereticornis	Nodal explants – coppice	Axillary bud proliferation	Patil and Kuruvinashetti, 1998
E. gunnii	Nodal explants	Axillary bud proliferation	Franclet and Boulay, 1982
E. dalrympleana	Nodal explants	Axillary bud proliferation	Franclet and Boulay, 1982
E. grandis & *E. nitens*	Nodal explants – seedling and coppice shoots	Axillary bud proliferation	Furze and Cresswell, 1985
E. viminalis	Nodal explants	Axillary bud proliferation	Wiechetek *et al.*. 1989
E. sideroxylon	Shoots	Direct organogenesis	Cheng *et al.*, 1992
E. tereticornis	Terminal buds	Shoot multiplication	Mascarenhas *et al.*,1982
E. globulus	Terminal buds	Shoot multiplication	Mascarenhas *et al.*,1982
E. citriodora	Terminal buds	Shoot multiplication	Mascarenhas *et al.*,1982
E. dunnii	Seeds	Somatic embryogenesis	Termignoni *et al.*, 1996
E. grandis	Zygotic embryos, hypocotyls	Somatic embryogenesis	Major *et al.*, 1997
E. grandis	*In vitro* shoots	Somatic embryogenesis	Watt *et al.*, 1991
E. citriodora	Decoated seeds	Somatic embryogenesis	Muralidharan *et al.*, 1989
E. grandis, nitens	Cotyledons, hypocotyledons	Somatic embryogenesis	Bandyopadhyay *et al.*, 1999
E. globulus Labill.	Zygotic embryos, floral tissue	Somatic embryogenesis	Nugent *et al.*, 1997

There are authors who contend that the use of tissue culture techniques to propagate *Eucalyptus* species is unnecessary, since the time for growth and morphology between micropropagated and macropropagated plants appears to be the same. Wilson (1996) pointed out that the time period for *in vitro* propagation of *Eucalyptus* plants and propagation through stem cuttings was about the same (56 weeks), and that the rapid multiplication observed *in vitro* was offset by the greater time required for 'pricking-out', acclimatisation and growth in a nursery. Moreover, comparing the morphology of micropropagated and macropropagated trees, workers have observed that there was no significant difference in tree height, volume, diameter at breast height between micropropagated plants of *Eucalyptus* species (Rockwood and Warrag, 1994; Yang *et al.*, 1995). However, in a study by Watt *et al.* (1995), which compared the growth of micropropagated and macropropagated *E. grandis* hybrids, it was concluded that micropropagated plants were superior to those of plants produced by rooted cuttings, in terms of survival, uniformity, annual growth and diameter at breast height.

In terms of the implementation of *in vitro* propagation methods, the most successful source material for woody plant tissue culture is juvenile tissue, however, the suitability of this approach for the propagation selected elite genotypes is questionable. The reason for this is that the selection of trees for superior wood traits can only occur once they have reached maturity, at which point researchers begin experiencing difficulties in manipulating them *in vitro* (McCown and Russell, 1987). However, techniques for overcoming the problem of maturity in proven genotypes, by stimulating rejuvenation through spraying with growth regulators, serial grafting on juvenile rootstock, coppicing and the use of orthotropic shoots, have been discussed and attempted by workers, with varying degrees of success (McComb and Bennet, 1982; Bonga, 1987). Explants obtained from such treated superior stock plants can then be subjected to *in vitro* manipulations via techniques such as axillary bud proliferation, which will be discussed further (section 3.1.3).

3.1.3 *In vitro* propagation via axillary bud proliferation and factors affecting this process

Axillary bud proliferation makes use of the normal route of lateral branch development from axillary meristems located in leaf axils. Each of these axillary buds is capable of developing into a lateral shoot, which is comparable with the main shoot (Hussey, 1978; Phillips and Hubstenberger, 1995).

Lateral shoots can be detached or layered for subsequent rooting. Each axillary shoot can in turn, produce its own axillary meristems and therein lies the potential for unlimited proliferation from a single meristem (Hussey, 1978). The axillary buds are treated with hormones to break dormancy and produce multiple shoot branches (Phillips and Hubstenberger, 1995). Since the young shoot apex is an active site for auxin biosynthesis, exogenous auxin is not always needed for bud-break, especially when relatively large explants from actively growing plants are used (Hu and Wang, 1983). Multiplication of the propagule is meant to achieve the maximum number of propagatory units and, according to Hu and Wang (1983), is the major economic criterion for successful commercial tissue culture propagation. It seems that the mechanism controlling the development of axillary buds appears to involve a combination of hormones (Nashar, 1989; George, 1993), and it has been observed that the application of cytokinin to cultured buds leads to rapid outgrowth and proliferation of otherwise inhibited buds (Hussey, 1978), since cytokinin helps in overcoming apical dominance (Zaerr and Mapes, 1982; Hu and Wang, 1983; Nashar, 1989; George, 1993).

After multiplication, the shoots are then separated and elongated before being rooted to produce plants. Alternatively, the shoots can be used as propagules for further propagation. Hu and Wang (1983) have observed that the elongation of shoots can often be inhibited by high residual cytokinin levels from the multiplication stage. Rooting depends on a complex interaction of stimulating and inhibiting substances in the buds as well as in the growing roots, and these substances have to be properly manipulated before successful rooting can be achieved (Bonga, 1977). Axillary bud proliferation typically

results in an average tenfold increase in shoot number per monthly culture passage, which makes it an attractive technique in the commercial propagation of forest tree species (Phillips and Hubstenberger, 1995).

The successful cultivation of viable plants *in vitro* via axillary bud proliferation depends on a number of factors that can impact either negatively or positively on the various stages in the *in vitro* propagation process (Thorpe, 1980; 1983; Ammirato, 1986). These include several parameters in the culture environment, such as chemical constituents, as well as physical factors affecting the culture environment. However, biological factors such as the source and preparation of the explant, together with sterilisation procedures to eradicate microbial contaminants also affect *in vitro* growth and development.

3.1.3.1 Biological factors

a) Selection of explant and responses to in vitro conditions

The first stage in the technique of propagating plants via axillary bud proliferation is the establishment of cultures using nodal explants. Even with the use of nodal explants as the starting material, several important factors have to be taken into consideration to achieve successful culture establishment. These include the season in which the explant is obtained, the size of the explant and the overall quality of the plant from which the explants are obtained (Thorpe, 1980; Cohen, 1986).

The position of the explant on the parent plant is another important factor to be considered in the choice of explant. Durand-Cresswell and Nitsch (1977) noted that the position of nodal explants on the parent tree played a vital role in determining the rooting ability of the cutting. Cohen (1986) was able to corroborate this by his assertion that explants may retain a memory of their position on the parent plant, which would affect their subsequent growth and development and is stable over several subcultures. Durand-Cresswell and Nitsch (1977) further observed that nodes from young plants were easy to root, but that in larger trees, only nodes in the region of actively growing,

photosynthesising leaves would be productive. Moreover, nodal explants harvested from the crown of the tree were the most successful in terms of rooting frequency, whereas nodes with senescing or abscissing leaves rarely rooted. Chang *et al.* (1992) observed that nodal explants from coppice shoots responded better to *in vitro* manipulations than explants taken from mature trees of *Eucalyptus radiata* ssp. *radiata*. This trend was confirmed in a study by Patil and Kuruvinashetti (1998), using *E. tereticornis*. The exudation of phenolics by the explant in response to sterilisation and cultural manipulations is also a serious problem and often leads to growth inhibition and even explant necrosis if untreated, however, authors have overcome this problem by including antioxidants in growth media (Jones and van Staden, 1991; George, 1993; Karkonen *et al.*, 1999).

b) Genotypic effects

From the earliest studies of morphogenesis in culture, it was recognised that certain groups of plants appeared to respond more readily in culture than others. Studies revealed that different species within a genus (Le Roux and van Staden, 1991a) and especially, different cultivars within a species responded differently in culture, and that there were genotype-dependant differences in the ease of plant regeneration (Ammirato, 1986). McComb and Bennet (1986) observed that multiplication rates of *Eucalyptus* differed, depending on the genotype and Laine and David (1994) recorded differences in growth regulator requirements for organogenesis between different clones of *E. grandis*. Trindade and Pais (1997) noted that different clones of *E. globulus* exhibited different requirements for successful rooting and furthermore, rejuvenation was achieved after different periods of culture for each clone. Yasodha *et al.* (1997) found that the availability of *in vitro* shoots of a height suitable for rooting was also genotype-dependant. Das and Mitra (1990) observed that for various clones of *E. tereticornis*, responses to standard conditions for each culture stage differed according to genotype. Grattapaglia *et al.* (1990) stated that one of the key factors contributing to the successful large-scale propagation of *Eucalyptus* species and hybrids was the intrinsic propagation potential of the clone. Yu and Reed (1993) observed that genotypic differences between clones of *Corylus avellana* resulted in variation in the number of sufficiently elongated

shoots that were available for rooting. Donald and Newton (1991) also observed clonal specificity in terms of the shoot yields achieved during multiplication of *Eucalyptus radiata*. This genotypically-dependant factor would give rise to differences in yield, despite such application of a standard micropropagation protocol. The fact that each genotype has distinct nutrient requirements *in vitro* could contribute to the high cost factor recognised by Biondi and Thorpe (1981) and render the application of *in vitro* techniques costly until this problem is overcome.

3.1.3.2 Sterilisation of the explant

Explant material harvested from the field, and even from stock plants grown under greenhouse conditions, carry with it a plethora of fungal and bacterial flora which must be eradicated before the successful establishment of cultures can be attempted. Although surface sterilisation procedures may eradicate surface contaminants, contamination may still occur at a later stage as a result of endogenous bacterial and fungal propagules within the explant. These microbial contaminants can not only prove detrimental to the *in vitro* plant, but can also modify the composition of the nutrient medium, producing anomalous results. It is thus imperative that these contaminants existing both on the surface of the explant and systemically be eradicated prior to any attempts to achieve successful *in vitro* propagation. Approaches to achieving asepsis have been discussed in Chapter 2.

3.1.3.3 Chemical Factors

Plant culture media are based on different concentrations of macro-and micro-nutrients, vitamins, carbon sources, growth regulators and gelling agents. It has been clearly established that the manipulation of these chemical factors has achieved various effects on the growth, development, yield and morphology of *in vitro* plants (Thorpe, 1980; Le Roux and van Staden, 1991a; George, 1993).

a) Macro- and micro-nutrients

Several different basal nutrient formulations containing different concentrations and organic and inorganic nutrients have been reviewed by various authors (Le Roux and van

Staden, 1991a; George, 1993). These form the basis of plant growth media and are distinct for different species, explants and even for various developmental stages. The most commonly-used formulation is MS (Murashige and Skoog, 1962) (Le Roux and van Staden, 1991a; George, 1993), but the use of other nutrient formulations has also been documented, as discussed. The efficacy of various macronutrient solutions in the absence of growth regulators was tested by Bon *et al.* (1998), and it was observed that Knop's solution was the least effective in inducing proliferative responses from both juvenile *Paraserianthes falcataria* and *Acacia mangium* explants, compared to MS medium (Murashige and Skoog, 1962). However, MS medium resulted in hyperhydricity in *Prunus maximowiczi* and *Prunus nipponica* cultures and both *Salix* species and *Lonicera chamissoi* were successfully cultured on nutrient media with reduced salt concentrations (Karkonen *et al.*, 1999). The *in vitro* elongation of shoots of *Auracaria* was successfullly carried out on White's medium (Maene and Debergh, 1987). This same medium was also used successfully to multiply shoots of *Dalbergia latifolia* (Raghava Swamy, 1992). Depending on the species, Mascarenhas *et al.* (1981) used either MS nutrients or White's basal medium to propagate *Tectona grandis*, *E. citriodora*, *Tamarindus indica* and *Punica granatum.* Yasodha *et al.* (1997) were able to use MS medium for shoot multiplication of *Eucalyptus* but transferred the shoots to medium with Knop's nutrients for root induction. The concentration of the nutrient medium is also important. It has been observed, for example that MS nutrients (Murashige and Skoog, 1962) at less than full strength is desirable for rooting elongated shoots in many tree species (McComb and Bennet, 1982; Raghava Swamy *et al*, 1992; Deshpande *et al.*, 1998; Sha Valli Khan *et al.*, 1999). Further, Franclet and Boulay (1982) achieved high frequency rooting of frost-resistant Eucalyptus species by using a diluted Knop's medium. A sequence of three different nutrient solutions was employed to root shoots of *E. grandis*, where shoots were transferred from White's medium to half-strength MS with charcoal, followed by half-strength MS liquid medium (Sankaro Rao and Venkateswara, 1985).

b) Vitamins

Growth and morphogenesis of plant tissue cultures can be improved by small amounts of organic compounds. Vitamins are required by animals in very small amounts as

necessary ancillary food factors, and many of these same compounds are also needed by plant cells as essential factors in metabolic processes. Although intact plants are able to produce their own requirements, *in vitro* plant cultures can become deficient in some factors, and growth and survival can then be improved by addition of vitamins to culture media (George, 1996).

c) Carbon sources

A source of carbon is an essential component of plant growth media, since most culture systems are heterotrophic or mixo-trophic (Kozai, 1988; George, 1993). Although George (1993) hazarded that the presence of sucrose in plant tissue culture media specifically inhibits chlorophyll and makes autotrophic growth less feasible (George, 1993), this carbohydrate has been found to be the best carbon source, together with glucose for supporting growth. The use of other monosaccharides (e.g. arabinose, xylose), disaccharides (e.g. cellobiose, maltose and trehalose) and polysaccharides, all of which are capable of being broken down to glucose and fructose has also been documented (George, 1993). Lou *et al.* (1996) observed that the concentration of sucrose played a major role in *in vitro* development - higher concentrations of sucrose appeared to weaken the response of cucumber cotyledonary explants to 2,4-D. Cheng *et al.* (1992) reported that the concentration of sucrose played a vital role in determining the rooting efficiency of *E. sideroxylon*, in that 2-6% favoured rooting, whilst 8-10% proved detrimental to cultures. Although previous studies showed that fructose was necessary for successful elongation of *Morus alba*, Karkonen *et al.* (1999) were able to use sucrose as a carbon source and did not experience inhibition of shoot elongation. Yu and Reed (1993) found that the proliferation response of *Coryllus avellana* was enhanced by the use of glucose as a carbon source.

d) Growth regulators

In manipulating the axillary bud proliferation, many growth regulators have been included in the culture medium. Plant growth regulators are essential in achieving the induction and control of morphogenesis in axillary bud proliferation (Thorpe, 1980; Zaerr and Mapes, 1982; Ammirato, 1986). A given plant growth regulator may have a range of

effects in different plant species and even in different organs. Without the addition of plant growth hormones, most tissues do not remain viable, much less grow in the particular manner desired (Zaerr and Mapes, 1982), although the culture of plants in the absence of growth regulators has also been reported (Bon *et al.*, 1998; Meszaros *et al.*, 1999). Several authors have observed that the ratio between auxin and cytokinin determines morphogenesis *in vitro* (Thorpe, 1980; Hu and Wang, 1983; Nashar, 1989; Trindade *et al.*, 1990; George, 1993). In the context of axillary bud proliferation, Trindade *et al.* (1990) found that exogenous cytokinin tended to be a major limiting and controlling factor in shoot multiplication of *E. globulus* and further, that BAP (benzylaminopurine) was more effective in stimulating multiplication than kinetin. This was confirmed in a study by Puddephat *et al.*(1997) on the *in vitro* establishment of *Quercus robur*. Lakshmi Sita (1986) observed that increasing concentrations of BAP resulted in increased shoot proliferation of *E. grandis*. However, in shoot multiplication of *E. sideroxylon*, low concentrations of BAP and NAA were more effective for stimulating multiplication than higher concentrations of the same growth regulators (Burger, 1987). Puddephat *et al.*(1997) observed that higher concentrations of IBA resulted in a higher number of roots produced per shoot, together with the production of basal callus in *Quercus robur*. Similarly, lower concentrations of IBA and NAA resulted in a higher percentage rooting with little or no callus formation in *E. grandis* (Lakshmi Sita, 1986). Also, Burger (1987) found that IBA was more effective in stimulating adventitious rooting of *E. sideroxylon* shoots than NAA.

Activated charcoal in tissue culture is usually used to adsorb metabolites that would otherwise inhibit growth and subsequent development *in vitro* (Fridborg *et al.*, 1978; Franclet and Boulay, 1982; Sankaro Rao and Venkateswara, 1985; Abdullah *et al.*, 1986; Jones and van Staden, 1991). Over and above the 'traditional' plant hormones used in tissue culture media, other growth regulators have also been successfully used to improve axillary bud proliferation. The use of thidiazuron was documented by Huetteman and Preece (1993), who noted that low concentrations induce greater axillary proliferation than other cytokinins, although inhibition of shoot elongation in woody species was a possible shortfall. Carmen-Feijoa and Iglesias (1998) noticed that the use of thidiazuron

rather than BAP (Benzylaminopurine) also produced satisfactory results in the *in vitro* propagation of *Gentiana lutea* via bud proliferation. Ibanez and Amo-Marco (1998) successfully used phloroglucinol to micropropagate *Mimuartia valentina* via axillary bud proliferation.

e) Gelling agents

Plant organs and tissues are most suitably retained above the surface of a culture medium by increasing its viscosity with some kind of gelling agent. Although several suitable gelling agents are available, results obtained on each have differed markedly. Hence, several considerations in terms of the use of gelling agents in media formulations have to be considered. Differences in morphology, yield and growth have been observed when different types of gelling agent have been used, and moreover, different brands of the same gelling agent. An additional consideration is whether or not gelling agents should be included in the medium, since this has also been observed to have significant effects on yield, morphology and even contamination of plant cultures. Agar has been traditionally employed as the preferred gelling agent for tissue cultures and various brands and grades are commercially available, each differing in the amounts of impurities they contain and the gelling capabilities (George, 1993). Many authors have observed that the use of different agar brands has had significant effects on yields and *in vitro* development of tissue cultures. In a study by Scholten and Pierik (1998), it was observed that agar quality affected the growth and development of axillary shoots and roots of *Rosa hybrida* and further, that different batches of the same agar actually resulted in colour differences of axillary shoots of *Quercus robur*. Debergh (1983) stated that brand and concentration of agar affected the chemical and physical characteristics of tissue culture media, with low concentrations of agar inducing hyperhydricity through the action of cytokinins.

Gorinova *et al.* (1993) compared the effect of using agar and microcrystal cellulose as gelling agents for *in vitro* cultures of *Nicotiana tabacum* and observed that the latter resulted in higher yields and proved a suitable and cheaper alternative to agar. Gelrite is a gellan gum, a heteropolysaccharide produced by the bacterium *Pseudomonas elodea*,

which has found increasing application in the preparation of tissue culture media as a cheaper alternative to agar (George, 1993). Further, it produces a clear gel which makes examinations of *in vitro* cultures easier. However, as with agar, low concentrations result in the occurrence of hyperhydricity (George, 1993). Huang *et al.* (1995) observed that Gelrite resulted in superior shoot proliferation and rooting of bamboo and *Ficus benjamina* compared to cultures grown in agar. Adventitious bud production in *Torenia fourneiri* was promoted by Gelrite and moderately by agarose of Bacto-agar, but bud induction was suppressed by agar (Huang *et al.*, 1995). Similar results, in terms of the superiority of Gelrite, compared to agar, were also observed in our laboratories. In a study by Macrae and van Staden (1990), the effect of the gelling agents agar, agarose and Gelrite on axillary bud proliferation of *Eucalyptus grandis* was examined. Results indicated that shoot multiplication as well as elongation was superior on Gelrite-containing media than on media that had been gelled with agar and further, rooting of elongated shoots on Gelrite-containing-media was better than on media containing agar (Macrae and van Staden, 1990). Other gelling agents, such as starch, have also been used. For example, Zimmerman *et al.* (1995) used a combination of Gelrite and cornstarch in media for the tissue culture of apple, raspberry and pear, and observed that this medium was superior in terms of stimulating shoot proliferation, than agar-gelled media.

3.1.3.4 Physical Factors

Further to the effect of chemical constituents on *in vitro* plant growth and development, various physical factors also impact on developmental process occurring during organogenesis via axillary bud proliferation. These include, light, temperature, ventilation and humidity.

a) Light and photoperiod

Light is a major factor in the culture environment and has been shown clearly to have an effect on organised development *in vitro*. The light requirements for development involve a combination of components, including intensity, photoperiod and quality, and these differ for various species and developmental processes. Zelena (2000) further observed

that light did not significantly affect the amount of IAA (indole-3-acetic acid) taken up by shoot segments, but did increase its rate of metabolism and stimulated the conversion of IAA into IAAsp (indole-3-acetyl aspartate). When explants of Pigeonpea were cultured under 16h light/8 h dark conditions, shoots were initiated after only 65 days in culture, but this ability was lost when explants were cultured under continuous light (Franklin *et al.*,2000). Puddephat *et al.* (1997) observed that daylength also influenced *in vitro* growth and development of shoots in *Quercus robur*. Karkonen *et al.* (1999) reported that photoperiod requirements of several japanese woody species depended on the latitude, with higher latitude species requiring longer photoperiods for apical growth cessation. Niimi *et al.* (1999) observed that, depending on the species of *Lillium*, either light or dark conditions stimulated growth of bulblets. The induction of root primordia can be stimulated in the dark, and Furze and Cresswell (1985) rooted shoots of *E. grandis* and *E. nitens* by subjecting them to dark conditions for 7-10 days prior to root elongation. Also, the activity of peroxidase during *in vitro* rooting of *Nothofagus* depended on the light and species, with peroxidase levels registering higher in light conditions, and the period for the induction of rooting was shorter in darkness (Calderon-Baltierra *et al.*, 1998). Noe *et al.* (1998) noted that *in vitro* growth and proliferation of *Vaccinium corymbosum* was affected by the wavelength and spectral composition of light to which the cultures were exposed: shoots exposed to a higher photosynthetic photon flux density (PPFD) showed dramatic reddening of leaves, and this effect was prevented by decreasing the wavelength shorter than 520nm. Further, the proliferation rate was generally depressed when wavelengths were between 650 – 760 nm (Noe *et al.*, 1998). Blomstedt *et al.* (1991) observed that a high light intensity tended to inhibit rooting of juvenile *E. regnans*. Horgan (1987) noticed that high light intensities allowed for better survival and growth of shoot clumps of *Pinus radiata*, following subculture. Ross-Karstens *et al.* (1998) also found that enhancing the light intensity resulted in an increase in stomatal density in coffee plantlets.

b) Temperature

Temperature effects on *in vitro* development have not been thoroughly evaluated and the general practise has been to maintain cultures at a constant temperature environment of

approximately 25°C. However, authors have observed that varying the temperature of the culture environment affects the rate of plant development. Puddephat *et al.* (1997) observed that higher temperatures stimulated shoot formation in *Quercus robur*. Niimi *et al.* (1999) also observed that temperature played a major role in stimulating the regeneration of bulblets. However, incubation at a lower temperature (15°C) of *E. citriodora* shoot cultures was essential for inducing shoot development in terminal buds (Gupta *et al.*, 1981). Franclet and Boulay (1982) observed that a lower temperature was necessary to induce rooting in frost resistant *Eucalyptus* species. Misra (1999) reported that, with regards to the elongation of shoots and roots in *E. nitens* and *E. globulus*, for both species, shoot and root elongation increased with an increase in temperature.

c) Ventilation, humidity and the in vitro gaseous environment

Confined conditions with culture vessels, together with the heating of the bottom of the vessel by artificial lighting contribute towards the high relative humidity in *in vitro* culture conditions (DeBergh *et al.*, 1992). The regulation of relative humidity, ventilation and gaseous composition within the micro-environment of the culture vessel has been shown to have an effect on the morphology and development of plants *in vitro*. Further, Thorpe (1980) suggested that regulating the relative humidity may be necessary for specific forms of organogenesis. Haisel *et al.* (1999) observed that the high humidity and low air turbulence within tightly sealed cultivation vessels induced that formation of plantlets with abnormal morphology, anatomy and physiology. Low carbon dioxide concentrations during exposure of *in vitro* plants to light also resulted in the limitation of photosynthetic rates and biomass production (Haisel *et al.*, 1999). Murphy *et al.* (1998), working with *Delphinium*, observed that multiplication rates *in vitro* and survival *ex vitro* were improved by the inclusion of small apertures with filters in the sides of plastic culture vessels. This result was confirmed by Zobayed *et al.* (1999), who noted that the ventilation of culture vessels could play a significant role in determining the *in vitro* growth and development of plants, since sealed vessels containing plants of *Brassica* exhibited poor shoot growth, with little leaf and shoot number, weight and callus volume. Ross-Karstens *et al.* (1998) reported that grape and coffee plantlets grown under *in vitro* conditions of continuous airflow with elevated carbon dioxide exhibited a reduced

stomatal density than those grown in hermetically sealed culture vessels. Of the five woody plant species tested, only *Betula* exhibited enhanced shoot length in high humidity micro-environments resulting from tight vessel closure (McClelland and Smith, 1990). In the other species tested by those authors (*Amelanchier, Malus, Forsythia* and *Acer*) this environment resulted in depressed growth and the prevalence of vitrified shoots. A great deal of interest has been generated about photoautotrophic growth *in vitro* and there have been suggestions that carbon dioxide is a major limiting factor during *in vitro* stages (DeBergh *et al.*, 1992). Jackson *et al.* (1991) observed that the accumulation of ethylene in culture vessels resulted in a reduction in leaf size by about 50% in *in vitro* plants of *Ficus lyrata*.

3.1.4 Aim

At the Mondi Mountain Home Laboratory (Hilton, Kwazulu-Natal, South Africa), the micropropagation, via axillary bud proliferation, of several elite and highly selected clones of *Eucalyptus* hybrids is carried out. As the clones have distinct requirements *in vitro*, the preparation of various media specific for each genotype is costly and time-consuming, which impacts negatively on the commercial application of the technique. The aim of this study was, therefore, to establish a single protocol that could be applied successfully for all the clones of three commercially important *Eucalyptus* hybrids, *viz. E. grandis* x *nitens* (GN), *E. grandis* x *nitens* (NH) and *E. grandis* x *urophylla* (GU). The approach used was to test various established media for each stage of the *in vitro* developmental process. These media were based on previously successful studies carried out on the species and hybrids under study. The objective was to determine a single high-yielding medium for each culture stage that could be used for all the clones of each hybrid.

3.2. Materials and Methods

3.2.1 Explant material

Nodal explants were harvested from stock plants of the tested hybrid clones maintained in the greenhouse, as outlined in Chapter 2 (section 2.2.2). These were sterilised according to the optimum protocol devised for all tested clones (Chapter 2).

3.2.2 Bud-break

Leaves on nodal explants were trimmed to roughly two-thirds their original size, before *in vitro* manipulations. Three different methods for effecting bud-break were tested. Method 1 involved placing four sterilised nodal explants per 50mm x 75 mm culture bottle, which contained 20ml of a standard bud-break medium, developed in our laboratories (MS nutrients, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose and 3.5g/l Gelrite, pH 6.2). Nodal explants were placed in culture bottles containing a multiplication medium specific for each hybrid (MS nutrients, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, 3.5 g/l Gelrite, pH 6.2 with either 0.2 mg/l BAP, 0.01 mg/l NAA and 25 g/l sucrose (GN and GU clones) or 0.2 mg/l BAP and 20g/l sucrose added (NH clones) (Method 2). Excised buds were also placed directly onto the above-mentioned multiplication medium (Method 3). Cultures were maintained for 2 weeks, at 25°C ± 2°C day/ 21°C night and a 16 h light/ 8 h dark photoperiod at a PPFD of 66μmol/m^2/s.

3.2.3 Multiplication

Shoots obtained after bud-break were used to test four different multiplication media. These all contained MS nutrients, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 20g/l or 25g/l sucrose and 3.5g/l Gelrite, pH 6.2, with various concentrations of growth regulators

(Table 3.4). Four shoots were maintained in each culture bottle containing 20ml of multiplication media, for six weeks with one sub-culture onto fresh medium of the same formulation after three weeks. Cultures were maintained for 6 weeks, at 25°C ± 2°C day/ 21°C night and a 16 h light/ 8 h dark photoperiod at a PPFD of 66μmol/m^2/s.

3.2.4 Elongation

Individual shoots were excised from shoot clumps obtained after multiplication. Four shoots were transferred to each culture bottle containing 20ml of elongation medium, consisting of MS nutrients, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, 0.35mg/l NAA, 0.1mg/l kinetin, 0.1mg/l IBA, 20g/l sucrose and 3.5g/l Gelrite (pH 6.2). Cultures were maintained for three to four weeks at 25°C ± 2°C day/21°C night, under a 16 h light/8 h dark photoperiod at a PPFD of 66μmol/m^2/s. Shoots were then subcultured onto fresh medium of the same formulation and maintained for a further three weeks, either under the same conditions outlined above, or cultures were kept in the dark at room temperature (~25°C).

Table 3.4. Formulations of tested multiplication media. All media contained MS nutrients, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, and 3.5g/l Gelrite® at pH 6.2 before autoclaving at 121°C for 20 minutes.

Medium	Formulation
M1	0.2mg/l BAP, 20g/l sucrose
M2	0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose
M3	0.5mg/l BAP, 0.2mg/l NAA, 20g/l sucrose
M4	0.1mg/l BAP, 0.01mg/l NAA, 0.2mg/l kinetin, 25g/l sucrose

3.2.5 Rooting

Suitably elongated shoots (≥ 20mm long) were each transferred to a 25mm x 100mm culture tube (1 shoot/tube) containing 10ml of the tested rooting medium, based on previous successful rooting studies in our laboratories (Table 3.5). All media contained ½ or ¼ MS nutrients (Makwarela, 1996; Mokotedi, 1999) 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 15g/l sucrose and 3.5g/l Gelrite, pH 5.8, with different concentrations and combinations of growth regulators and inorganic additives. Cultures were initially maintained in the dark at room temperature (~25°C for 72 hours, before being transferred to a 16 h light/ 8 h dark photoperiod and a PPFD of 37 μmol/m^2/s at 24°C day/ 21°C night for seven days. Following this, cultures were transferred to a 16 h light/ 8 h dark photoperiod and a PPFD of 66μmol/m^2/s at 25°C ± 2°C day/ 21°C night for 28 days.

Table 3.5. Formulations of tested rooting media. Unless otherwise stated, all media contained ½ MS nutrients, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, and 3.5g/l Gelrite at pH 5.8 before autoclaving.

Medium	Formulation
RM 1	1 mg/l IBA
RM 2	0.1 mg/l IBA
RM 3	0.1 mg/l IBA, 0.5 mg/l NAA
RM 4	¼ MS, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$, 0.1mg/l IBA

3.2.6 Analysis of results and photography

The mean and standard error was determined from an average of three replicates. Data were analysed using a one way analysis of variance (ANOVA) and means were contrasted using a Scheffe's multiple range test (95% confidence interval). Photographs were recorded with a Nikon FM2 camera, fitted with a 60mm Mikro Nikkor lens.

3.3 Results and Discussion

As discussed previously in this chapter, *in vitro* propagation via axillary bud proliferation can realise high plantlet yield returns. This method involves the initiation of axillary buds from leaf axils of sterilised nodal explants for 1-2 weeks (Figure 3.1), which are then excised from the nodal explant and are induced to proliferate on a multiplication medium usually containing auxins and cytokinins, for 3-4 weeks (Figure 3.2). Once established, individual shoots are excised from the shoot clump, elongated *in vitro* (Figure 3.3), and then rooted for 3-4 weeks (Figure 3.4). Finally, the established plantlets are hardened-off in the greenhouse (Figure 3.5). Each *in vitro* culture stage is usually characterised by specific growth media and culture conditions, a situation that may be complicated if different genotypes have distinct nutrient requirements. This study aimed to establish high-yielding media for each of the *in vitro* culture stages that could be applied successfully to all clones of each of three *Eucalyptus* hybrids under study. The first stage in the study involved devising a suitable surface sterilisation method for all tested clones, as presented in Chapter 2. In this study, therefore, explants of all clones were sterilised according to the established protocol (Section 2.3.3).

Figure 3.1. **The initiation of axillary buds from sterile nodal explants.** Bar= 1cm

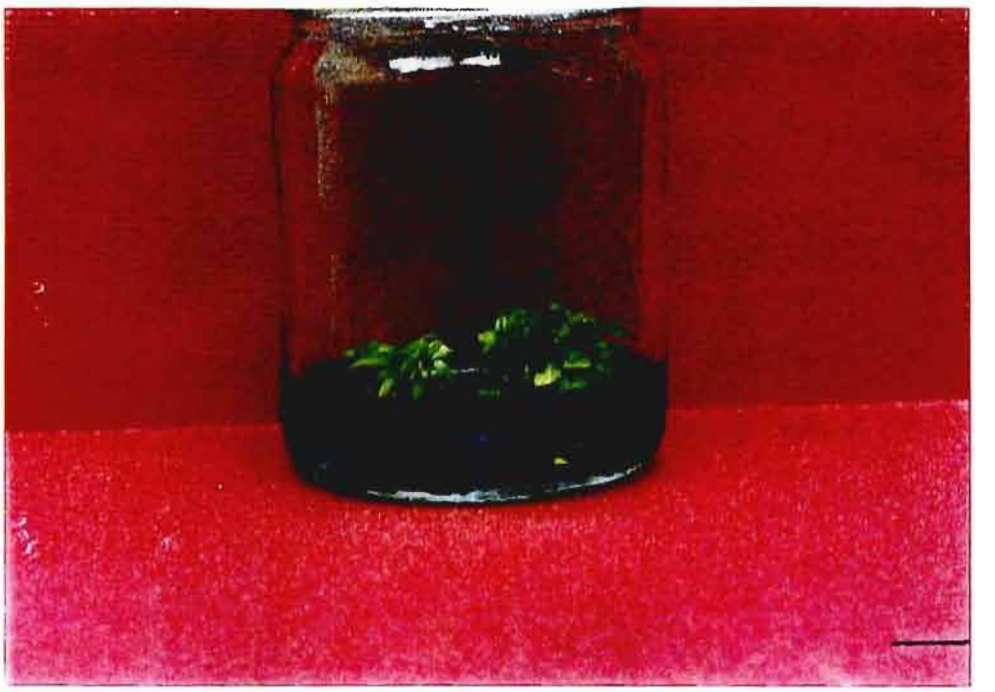

Figure 3.2. **Proliferation of excised axillary buds.** Bar= 1.2 cm

Figure 3.3. **Elongation of individual shoots.** Bar= 1 cm

Figure 3.4. ***In vitro*** **rooting of elongated shoots.** Bar= 1.25 cm

Figure 3.5. **Hardened-off plantlet**. Bar= 2 cm

3.3.1 The effect of bud-break method on the initiation of axillary buds

Following the surface sterilisation of nodal explants, the next step in the process of axillary bud proliferation, as outlined above, involves the initiation of axillary buds from the axils of nodal explants. This process is achieved by subjecting the nodal explants to culture media and growth conditions that encourage the outgrowth of axillary buds, which are then excised and multiplied (Hussey, 1978; Hu and Wang, 1983; Le Roux and van Staden, 1991a; George, 1993; Phillips and Hubstenberger, 1995).

In this study, three different bud-break methods were tested to examine their efficacy in inducing axillary bud-break, and their effect on shoot/bud yields three weeks after transfer to multiplication medium. Method 1 involved placing the nodal explants on a distinct bud-break medium (MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose, 3.5g/l Gelrite) for 1-2 weeks then transferring the excised buds to a different medium for shoot multiplication. Both the bud-break (using nodal explants) and multiplication stages were carried out on a single medium in method 2. Method 3 followed the same protocol as method 2, differing only in that excised axillary buds rather than nodal explants, were used as primary explants.

Table 3.6 shows the bud-break, necrosis and shoot yields for five clones of *E. grandis* x *nitens* (GN), achieved in response to the three tested bud-break methods. Necrosis, bud-break and shoot yields were compared among clones for each bud-break method. Results indicated that with two exceptions, for all the methods, these three parameters did not differ significantly among clones. The exceptions were necrosis and bud-break percentages (67%-85% among clones) obtained using method 2. Method 3 failed to elicit any bud-break response from all five clones (0.0%) and necrosis levels were high in some clones (4.8-41.1%). Of all the three methods, method 1 resulted in low necrosis (0.0-6.9%) and high bud-break levels (87.5-90.3%) and moderate shoot yields (2-4 shoots/bud) in all the clones.

An examination of the effect of all three bud-break methods on each clone confirmed that method 1 was the best choice for bud-break of *E. grandis* x *nitens* (GN) clones. Bud-break values for GN 9 (90.27 ± 1.38 %; p= 0.0000), GN 15 (87.5 ± 2.41 %; p= 0.0000) and GN 121 (90.27 ± 1.38 %; p= 0.0000) were significantly higher at the 95% confidence interval, using method 1, compared to values achieved using methods 2 and 3. However, there was no significant difference in bud-break values achieved using method 1 and 2, for GN 1 (90.3 ± 3.67 % for method 1, 85.5 ± 1.42 % for method 2; p= 0.0000) and GN 108 (88.9 ± 1.38 % for method 1, 82.6 ± 3.08 % for method 2; p= 0.0000). Method 3 (excised buds) completely failed to elicit any bud-break response from all tested GN clones (0.0%) and also resulted in necrosis levels in all clones (4.8 ± 4.76 %, p= 0.5198 for GN 1; 5.7 ± 2.67 %, p= 0.0763 % for GN 9; 16.6 ± 0.36 %, p= 0.1036 for GN 15; 36.1 ± 16.0 %, p= 0.06099 for GN 108; 41.1 ± 8.84 %, p= 0.0106 for GN 121) that were higher than those achieved for methods 1 and 2 (Table 3.6). Shoot yields were recorded three weeks after transfer to multiplication medium (0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose) and results showed that, for each of the five clones, there was no significant difference in shoot yields between method 2 and 3. However, for all five clones, the highest shoot yields were obtained using method 1 (2-4 shoots/bud among clones). Thus, method 1 was the option selected for initiation of axillary buds for clones of *E. grandis* x *nitens* (GN).

A similar approach was used for *E. grandis* x *nitens* (NH) clones (Table 3.7). The results indicated that for method 1, necrosis did not differ significantly among clones, and further, with the exception of NH 0, bud-break and shoot yields were not statistically different among clones (Table 3.7). The same trend was observed using method 2. For method 3, necrosis levels did differ significantly among clones, with NH 69 and NH 70 exhibiting significantly higher necrosis levels than NH 0 and NH 58 clones (Table 3.7). This method did not result in bud-break values and shoot yields that differed significantly among clones (Table 3.7). Of the three methods, method 1 resulted in the lowest necrosis levels (6.9-27.8 %) and moderately low to relatively high bud-break values (17.4-75 %) for all the clones.

In terms of comparing the efficacy of the three bud-break methods for each clone, method 1 resulted in the highest bud-break values for all four clones (75 ± 9.6 % for NH 0; 33.3 ± 4.2 % for NH 58; 17.4 ± 1.3 % for NH 69; 20.2 ± 8.1 % for NH 70), however, these values were not significantly different to those obtained using method 2 (66.7 ± 7.2 %, p= 0.0026 for NH 0; 22.2 ± 11.9 %, p= 0.2858 for NH 58; 8.8 ± 3.9 %, p= 0.0120 for NH 69; 9.7 ± 5.0 %, p= 0.1784 for NH 70). However, the highest necrosis levels were recorded using method 2 for clones NH 0 (20.8 ± 4.2 %; p=0.0312) and NH 58 (41.7 ± 4.8 %; p=0.0054). Clones NH 69 and NH 70 exhibited no significant difference in necrosis among the three methods (26.2 ± 8.5 % for method 1, 26.3 ± 2.9 % for method 2, 50.0 ± 7.2 % for method 3; p= 0.0712 for NH 69) (26.8 ± 3.3 % for method 1, 34.7 ± 9.7 % for method 2, 34.7 ± 3.7 % for method 3; p= 0.6148 for NH 70). Method 1 gave the highest shoot yield only for NH 0 (2 ± 0.14 shoots/bud), whereas shoot yields for NH 58, NH 69 and NH 70 did not differ significantly for the three methods.

Hence, despite bud-break and shoot yields that were highly genotype-dependent and varied from clone to clone, bud-break method 1 was selected as the method of choice for clones of *E. grandis* x *nitens* (NH) clones. However, it is recognised that further study may be necessary to establish a better bud-break medium that can be used successfully for all clones of this hybrid.

Results from similar bud-break experiments with five clones of *E. grandis* x *urophylla* (GU) are presented in Table 3.8. Using method 1, necrosis values did not differ significantly among clones (0.0%) with the exception of GU 21 (2.8 ± 1.38 %; p= 0.519) (Table 3.8). Similarly, for bud-break, there was no significant difference in values among three of the four clones, with only GU 21 exhibiting higher bud-break values (97.2 ± 1.38 %; p= 0.1817) (Table 3.8). Further, there was no significant difference in shoot yield using method 1. For method 2, both necrosis and bud-break values did not differ significantly among clones, whilst shoot yield differed only for GU 244 (3 ± 0.35 shoots/bud) (Table 3.8). Method 3 was the only method for which all three parameters were not statistically different among all four clones (Table 3.11). Method 2 resulted in

low necrosis values (1.4-8.3 %) and moderate bud-break values (80.6-91.7 %) and shoot yields (1-3 shoots/bud) for all the clones and was thus selected as the bud-break method for this hybrid.

An assessment of the effect of the three bud-break methods on each clone, indicated that bud-break for three clones did not differ significantly among methods 1, 2 and 3: GU 21 (97.2 ± 1.38 % for method 1, 91.6 ± 4.8 % for method 2, 73.7 ± 13.15 % for method 3; p= 0.1842), GU 151 (84.7 ± 6.94 % for method 1, 80.6 ± 7.35% for method 2, 55.5 ± 7.34 % for method 3; p= 0.0574), and GU 297 (93.3 ± 1.67 % for method 1, 86.6 ± 3.3 % for method 2, 81.7 ± 4.4 % for method 3; p= 0.1199). However, only GU 244 exhibited bud-break values for method 1 (90.6 ± 0.56 %) and 2 (91.7 ± 4.81 %) that were significantly higher compared to method 3 (59.7 ± 9.7 %; p= 0.0184). Necrosis did not vary significantly among the three bud-break techniques for all the clones (2.8 ± 1.38 % for method 1, 2.7 ± 2.70 % for method 2, 8.2 ± 2.14 % for method 3; p=0.2071 for GU 21) (0.0 ± 0.00 % for method 1, 1.4 ± 1.38 % for method 2, 0.0 ± 0.00 % for method 3; p=0.4219 for GU 151); (0.0 ± 0.00 % for method 1, 8.3 ± 8.30 % for method 2, 4.2 ± 0.00 % for method 3; p= 0.5120 for GU 244); (0.0 ± 0.00 % for method 1, 4.2 ± 4.16 % for method 2, 1.4 ± 1.38 % for method 3; p= 0.5330 for GU 297), although the lowest values were recorded using method 1 (0.0-2.8%). Similarly, shoot yields for all four clones did not differ significantly among the three methods, although the best response was obtained using method 2 (1-3 shoots/bud).

Since there was no significant differences in bud break, necrosis and shoot yield between methods 1 and 2, and the objective was to reduce the number of different media used during the culture process, method 2 was chosen for initiating axillary buds for clones of this hybrid.

In summary, both the *E. grandis* x *nitens* (GN and NH) hybrid clones behaved similarly in that their highest bud-break values were achieved using method 1 (nodal explants; 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose) (87.5-90.3% bud-break

Table 3.6. Effect of different bud-break methods (methods 1-3) on necrosis, bud break percentages and shoot yields in five clones of *E. grandis* x *nitens* (GN). Results for bud-break were recorded after two weeks. Shoot yields were determined three weeks after shoots were transferred to multiplication medium (MS nutrients, 0.1mg/l biotin, 0.1 mg/l calcium pantothenate, 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose and 3.5g/l Gelrite). Cultures were maintained at 25 ± 2°C day/ 21°C night and a 16 h light/ 8 h dark photoperiod and a PPFD of 66μM/m^2/s.

Method 1 = nodal explants on MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose, 3.5g/l Gelrite

Method 2 = nodal explants on MS nutrients, 0.1mg/l biotin, 0.1mg/lcalcium pantothenate, 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose and 3.5g/l Gelrite

Method 3 = excised buds on same medium as method 2

Clone	Method 1			Method 2			Method 3		
	% Necrosis [z]	% Bud Break [z]	# Shoots/bud [z]	% Necrosis [z]	% Bud Break [z]	# Shoots/bud [z]	% Necrosis [z]	% Bud Break [z]	# Shoots/bud [z]
GN 1	0.0 ± 0.00 [a]	90.3 ± 3.67 [a]	2 ± 0.14 [a]	1.4 ± 1.38 [a]	85.5 ± 1.42 [b]	1 ± 0.04 [a]	4.8 ± 4.76 [a]	0.0 ± 0.00 [a]	1 ± 0.05 [a]
GN 9	1.4 ± 1.38 [a]	90.3 ± 1.38 [a]	3 ± 0.19 [a]	3.8 ± 0.13 [a]	79.8 ± 0.64 [ab]	1 ± 0.08 [a]	5.7 ± 2.67 [a]	0.0 ± 0.00 [a]	1 ± 0.05 [a]
GN 15	1.4 ± 1.38 [a]	87.5 ± 2.41 [a]	2 ± 0.10 [a]	7.3 ± 3.18 [ab]	72.9 ± 3.18 [ab]	1 ± 0.21 [a]	16.6 ± 0.36 [a]	0.0 ± 0.00 [a]	1 ± 0.03 [a]
GN 108	2.8 ± 1.38 [a]	88.9 ± 1.38 [a]	3 ± 1.08 [a]	2.7 ± 1.34 [a]	82.6 ± 3.08 [b]	1 ± 0.10 [a]	36.1 ± 16.0 [a]	0.0 ± 0.00 [a]	1 ± 0.10 [a]
GN 121	6.9 ± 2.78 [a]	90.3 ± 1.38 [a]	4 ± 0.26 [a]	15.7 ± 1.62 [b]	67.1 ± 2.44 [a]	2 ± 0.17 [a]	41.1 ± 8.84 [a]	0.0 ± 0.00 [a]	1 ± 0.02 [a]

[z] a-b = mean separation within columns, Sheffe's multiple range test (n=3, $p \leq 0.05$).

Table 3.7. Effect of different bud-break methods (methods 1-3) on necrosis, bud break percentages and shoot yields in four clones of *E. grandis* x *nitens* (NH). Results for bud-break were recorded after two weeks. Shoot yields were determined three weeks after shoots were transferred to multiplication medium (MS nutrients, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.2mg/l BAP, 25g/l sucrose and 3.5g/l Gelrite). Cultures were maintained at 25 ± 2°C day/ 21°C night and a 16 h light/ 8 h dark photoperiod and a PPFD of 66μM/m^2/s.

Method 1 = nodal explants on MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose, 3.5g/l Gelrite

Method 2 = nodal explants on MS nutrients, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.2mg/l BAP, 20g/l sucrose and 3.5g/l Gelrite

Method 3 = excised buds on same medium as method 2

Clone	Method 1			Method 2			Method 3		
	% Necrosis [z]	% Bud break [z]	# Shoots/bud [z]	% Necrosis [z]	% Bud break [z]	# Shoots/bud [z]	% Necrosis [z]	% Bud break [z]	# Shoots/bud [z]
NH 0	6.9 ± 1.39 [a]	75 ± 9.6 [b]	2 ± 0.14 [b]	20.8 ± 4.2 [a]	66.7 ± 7.2 [b]	2 ± 0.20 [b]	5.6 ± 3.7 [a]	13.9 ± 5.6 [a]	2 ± 0.19 [a]
NH 58	27.8 ± 3.7 [a]	33.3 ± 4.2 [a]	1 ± 0.04 [a]	41.7 ± 4.8 [a]	22.2 ± 11.9 [a]	1 ± 0.14 [a]	8.3 ± 4.8 [a]	15.3 ± 1.4 [a]	1 ± 0.00 [a]
NH 69	26.2 ± 8.5 [a]	17.4 ± 1.3 [a]	1 ± 0.04 [a]	26.3 ± 2.9 [a]	8.8 ± 3.9 [a]	1 ± 0.00 [a]	50.0 ± 7.2 [b]	1.4 ± 1.4 [a]	1 ± 0.00 [a]
NH 70	26.8 ± 3.3 [a]	20.2 ± 8.1 [a]	2 ± 0.03 [ab]	34.7 ± 9.7 [a]	9.7 ± 5.0 [a]	1 ± 0.21 [a]	34.7 ± 3.7 [b]	2.8 ± 2.8 [a]	1 ± 0.03 [a]

[z] a-b = mean separation within columns, Sheffe's multiple range test (n=3, p≤ 0.05).

Table 3.8. Effect of different bud-break methods (methods 1-3) on necrosis, bud break percentages and shoot yields in four clones of *E. grandis* x *urophylla* (GU). Results for bud-break were recorded after two weeks. Shoot yields were determined three weeks after shoots were transferred to multiplication medium (MS nutrients, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose and 3.5g/l Gelrite). Cultures were maintained at 25 ± 2°C day/ 21°C night and a 16 h light/ 8 h dark photoperiod and a PPFD of 66μM/m^2/s.

Method 1 = nodal explants on MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose, 3.5g/l Gelrite

Method 2 = nodal explants on MS nutrients, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose and 3.5g/l Gelrite

Method 3 = excised buds on same medium as method 2

Clone	Method 1			Method 2			Method 3		
	% Necrosis [z]	% Bud Break [z]	# Shoots/bud [z]	% Necrosis [z]	% Bud Break [z]	# Shoots/bud [z]	% Necrosis [z]	% Bud Break [z]	# Shoots/bud [z]
GU 21	2.8 ± 1.38 [b]	97.2 ± 1.38 [b]	2 ± 0.31 [a]	2.7 ± 2.70 [a]	91.6 ± 4.8 [a]	2 ± 0.42 [ab]	8.2 ± 2.14 [a]	73.7 ± 13.15 [a]	2 ± 0.03 [a]
GU 151	0.0 ± 0.00 [a]	84.7 ± 6.94 [a]	2 ± 0.31 [a]	1.4 ± 1.38 [a]	80.6 ± 7.35 [a]	2 ± 0.11 [a]	0.0 ± 0.00 [a]	55.5 ± 7.34 [a]	1 ± 0.08 [a]
GU 244	0.0 ± 0.00 [a]	90.6 ± 0.56 [ab]	1 ± 0.17 [a]	8.3 ± 8.30 [a]	91.7 ± 4.81 [a]	3 ± 0.35 [b]	4.2 ± 0.00 [a]	59.7 ± 9.7 [a]	2 ± 0.53 [a]
GU 297	0.0 ± 0.00 [a]	93.3 ± 1.67 [ab]	2 ± 0.31 [a]	4.2 ± 4.16 [a]	86.6 ± 3.3 [a]	1 ± 0.21 [a]	1.4 ± 1.38 [a]	81.7 ± 4.4 [a]	1 ± 0.03 [a]

[z] a-b = mean separation within columns, Sheffe's multiple range test (n=3, p≤ 0.05).

for GN clones; Table 3.6), (17.4-75% bud-break for NH clones; Table 3.7). No significantly different bud-break results were obtained for clones of *E. grandis* x *urophylla* (GU) using methods 1 (84.7-97.2% bud-break) and 2 (80.6-91.7% bud-break), but method 2 (nodal explants; 0.2mg/l BAP, 0.01mg/l NAA, 20g/l sucrose) was selected for this hybrid. The reason for this was that the medium used in method 2 could be used to effect both bud-break and shoot multiplication (Table 3.8). Although contamination levels in clones of all three hybrids were lower using method 3 (excised buds), the labour intensity involved in handling excised buds, their poor bud-break and shoot yields, and damage and necrosis, precludes the viable use of this explant to effect bud-break in a commercial setting.

Most authors have found it necessary to effect bud-break on a medium that is distinct from multiplication medium (Lakshmi Sita and Shobha Rani, 1985: *Eucalyptus grandis*; Fantini Jr. and Cortezzi Graca, 1986: *Eucalyptus dunni* x *Eucalyptus* spp.; Jones and van Staden, 1991: *Eucalyptus* spp.; Le Roux and van Staden, 1991b: *Eucalyptus* spp.; Mederos Molina *et al.*, 1997: *Salvia canariensis*; Patil and Kuruvinashetti, 1998: *Eucalyptus*; Vijaya Chitra and Padmaja, 1999: *Morus indica*). In terms of the present study, the requirement of a distinct bud-break medium for clones of *E. grandis x nitens* (GN and NH) appears to be consistent with these findings. On the other hand, results for *E. grandis* x *urophylla* (GU) parallel those of other authors who observed that both the bud-break and multiplication stages in some species can be successfully carried out using the same medium formulation, with sub-culturing onto fresh medium at regular intervals (Horgan, 1987: *Pinus radiata*; Blomstedt *et al.*, 1991: *Eucalyptus regnans*; Donald and Newton, 1991: *Eucalyptus radiata*; Beck *et al.*, 1998: *Acacia mearnsii*; Makwarela, 1996: *Eucalyptus* hybrid clones). Hence, although most authors prefer to use distinct media for bud-break and multiplication, it is possible to reduce costs and time associated with the preparation of two different media formulations for each step, by using a single medium for both stages, for certain clones, *e.g.* clones of *E. grandis* x *urophylla* (GU).

Bud-break yields similar to those achieved for the three hybrids in this study: 87.5-90.3% for GN clones, 17.4-75% for NH clones and 84.7-97.2% for GU clones, have also been reported by some authors. Le Roux and van Staden (1991b) obtained 90% bud-break in two *E. macarthurii* hybrids, which is comparable to the values achieved for GN and GU clones. Patil and Kuruvinashetti (1998) achieved 22% and 63% bud-break from nodal explants from epicormic and coppice shoots respectively, of *E. tereticornis*, whilst Boulay (1983) reported that only 2-40% bud-break could be obtained from 25 frost resistant *Eucalyptus* clones. Gupta and Mascarenhas (1987) reported bud-break values of 35-40% from five species of *Eucalyptus*, *viz. E. camaldulensis*, *E. citriodora*, *E. globulus*, *E. tereticornis* and *E. torrelliana*. These values are close to those achieved for clones of *E. grandis* x *nitens* (NH ; a cold-tolerant hybrid) in this study.

3.3.2 The effect of medium composition on the *in vitro* proliferation of axillary buds

The third stage in the process of axillary bud proliferation involves the *in vitro* multiplication of axillary buds following bud-break. The main objective of this culture stage is to produce the maximum number of viable plant propagules, and this shoot multiplication response arises through the application of cytokinins to overcome the apical dominance of shoots, and encourage the growth of lateral shoots (Hussey, 1978; Hu and Wang, 1983; George, 1993).

Shoots were initiated from nodal explants according to the optimised bud-break methods determined for each hybrid (3.3.1). These shoots were used subsequently to test four different multiplication media. Cultures were maintained at 25°C ± 2°C day/ 21°C night with a 16 h light/ 8 h dark photoperiod (PPFD 66μmol/m^2/s) for 6 weeks with one subculture onto fresh medium after 3 weeks. Shoot yields were recorded for each clone on each multiplication medium after 6 weeks.

Table 3.9 shows shoot yields of five clones of *E. grandis* x *nitens* (GN) after 6 weeks, in response to the tested multiplication media. Shoot yields on each multiplication medium

were compared among clones, *i.e.* by columns. Results indicate all four media resulted in shoot yields that reflected a measure of clonal specificity in terms of shoot multiplication response. Mediums M1 and M2 yielded values that were high and fairly consistent among clones (4-12 shoots/bud; p= 0.0182 for M1) (4-13 shoots/bud; p= 0.0009 for M2) (Table 3.9). In M1, yields for GN 1 (8 ± 1.08 shoots/bud), GN 15 (8 ± 0.99 shoots/bud), and GN108 (6 ± 1.28 shoots/bud) did not differ significantly from one another and from GN 9 (12 ± 2.14 shoots/bud) and GN 121 (4 ± 0.63 shoots/bud), however, the shoot yield for GN 121 was significantly lower than that achieved for GN 9 (Table 3.9). In M2, there was no significant difference in shoot yields of GN9 (11 ± 1.48 shoots/bud), GN 15 (13 ± 1.51 shoots/bud), GN 108 (11 ± 0.41 shoots/bud) and GN 121 (8 ± 0.33 shoots/bud), but GN 1 exhibited a significantly lower shoot yield in this medium (4 ± 0.92 shoots/bud) (Table 3.9). Shoot yields in M3 were not significantly different among GN 1 (2 ± 0.52 shoots/bud), GN 9 (4 ± 0.19 shoots/bud), GN 15 (4 ± 0.20 shoots/bud), and GN 121 (2 ± 0.27 shoots/bud), but GN 108 shoot yields (5 ± 0.60 shoots/bud) were significantly higher than those of GN 1 and GN 121 (Table 3.9). Genotypic effects were also evident for medium M4: GN 9 (6 ± 0.57 shoots/bud) and GN 15 (6 ± 0.75 shoots/bud) shoot yields were not significantly different to GN 1 (3 ± 0.67 shoots/bud) and GN 108 (5 ± 0.42 shoots/bud), but were significantly higher compared to GN 121 (2 ± 0.44 shoots/bud) (Table 3.9). Since medium M2 resulted in consistent and high yields for most of the clones, this medium was selected for multiplying clones of this hybrid.

A comparison of all four media on each clone individually showed that highest shoot yields for GN 15 (13 ± 1.5 shoots/bud; p= 0.0015), GN 108 (11 ± 0.4 shoots/bud; p=0.0010) and GN 121 (8 ± 0.3 shoots/bud; p= 0.0001), were achieved on medium M2 (Figure 3.6). Shoot yields for GN 9 were not significantly different between M1 (12 ± 2.1 shoots/bud) and M2 (11 ± 1.5 shoots/bud), but significantly better than those yields on medium M3 (4 ± 0.2 shoots/bud) and M4 (6 ± 0.6 shoots/bud; p=0.0000 [p<0.05]). For GN 1, the shoot yield in medium M1 (8 ± 1.1 shoots/bud) was significantly higher than that achieved in medium M3 (2 ± 0.5 shoots/bud) and M4 (3 ± 0.7 shoots/bud;

p=0.0067). Hence, despite some genotypic differences, M2 was selected as the best medium for the multiplication of clones of this hybrid.

Table 3.9. The effect of medium composition on shoot/bud yield from five clones of *E. grandis* x *nitens* (GN). Axillary buds were initiated on MS nutrients, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose and 3.5g/l Gelrite over a period of 1-2 weeks. Then shoots were transferred to multiplication media (see below), and maintained under a 16h light/8h darkness photoperiod, and a PPFD of 66μM/m^2/s at 25 ± 2°C day/ 21°C night for six weeks, with one subculture onto fresh medium of the same formulation after three weeks. Results were recorded after six weeks in culture. All tested multiplication media contained MS nutrients, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 3.5g/l Gelrite, and

M1 = 0.2mg/l BAP, 20g/l sucrose,

M2 = 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose,

M3 = 0.5mg/l BAP, 0.2mg/l NAA, 20g/l sucrose,

M4 = 0.1mg/l BAP, 0.01mg/l NAA, 0.2mg/l kinetin, 25g/l sucrose.

Clone	# shoots/bud			
	M1 [z]	M2 [z]	M3 [z]	M4 [z]
GN 1	8 ± 1.077 [ab]	4 ± 0.918 [a]	2 ± 0.515 [a]	3 ± 0.667 [ab]
GN 9	12 ± 2.141 [b]	11 ± 1.475 [b]	4 ± 0.199 [ab]	6 ± 0.568 [b]
GN 15	8 ± 0.994 [ab]	13 ± 1.514 [b]	4 ± 0.203 [ab]	6 ± 0.753 [b]
GN 108	6 ± 1.281 [ab]	11 ± 0.409 [b]	5 ± 0.602 [b]	5 ± 0.415 [ab]
GN 121	4 ± 0.632 [a]	8 ± 0.332 [ab]	2 ± 0.267 [a]	2 ± 0.444 [a]

[z] a-b = mean separation within columns, Scheffe's multiple range test (n=3, p≤ 0.05).

Similarly, with clones of *E. grandis* x *nitens* (NH) (Table 3.10), results show medium M2 was the only medium for which shoot yields did not differ significantly among clones (4-9 shoots/bud; p= 0.0242). In medium M1, shoot yields for NH 0 (21 ± 3.82 shoots/bud) and NH 70 (20 ± 1.27 shoots/bud) were not significantly different to each other, but both

these values were significantly higher compared to NH 58 (4 ± 0.36 shoots/bud) and NH 69 (1 ± 0.04 shoots/bud; p= 0.0002). This trend was repeated for M3 and M4, where shoot yield values generally did not differ significantly between NH 0 (5 ± 0.50 shoots/bud for M3; 6 ± 0.80 shoots/bud for M4) and NH 70 (2 ± 0.23 shoots/bud for M3; 7 ± 1.42 shoots/bud for M4), but both these values being higher than those obtained for NH 58 (2 ± 0.11 shoots/bud for M3; 3 ± 0.31 shoots/bud for M4) and NH 69 (2 ± 0.08 shoots/bud for M3; 1 ± 0.22 shoots/bud for M4) (Table 3.10). Hence, a single high-yielding medium for all the clones could not be determined.

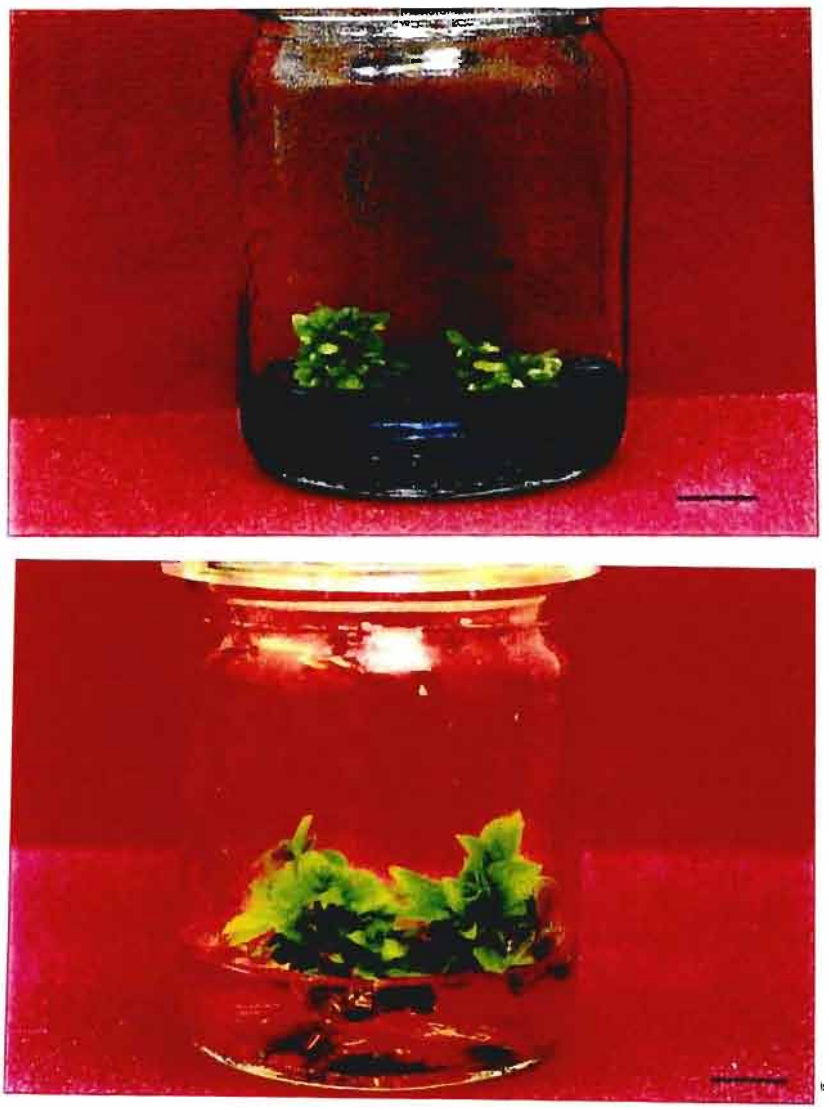

Figure 3.6 **The effect of medium composition on *in vitro* shoot multiplication of GN121, a clone of *E. grandis* x *nitens* (GN).** Shoots on the top picture were multiplied in medium M1 (0.2mg/l BAP and 20g/l sucrose) over 6 weeks. Bar= 1.1 cm. Shoots on the bottom picture were multiplied over the same time period in medium M2, containing 0.2mg/l BAP, 0.01mg/l NAA and 25g/l sucrose. Bar= 1 cm.

An assessment of the effect of all four media on each clone individually indicated that the highest shoot yields for NH 0 (21 ± 3.8 shoots/bud; p=0.0009) and NH 70 (20 ± 1.3 shoots/bud; p= 0.0003) were achieved on medium M1 (0.2m/l BAP, 20g/l sucrose). Shoot yield for NH 69 was consistently poor for all tested media and further, did not differ significantly among the four tested media (p=0.1434) Clone NH 69 was thus not included in further studies. The best medium for NH 58 (9 ± 1.4 shoots/bud) was medium 2 (0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose) (p= 0.0004). Despite shoot yields that were highly genotype specific, medium M1 (0.2mg/l BAP, 20g/l sucrose) was selected due to the high shoot yields obtained for NH 0 and NH 70, and the fact that there was no significant difference in shoot yields among media for NH 69. However, it is recognised that due to the clonal specificity in responses, a single high yielding multiplication medium suitable for all the clone of this hybrid could not be determined.

Table 3.10. The effect of medium composition on shoot/bud yield from four clones of *E. grandis* x *nitens* (NH). Axillary buds were initiated on MS nutrients, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose and 3.5g/l Gelrite over a period of 2 weeks. Then shoots were transferred to multiplication media (see below), and maintained under a 16h light/8h darkness photoperiod, and a PPFD of 66μM/m^2/s at 25 ± 2°C day/ 21°C night for six weeks, with one subculture onto fresh medium of the same formulation after three weeks. Results were recorded after six weeks in culture. All tested multiplication media contained MS nutrients, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 3.5g/l Gelrite, and

M1 = 0.2mg/l BAP, 20g/l sucrose,

M2 = 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose,

M3 = 0.5mg/l BAP, 0.2mg/l NAA, 20g/l sucrose,

M4 = 0.1mg/l BAP, 0.01mg/l NAA, 0.2mg/l kinetin, 25g/l sucrose.

Clone	# shoots/bud			
	M1[z]	M2[z]	M3[z]	M4[z]
NH 0	21 ± 3.823[b]	4 ± 0.317[a]	5 ± 0.504[b]	6 ± 0.802[b]
NH 58	4 ± 0.355[a]	9 ± 1.400[a]	2 ± 0.112[ab]	3 ± 0.305[a]
NH 69	1 ± 0.042[a]	2 ± 0.398[a]	2 ± 0.083[a]	1 ± 0.224[a]
NH 70	20 ± 1.273[b]	9 ± 2.485[a]	2 ± 0.231[b]	7 ± 1.421[b]

[z] a-b = mean separation within columns, Scheffe's multiple range test (n=3, p≤ 0.05).

Similar multiplication experiments were conducted on four clones of *E. grandis* x *urophylla* (GU), and results are presented in Table 3.11. Shoot yields were compared among clones for each medium. Results indicated that the only medium for which there was no significant difference in shoot yields among clones was medium M3 (3-5 shoots/bud; p= 0.2984) (Table 3.11). In M1, the shoot yields for GU 151 (4. ± 0.45 shoots/bud) and GU 297 (3 ± 0.27 shoots/bud) were not significantly different to yields for GU 244 (5 ± 0.84 shoots/bud) and GU 21 (2 ± 0.32 shoots/bud), however, GU 21 exhibited significantly lower shoot yields compared to GU 244 (p= 0.0213) (Table 3.11). In M2, shoot yields of GU 21 (2 ± 0.11 shoots/bud) and GU 244 (3 ± 0.13 shoots/bud) were not significantly different to each other, but were significantly lower compared to GU 151 (5 ± 0.41 shoots/bud) and GU 297 (6 ± 0.39 shoots/bud; p= 0.0000[p< 0.05]) (Table 3.11). In medium M4, shoot yields did not differ significantly between GU 21 (4 ± 0.33 shoots/bud) and GU 151 (3 ± 0.21 shoots/bud), however, these values were significantly lower compared to GU 297 (7 ± 0.86 shoots/bud), and fairly similar to GU 244 (5 ± 0.52 shoots/bud; p= 0.0152) (Table 3.11). Although medium M4 appeared to result in higher shoot yields, M2 was selected as the medium for multiplying clones of this hybrid.

The effect of all four media on shoot yields of each clone was also assessed. In this regard, for all four clones, there was no significant difference in shoot yields among the four tested media, *i.e.* medium composition did not affect shoot yield for each clone (p=0.0309 for GU 21; p=0.1411 for GU 151; p=0.0818 for GU 244; p=0.0619 for GU 297). It is noted that shoot yields on M4 (4-7 shoots/bud), although not significantly different, were higher compared to those achieved on other media (2-5 shoots/bud for M1, 2-6 shoots/bud for M2, 3-5 shoots/bud for M3), however, medium M2 was selected as the multiplication medium for this hybrid. This was in keeping with the objective of reducing the total number of different media used during the culture process, since M2 had been selected for GN clones, and further, could also be used to effect bud-break.

Table 3.11. The effect of medium composition on shoot/bud yield in four clones of *E. grandis* x *urophylla* (GU). Axillary buds were initiated on MS nutrients, 0.1mg/l calcium pantothenate, 0.1mg/l biotin, 0.04mg/l NAA, 0.11mg/l BAP, 0.05mg/l kinetin, 20g/l sucrose and 3.5g/l Gelrite over a period of 1-2 weeks. Then shoots were transferred to multiplication media (see below), and maintained under a 16h light/8h darkness photoperiod, and a PPFD of 66μM/m^2/s at 25 ± 2°C day/ 21°C night for six weeks, with one subculture onto fresh medium of the same formulation after three weeks. Results were recorded after six weeks in culture. All tested multiplication media contained MS nutrients, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 3.5g/l Gelrite, and

M1 = 0.2mg/l BAP, 20g/l sucrose,

M2 = 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose,

M3 = 0.5mg/l BAP, 0.2mg/l NAA, 20g/l sucrose,

M4 = 0.1mg/l BAP, 0.01mg/l NAA, 0.2mg/l kinetin, 25g/l sucrose.

Clone	# shoots/bud			
	M1 [z]	M2 [z]	M3 [z]	M4 [z]
GU 21	2 ± 0.32^{a}	2 ± 0.11^{a}	3 ± 0.76^{a}	4 ± 0.33^{a}
GU 151	4. ± 0.45^{ab}	5 ± 0.41^{b}	4 ± 0.19^{a}	3 ± 0.21^{a}
GU 244	5 ± 0.84^{b}	3 ± 0.13^{a}	5 ± 0.28^{a}	5 ± 0.52^{ab}
GU 297	3 ± 0.27^{ab}	6 ± 0.39^{b}	5 ± 1.26^{a}	7 ± 0.86^{b}

[z] a-b = mean separation within columns, Scheffe's multiple range test (n=3, p≤ 0.05).

In summary, this study, indicated that with references to shoot multiplication, GN clones generally respond best to a medium containing 0.2mg/l BAP, 0.01mg/l NAA and 25g/l sucrose (M2). Clones of *E. grandis* x urophylla (GU) achieved higher shoot yields (3-7 shoots/bud) on multiplication medium M4 (0.1mg/l BAP, 0.01mg/l NAA, 0.2mg/l kinetin, 25g/l sucrose) but these values were not significantly different to those obtained on medium M2 (2-6 shoots/bud) (Table 3.11). The latter medium (M2) was selected for multiplication of GU clones since it could be used for bud-break as well and it was also selected for GN clones. Thus, it would appear that clones of *E. grandis* x *nitens* (GN) and *E. grandis* x *urophylla* (GU) behaved similarly in terms of their growth regulator requirements for multiplication. A single high-yielding multiplication medium for all clones of *E. grandis* x *nitens* (NH) could not be determined. There were vast differences

within *E. grandis* x *nitens* (NH), with clones responding very specifically to the various media; NH 58 achieved the highest multiplication yield (9 shoots/bud) on a medium M2 (0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose) rather than on the medium that achieved the highest yields for NH 0 (21 shoots/bud) and NH 70 (20 shoots/bud), *i.e.* M1 (0.2mg/l BAP, 20g/l sucrose). This clonal specificity with regards to multiplication media, has also been observed by several authors in similar studies on axillary bud proliferation (McComb and Bennet, 1986; Donald and Newton, 1991; Merkle and Gotz, 1990).

The tested multiplication media in the current investigation were based on previous work on the hybrids in this study (Furze and Cresswell, 1987; Makwarela, 1996; McAlister, *pers. comm.*). Genotypic differences among the three hybrids resulted in the observed differences in response to these media. As stated earlier, GN clones responded best in a medium containing a combination of auxin (0.01mg/l NAA) and cytokinin (0.2mg/l BAP). Other authors have also achieved maximum multiplication yields by using media containing a combination of auxins and cytokinin (Sankara Rao and Venkateswara; 1985; Lakshmi Sita, 1986; Burger, 1987; Trindade *et al.*, 1990; Le Roux and van Staden, 1991b; Yang *et al.*, 1995; Lux *et al.*, 1997/98; Deshpande *et al.*,1998; Liew and Teo, 1998; Karkonen *et al.*, 1999).

However, high multiplication yields achieved in two clones of *E. grandis* x *nitens* (NH 0 and NH 70; 21 and 20 shoots/bud respectively) when cytokinin was used singly (0.2mg/l BAP). Similarly, reports of high shoot yields achieved on media containing only cytokinin, have also been documented in other tree species (Trindade *et al.*, 1990; Warrag *et al.*, 1990; Jokinen and Tormala, 1991; Yu and Reed, 1993; McKellar *et al.*, 1994; Puddephat *et al.*, 1997; Sudhakar Johnson *et al.*, 1997; Beck *et al.*, 1998; Donovan *et al.*, 1999).

Authors have also obtained high shoot yields using a combination of different cytokinins in multiplication media (Mascarenhas *et al.*, 1982; Abdullah *et al.*, 1986; Raghava Swamy *et al.*, 1992; Yasodha *et al.*, 1997). In the present study, a similar media formulation (M4: 0.1mg/l BAP, 0.1mg/l kinetin, 0.01mg/l NAA, and 25g/l sucrose)

achieved moderately high yields in clones of *E. grandis* x *urophylla* (GU) (3-7 shoots/bud), but not in GN and NH clones.

The established media for GN and GU clones resulted in shoot yields of 4-13 shoots/bud for GN clones and 2-6 shoots/bud for GU clones. Yields of 21 shoots/bud and 20 shoots/bud were also obtained for clones NH 0 and NH 70 respectively. Similar multiplication yields have been reported by other authors (Franclet and Boulay, 1982: *E. gunnii, E. dalrympleana*: 10 shoots/explant; Wiecheteck *et al.*, 1989: *E. viminalis*: 506 shoots/explant; Fantini Jr. *et al.*, 1989: *E. dunnii* x *Eucalyptus* spp.: 8 shoots/explant; Jones and van Staden, 1994: *E. grandis* hybrids: 6-10 shoots/explant; Patil and Kuruvinashetti, 1998: *E. tereticornis*: 8-10 shoots/explant). Shoot yields closer to those achieved for NH 0 and NH 70 have also been documented (Mascarenhas *et al.*, 1982: *Eucalyptus* species: 8-20 shoots/explant). Higher shoot yields have, however, been obtained in forest tree species. Chang *et al.*(1992) reported a yield of 50-70 shoots/nodal culture for *Eucalyptus radiata*. Cortezzi-Graça and Mendes (1989) obtained up to 35 shoots/explant in *E. dunnii* after 60 days. Sankara Rao and Venkateswara (1985) obtained an average of 40 shoots/explant in *E. grandis* and Lakshmi Sita (1986) achieved 30-50 shoots/nodal culture of *E. grandis*. Comparing these high yields, the yields in this study, even on the best media, are low.

3.3.3 The effect of light on the elongation of shoots

During multiplication, not all shoots elongate sufficiently to allow rooting and individual shoots are usually transferred to a specific medium for elongation prior to rooting (Furze and Cresswell, 1985; Cortezzi-Graça and Mendes, 1989; Le Roux and van Staden, 1991b; George, 1993). In this study, after an initial three week period on elongation medium (MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.35mg/l NAA, 0.1mg/l kinetin, 0.1mg/l IBA, 20g/l sucrose, 3.5g/l Gelrite, 16h light/8h dark photoperiod, PPFD of 66µmol/m^2/s, 25°C ± 2°C day/21°C night), shoots from all tested hybrid clones had not elongated sufficiently for use in rooting trials, as they were less than 10mm in length.

Consequently, shoots were sub-cultured onto fresh medium and subjected to two different culture conditions for a further three weeks. Cultures were placed in complete darkness at room temperature (~25°C) for the three week interval (dark treatment) or maintained under the same conditions applied for the first three weeks in elongation medium (light treatment).

As stated above, shoots of *E. grandis* x *nitens* (GN) clones failed to elongate after the first three weeks in elongation medium (passage 1) (change in shoot length after passage 1: 0.1-0.4mm)' (Table 3.12). After subculture onto the same medium, shoot were subjected to either light (16h light/8h dark photoperiod at 25°C ± 2°C day/21°C night and a PPFD of 66μmol/m^2/s) or dark conditions (~25°C). The effect on elongation of these treatments is presented in Table 3.12. For all five clones, the exposure of subcultured shoots to complete darkness at room temperature did not elicit elongation (shoot length did not exceed 8.9mm) (change in shoot length after passage 2[dark]: 0.2-0.9 mm) (Table 3.12). However, light, conditions had a significantly positive effect on the elongation of shoots of all the clones (change in shoot length after passage 2[light]: 22.9-35.2mm) (Table 3.12). In addition, necrosis and contamination of shoots was greater when shoots were subjected to complete darkness, rather than under a photoperiod (results not shown).

Tested clones of *E. grandis* x *nitens* (NH) and GN clones yielded similar results; passage 1 failed to elicit elongation (change in shoot length after passage 1: 0.1-0.3 mm) and so did a further three-week period in total darkness (change in shoot length after passage 2[dark]: 0.1-1.8 mm) (Table 3.13). Light had a positive effect on all GN clones, but with reference to NH hybrid, only shoots of clones NH 0 and NH 70 elongated significantly (change in shoot length after passage 2[light]: 32.2 mm for NH 0; 24.7 mm for NH 70) (Table 3.13) (Figure 3.7). A further study was thus conducted on NH58, which involved the transfer of shoots in elongation medium back onto multiplication medium (0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose) for a further culture passage before attempting elongation once more. After three weeks, shoots elongated by 20.2mm compared to their initial length (Table 3.13).

Clones of *E. grandis* x *urophylla* (GU) underwent similar elongation treatments and the effect of the culture treatments on elongation was assessed for each clone. The results of these experiments are presented in Table 3.14, and are similar to those recorded for GN and NH clones. Shoot elongation after three weeks (passage 1) was negligible (change in shoot length after passage 1: 0.2-0.3 mm) (Table 3.14). Similarly, during passage 2, darkness had no effect on elongation (change in shoot length after passage 2[dark]: 0.3-0.9 mm) but the light treatment resulted in a significant increase in shoot length (change in shoot length after passage 2[light]: 21.6-29.3 mm) (Table 3.14).

In conclusion, all tested clones of all three tested *Eucalyptus* hybrids required light (66μM/m^2/s PPFD) under photoperiod conditions (16 h light/ 8 h dark, 25 ± 2°C day/ 21°C night) for 6 weeks, for successful elongation of shoots.

Table 3.12. The effect of elongation treatments on shoot elongation of five clones of *E. grandis* x *nitens* (GN). Shoot length (mm) was determined after multiplication (initial), and after three weeks in MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.35mg/l NAA, 0.1mg/l kinetin, 0.1mg/l IBA, 20g/l sucrose and 3.5g/l Gelrite, at 25 ± 2°C day/ 21°C night, 16 h light/ 8 h dark photoperiod, PPFD of 66μM/m^2/s (passage 1). Subcultured shoots were subsequently subjected to growth conditions outlined above (Light treatment) or maintained at ~25°C in total darkness for three weeks, at which point shoot length was again recorded.

Clone	Shoot Length (mm)			
	Initial	After passage 1 (3 weeks)	After passage 2 (3 weeks)	
			Dark	Light
GN 1	4.9 ± 0.09[a]	5.1 ± 0.15[a]	5.8 ± 0.06[a]	40.3 ± 1.50[b]
GN 9	4.4 ± 0.24[a]	4.7 ± 0.17[a]	5.6 ± 0.20[a]	32.3 ± 1.45[b]
GN 15	5.6 ± 0.27[a]	5.7 ± 0.25[a]	6.2 ± 0.18[a]	28.6 ± 1.40[b]
GN 108	8.1 ± 0.49[a]	8.5 ± 0.21[a]	8.9 ± 0.13[a]	33.0 ± 0.78[b]
GN 121	8.1 ± 0.62[a]	8.2 ± 0.55[a]	8.4 ± 0.59[a]	35.1 ± 3.20[b]

a-b = mean separation between columns, Scheffe's multiple range test (n=3, p≤ 0.05).

Table 3.13. The effect of elongation treatments on shoot elongation of three clones of *E. grandis* x *nitens* (NH). Shoot length (mm) was determined after multiplication (initial), and after three weeks in MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.35mg/l NAA, 0.1mg/l kinetin, 0.1mg/l IBA, 20g/l sucrose and 3.5g/l Gelrite, at 25 ± 2°C day/ 21°C night, 16 h light/ 8 h dark photoperiod, PPFD of 66μM/m^2/s (passage 1). Subcultured shoots were subsequently subjected to growth conditions outlined above (Light treatment) or maintained at ~25°C in total darkness for three weeks, at which point shoot length was again recorded.

	Shoot Length (mm)			
	Initial	After passage 1 (3 weeks)	After passage 2 (3 weeks)	
Clone			Dark	Light
NH 0	5.6 ± 0.19[a]	5.9 ± 0.17[a]	7.7 ± 0.33[a]	38.1 ± 1.27[b]
NH 70	5.7 ± 0.09[a]	5.9 ± 0.12[a]	6.0 ± 0.58[a]	30.6 ± 0.99[b]
NH 58 (a)	5.3 ± 0.21[a]	5.4 ± 0.22[a]	5.7 ± 0.67[a]	7.3 ± 0.33[b]
NH 58 (b)	6.2 ± 0.24[a]	26.4± 0.81[b]		

a-b = mean separation between columns Scheffe's multiple range test (n=3, p≤ 0.05).

Figure 3.7 **A comparison of the effect of lighting on elongation of shoots from clone NH 0 from *E. grandis* x *nitens* (NH), after six weeks.** Shoots on the left vessel were maintained in complete darkness whereas shoots on the right vessel were maintained under a 16 h light/ 8 h dark photoperiod at 25°C ± 2°C day/21°C night and a PPFD of 66μmol/m^2/s for three weeks. Bar= 1.8 cm

Table 3.14. The effect of elongation treatments on shoot elongation of four clones of *E. grandis* x *urophylla* (GU). Shoot length (mm) was determined after multiplication (initial), and after three weeks in MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.35mg/l NAA, 0.1mg/l kinetin, 0.1mg/l IBA, 20g/l sucrose and 3.5g/l Gelrite, at 25 ± 2°C day/ 21°C night, 16 h light/ 8 h dark photoperiod, PPFD of 66μM/m^2/s (passage 1). Subcultured shoots were subsequently subjected to growth conditions outlined above (Light treatment) or maintained at ~25°C in total darkness for three weeks, at which point shoot length was again recorded.

Clone	Shoot Length (mm)			
	Initial	After Passage 1 (3 weeks)	After passage 2 (3 weeks)	
			Dark	Light
GU 21	5.3 ± 0.09[a]	5.5 ± 0.06[a]	6.4 ± 0.48[a]	28.7 ± 1.92[b]
GU 151	5.4 ± 0.09[a]	5.7 ± 0.09[a]	6.5 ± 0.83[a]	30.8 ± 0.82[b]
GU 244	5.9 ± 0.15[a]	6.1 ± 0.21[a]	6.4 ± 0.69[a]	27.7 ± 2.03[b]
GU 297	5.8 ± 0.03[a]	6.0 ± 0.09[a]	6.4 ± 0.78[a]	35.3 ± 2.34[b]

a-b = mean separation between columns, Scheffe's multiple range test (n=3, p≤ 0.05).

The rationale behind subjecting the shoots to complete darkness was that elongation would occur as a result of etiolation. This was based on a study by Mandal (1989), which indicated that shoots of *Populus* that were subjected to total darkness for two weeks exhibited superior elongation than those that had been maintained in a growth chamber under light and photoperiod conditions. In addition, Fantini Jr. and Cortezzi-Graça (1989) observed that shoots of *E. dunnii* hybrids elongated better in the dark after 60 days than those that had been maintained under a photoperiod. In the present study, light and photoperiod conditions proved to be the most successful in terms of inducing an elongation response in clones of all three hybrids. Similar observations have been also reported by other authors attempting to elongate shoots of forest tree species (Franclet and Boulay, 1982; Furze and Cresswell, 1985; Lakshmi Sita and Shobha Rani, 1985; Blomstedt *et al.*, 1991; Le Roux and van Staden, 1991b; Puddephat *et al.*, 1997; Deshpande *et al.*, 1998). These observations indicate that *in vitro* shoots of certain

species should be elongated under light and photoperiod conditions than under conditions of total darkness. With reference to the difficulties experienced in elongating clone NH 58, it appears that genotype played a significant role in determining the response of this clone to elongation conditions. Similarly, Yasodha *et al.* (1997) observed that different genotypes of difficult-to-propagate *Eucalyptus* clones of *E. camaldulensis* required specific *in vitro* conditions for successful elongation, and that under a given set of conditions, the percentage of shoots that elongated ranged from 15%-56% among the clones. Further, Yu and Reed (1993) reported differences in shoot length following elongation, as a result of genotypic effects among cultivars of *Corylus avellana* (hazelnut).

In this study, elongation occurred over a period of six weeks. Some workers have documented culture passages for elongation comparable to the 6 weeks necessary in the present study (Fantini Jr. and Cortezzi-Graça, 1989: *E. dunnii* x *Eucalyptus* species, ~9 weeks). Das and Mitra (1990) achieved maximum elongation of *E. tereticornis* only after the third subculture on elongation medium (12 weeks). However, other authors working with *Eucalyptus* species have reported shorter elongation periods of 8-10 days (Gupta and Mascarenhas, 1987: *E. camaldulensis*, *E. citriodora*, *E. globulus*, *E. tereticornis* and *E. torrelliana*), 2 weeks (Cortezzi Graca and Mendes, 1989: *E. dunnii*), 10-15 days (*Mascarenhas et al.*, 1982: *E. citriodora*, *E. tereticornis*, *E. globulus*), 2-3 weeks (Franclet and Boulay, 1982: frost-tolerant *Eucalyptus* species), 2-4 weeks (Cid *et al.*, 1999: *E. grandis* x *urophylla*) and 4 weeks (Warrag *et al.*, 1990: *E. grandis* hybrids).

Authors, working with *Eucalyptus*, that were able to effect elongation over a shorter period of time (≤ 4 weeks), have generally included gibberellic acid in the medium (Cortezzi Graca and Mendes, 1989; Cid *et al.*, 1999), sometimes in combination with activated charcoal (Franclet and Boulay, 1982; Wiechetek *et al.*, 1989; Yasodha *et al.*, 1997). Similarly, authors working with other tree species have effected shoot elongation over periods of less than 4 weeks, by using gibberellic acid and/or activated charcoal (Ball, 1987: *Sequoia*; Deshpande *et al.*, 1998: *Ficus religiosa*; Mederos Molina and Trujillo, 1999b: *Pistachia*; Vijaya Chitra and Padmaja, 1999: *Morus indica*). Based on

this, the elongation passage for clones in this study might be reduced by the use of an elongation medium that contains gibberellic acid and/or activated charcoal. The reduction of the culture period for elongation is important, since it would make sufficiently elongated shoots available for rooting within a shorter space of time, and thus allow a greater output of viable plantlets within the production period.

3.3.4 The effect of medium composition on the rooting of shoots

Following elongation, shoots, are usually transferred to a specially-formulated medium, making use of certain methods to induce *in vitro* rooting. To reduce the costs of micropropagation, some laboratories opt for *ex vitro* rooting (Constantine, 1986; George, 1993; Chang and Donald, 1992; Watt *et al.*, 1995). However, most authors rely on *in vitro* rooting of micropropagated plantlets prior to transfer to *ex vitro* conditions for acclimatisation (Gupta *et al.*, 1981; McComb and Bennett, 1982; Burger, 1987; Lakshmi Sita, 1986; Das and Mitra, 1990; Warrag *et al.*, 1990; Blomstedt *et al.*, 1991; Donald and Newton, 1991; Yasodha *et al.* 1997; Patil and Kuruvinashetti, 1998).

In this study, selected clones were subjected to four different rooting media, and maintained for 72 hours in complete darkness at room temperature (25°C) followed by transfer to a 16 h light/ 8 h dark photoperiod at a PPFD of 37 $\mu mol/m^2/s$ and at 24°C day/ 21°C night for seven days. Cultures were subsequently transferred to a 16 h light/ 8 h dark photoperiod and a PPFD of 66$\mu mol/m^2/s$ at 25°C ± 2°C day/ 21°C night for 21 days, at which time rooting frequency (% rooting) and root length was recorded.

Table 3.15 shows % rooting and root lengths for *E. grandis* x *nitens* (GN) clones, in response to the four tested rooting media. Root length and % rooting were compared among clones for each medium. Results indicated that genotype played a significant role in affecting these parameters, as for the previous culture stages. The only two media for which % rooting did not vary significantly among clones, were RM 1 (½ MS, 1mg/l IBA) (63-86%) and RM 4 (¼ MS, 0.1mg/l IBA, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$)

(56-93%) (Table 3.15). Genotypic effects resulted in significant differences in % rooting among clones in mediums RM 2 (½ MS, 0.1mg/l IBA) (40-73.3%) and RM 3 (½ MS, 0.1mg/l IBA, 0.5mg/l NAA) (6.7-80%) (Table 3.15). Similarly, root length differed significantly among clones that were rooted in mediums RM 1 (5.9-41.1 mm) and RM 2 (7.9-53.2 mm) (Table 3.15). The only two media that did not result in vast differences in root lengths among clones were RM 3 (2.3- 19.1 mm) and RM 4 (38.7-47.1 mm) (Table 3.15). Based on the high % rooting (56.7-93.3%) and longer root lengths (38.7-47.1 mm) obtained on RM 4, this medium was chosen as the best medium for rooting clones of this hybrid.

An assessment of the effect of all four rooting media on % rooting and root length of each clone, confirmed the choice of RM 4 as the best rooting medium. The response of individual clones to the media was consistent, in that % rooting for four out of five clones, % rooting did not differ significantly among the four media. The exception was GN 15, in which the highest % rooting was achieved in RM 1 (86.7 ± 6.0 %; p=0.0004). Root length was significantly greater in RM 4 for all clones except GN 1, in which there was no significant difference in root length among media RM 1, RM 2 and RM 4 (Figure 3.8). Hence, RM 4 was the medium selected for clones of this hybrid.

Three clones of *E. grandis* x *nitens* (NH) also underwent rooting trials, and % rooting and root length were compared in the same manner as outlined for GN clones, and the results are presented in Table 3.16. Again, genotypic differences among clones resulted in significant differences in the response of each clone to the tested media. Generally % rooting did not differ significantly between NH 0 and NH 70 for all tested media, but results for NH 58 did vary significantly from these two clones (Table 3.16). However, root length in all media was not significantly different between NH 58 and NH 70, which together, were significantly different to NH 0.

With regards to the response of individual clones to the four media, there was no significant difference in % rooting among the four rooting media for NH 0 (p=0.0809), but NH 70 exhibited the highest rooting frequency on medium RM 4 (76.7 ± 3.33 %;

p=0.0016). Similarly, NH 58 achieved the highest % rooting on RM 4 (36.7 ± 3.33 %), although these were not significantly different to rooting percentages achieved using the other three media (p=0.0637). Root length for all three clones was significantly higher using RM 4 (¼ MS, 0.1mg/l IBA, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$) than any of the other three rooting media. Based on these results, this medium was deemed the most appropriate for rooting clones of this hybrid.

Figure 3.8. **The effect of medium composition on root length of GN 15, a clone of *E. grandis* x *nitens* (GN).** Shoots in RM 1 (top picture) (½ MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 1mg/l IBA, 15g/l sucrose, 3.5g/l Gelrite) produced short stunted roots, with callus as the base of the shoot. Bar= 1.1 cm. Shoots rooted on RM 4 (bottom picture) (¼ MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.1mg/l IBA, 15g/l sucrose, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$, 3.5g/l Gelrite) produced long, well-developed roots without callus. Bar = 0.9 cm

Similarly, % rooting and root lengths were compared for each medium among four clones of *E. grandis* x *urophylla* (GU), and the results are presented in Table 3.17. In RM 1 (½ MS, 1mg/l IBA) and RM 2 (½ MS, 0.1mg/l IBA), % rooting did not differ significantly among GU 151, GU 244 and GU 297 only (Table 3.17). There was no significant difference in % rooting among all four clones in RM 3 and RM 4 (Table 3.17). In terms of root length, only RM 1 and RM 2 resulted in significant differences in root length among the four clones. With regards to the response of individual clones to the four rooting media, GU 21 generally performed poorly on RM 1, 2 and 3, but RM 4 yielded a more favourable rooting response (46.7 ± 8.82 % rooting), which was significantly better than the % rooting values on the other media ($p=0.0022$). For GU 151 and GU 244, there was no significant difference in % rooting among the four media, whereas, for GU 297, % rooting on RM 4 (96.7 ± 3.33%) was significantly higher only compared to RM 3 (63.3 ± 8.82 %) ($p=0.0156$). Significantly higher root lengths for all four clones were achieved on RM 4. Hence, based on these results, as well as the fact that this medium was selected for *E. grandis* x *nitens* (GN and NH) hybrid clones, RM 4 was chosen for clones of *E. grandis* x *urophylla* (GU).

In summary, a single, common rooting medium was possible for all tested clones of the three hybrids. Although the % rooting and root lengths varied among clones within each hybrid, the most appropriate medium for rooting of all clones of the three hybrids consisted of ¼ MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$ 0.1mg/l IBA, 15g/l sucrose, 3.5g/l Gelrite, pH 5.8 (Tables 3.15, 3.16, 3.17).

Table 3.15. The effect of medium composition (RM1-RM4) on % rooting and root length in five clones of *E. grandis* x *nitens* (GN). Elongated shoots (≥ 20mm) were transferred to rooting media (see below). Cultures were initially subjected to a three day period of total darkness at room temperature (~25°C), before being transferred to a 16h light/8h darkness photoperiod at a PPFD of 37 µmol/m^2/s at 24°C day/ 21°C night for seven days. This was followed by a 16 h light/ 8 h dark photoperiod and a PPFD of 66µmol/m^2/s at 25°C ± 2°C day/ 21°C night for 28 days, at which time results were obtained. Unless otherwise stated, all media contained ½ MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 15g/l sucrose and 3.5g/l Gelrite.

RM 1 = 1 mg/l IBA,

RM 2 = 0.1mg/l IBA,

RM 3 = 0.1mg/l IBA, 0.5mg/l NAA and

RM 4 = ¼ MS, 0.1mg/l IBA, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$

Clone	RM 1		RM 2		RM 3		RM 4	
	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]
GN 1	66.7 ± 14.53 [a]	41.1 ± 2.98 [c]	40.0 ± 5.77 [a]	53.2 ± 8.41 [a]	43.3 ± 8.82 [ab]	12.4 ± 0.64 [a]	63.3 ± 3.33 [a]	47.1 ± 4.62 [a]
GN 9	63.3 ± 8.82 [a]	18.1 ± 0.97 [b]	60.0 ± 5.77 [ab]	17.4 ± 1.03 [b]	16.7 ± 8.82 [a]	8.6 ± 4.38 [a]	56.7 ± 12.02 [a]	45.9 ± 6.18 [a]
GN 15	86.7 ± 6.0 [a]	8.4 ± 0.7 [a]	40.0 ± 5.8 [a]	7.9 ± 2.1 [b]	6.7 ± 6.67 [a]	2.3 ± 2.33 [a]	60.0 ± 10.00 [a]	39.4 ± 4.56 [a]
GN 108	70.0 ± 10.0 [a]	5.9 ± 0.40 [a]	73.3 ± 8.82 [b]	8.2 ± 1.64 [b]	80.0 ± 15.28 [b]	15.5 ± 3.61 [a]	93.3 ± 6.67 [a]	41.2 ± 6.43 [a]
GN 121	76.7 ± 6.7 [a]	15.4 ± 1.3 [b]	73.3 ± 8.8 [b]	11.4 ± 3.2 [b]	76.7 ± 8.8 [b]	19.1 ± 3.83 [a]	86.7 ± 6.67 [a]	38.7 ± 3.02 [a]

[z] a-b = mean separation within columns, Scheffe's multiple range test (n=3, p≤ 0.05).

Table 3.16. The effect of medium composition (RM1-RM4) on % rooting and root length of three clones of *E. grandis* x *nitens* (NH). Elongated shoots (≥ 20mm) were transferred to rooting media (see below). Cultures were initially subjected to a three day period of total darkness at room temperature (~25°C), before being transferred to a 16h light/8h darkness photoperiod at a PPFD of 37 µmol/m^2/s at 24°C day/ 21°C night for seven days. This was followed by a 16 h light/ 8 h dark photoperiod and a PPFD of 66µmol/m^2/s at 25°C ± 2°C day/ 21°C night for 28 days, at which time results were obtained. Unless otherwise stated, all media contained ½ MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 15g/l sucrose and 3.5g/l Gelrite.

RM 1 = 1 mg/l IBA,

RM 2 = 0.1mg/l IBA,

RM 3 = 0.1mg/l IBA, 0.5mg/l NAA and

RM 4 = ¼ MS, 0.1mg/l IBA, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$

Clone	RM 1		RM 2		RM 3		RM 4	
	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]
NH 0	60.0 ± 15.28 [b]	26.2 ± 2.94 [b]	46.7 ± 12.02 [a]	27.9 ± 5.11 [b]	90.0 ± 5.77 [b]	28.6 ± 3.59 [b]	73.3 ± 3.33 [b]	39.4 ± 4.88 [b]
NH 58	10.0 ± 5.78 [a]	5.2 ± 2.59 [a]	13.3 ± 6.67 [a]	2.7 ± 1.59 [a]	26.7 ± 8.82 [a]	7.9 ± 0.91 [a]	36.7 ± 3.33 [a]	16.9 ± 0.44 [a]
NH 70	46.7 ± 3.33 [ab]	12.2 ± 1.51 [a]	43.3 ± 3.33 [a]	12.7 ± 1.17 [a]	60.0 ± 5.77 [b]	17.7 ± 1.26 [a]	76.7 ± 3.33 [b]	27.4 ± 1.94 [ab]

[z] a-b = mean separation within columns, Scheffe's multiple range test (n=3, p≤ 0.05).

Table 3.17. The effect of medium composition (RM1-RM4) on % rooting and root length in four clones of *E. grandis* x *urophylla* (GU). Elongated shoots (≥ 20mm) were transferred to rooting media (see below). Cultures were initially subjected to a three day period of total darkness at room temperature (~25°C), before being transferred to a 16h light/8h darkness photoperiod at a PPFD of 37 µmol/m^2/s at 24°C day/ 21°C night for seven days. This was followed by a 16 h light/ 8 h dark photoperiod and a PPFD of 66µmol/m^2/s at 25°C ± 2°C day/ 21°C night for 28 days, at which time results were obtained. Unless otherwise stated, all media contained ½ MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate, 15g/l sucrose and 3.5g/l Gelrite.

RM 1 = 1 mg/l IBA,

RM 2 = 0.1mg/l IBA,

RM 3 = 0.1mg/l IBA, 0.5mg/l NAA and

RM 4 = ¼ MS, 0.1mg/l IBA, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$

Clone	RM 1		RM 2		RM 3		RM 4	
	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]	% Rooting [z]	Root length (mm) [z]
GU 21	13.3 ± 3.33 [a]	13.8 ± 7.70 [a]	3.3 ± 3.33 [a]	2.0 ± 2.00 [a]	23.3 ± 3.33 [a]	13.6 ± 2.09 [a]	46.7 ± 8.82 [a]	39.4 ± 4.88 [a]
GU 151	63.3 ± 6.67 [b]	37.0 ± 2.61 [b]	46.7 ± 3.33 [b]	37.9 ± 4.54 [c]	36.7 ± 13.33 [a]	37.9 ± 6.86 [b]	60.0 ± 20.81 [a]	38.1 ± 3.89 [a]
GU 244	76.7 ± 3.33 [b]	8.2 ± 0.83 [a]	66.7 ± 12.02 [bc]	8.0 ± 0.17 [ab]	56.7 ± 3.33 [a]	29.9 ± 2.51 [ab]	76.7 ± 3.33 [a]	28.1 ± 2.61 [a]
GU 297	70.0 ± 5.77 [b]	20.8 ± 1.84 [ab]	90.0 ± 5.77 [c]	21.5 ± 3.30 [b]	63.3 ± 8.82 [a]	15.4 ± 0.67 [a]	96.7 ± 3.33 [a]	30.2 ± 1.21 [a]

[z] a-b = mean separation within columns, Scheffe's multiple range test (n=3, p≤ 0.05).

According to George (1996), it is common practise to transfer shoots to be rooted from high to low strength media, with the favourable effect of reduced nutrients being due to the lower optimum concentration of nitrogen ions being necessary for root formation than for shoot formation and growth. In this study, rooting media containing MS nutrients at ½ strength and ¼ strength were tested. For GN clones, % rooting was 6.7-86.7% on media containing ½ MS, NH clones 10-90% and GU clones 3.3-90%. Similarly, authors have achieved successful rooting using basal nutrients at half-strength (Lakshmi Sita, 1986: *E. grandis*; Karkonen *et al.*, 1999: *Callicarpa, Chosenia, Lonicera, Maackia, Morus, Populus, Prunus, Ribes, Salix, Tiosusu*; Raghava Swamy *et al*, 1992: *Dalbergia latifolia*; Puddephat *et al.*, 1999: *Quercus robur*; Sha Valli Khan *et al.*, 1999: *Syzygium alternifolium*; Lakshmi Sita and Shobha Rani, 1985; *E. grandis*; Jones and van Staden, 1994: *Eucalyptus grandis x urophylla*; Le Roux and van Staden, 1991b: *Eucalyptus* spp.)

The results of the present study indicated that the best medium for efficient rooting of all clones contained ¼ MS. This outcome parallels reported studies which achieved similar rooting percentages using MS nutrients at ¼-strength. Cortezzi-Graça and Mendes (1989) achieved the highest rooting percentages (60%) in *E. dunnii* using ¼ MS in the rooting medium. Warrag *et al.* (1990) obtained 98% rooting of *E. grandis* hybrids on a medium containing ¼ MS, and suggested that this medium was the most economical to use, since it gave rooting percentages that were comparable to those achieved with MS and ½ MS. After exhaustive testing of various media formulations, Mokotedi (1999) concluded that the most efficient medium for rooting of two *E, grandis x nitens* (GN) clones contained ¼ MS, and the optimum media determined in the present study was one that was devised by that author.

In terms of growth regulator requirements, a higher ratio of auxin to cytokinin is necessary for rooting, and in attempts to induce rhizogenesis, several different auxins have been employed by workers. However, it is generally agreed that IBA is the best auxin for inducing rooting (Burger, 1987; Zaerr and Mapes, 1988; Pelosi *et al.*, 1995; Puddephat *et al.*, 1999), although in a study by Mederos *et al* (1997/98), both NAA and IBA proved to be equally effective in inducing roots in *Plantago major*. Sudhakar

Johnson *et al* (1998) achieved optimum rooting in *Saussurea lappa* using NAA (1.07μM), and Lakshmi Sita (1986) observed 70% rooting of *E. grandis* using IAA (0.5mg/l) which proved superior to IBA (~30% rooting).

Authors have generally achieved highly successful rooting using IBA alone at a concentration of 1mg/l (Franclet and Boulay, 1982: *Eucalyptus* spp.; Cortezzi Graca and Mendes, 1989: *E. dunnii*). Furze and Cresswell (1985) achieved 90% and 80% rooting of *E. grandis* and *E. nitens* respectively, using 1mg/l IBA. The same concentration achieved 82% rooting of *E. dunnii x Eucalyptus* spp. (Fantini Jr. and Cortezzi Graca, 1989), and 80% rooting of *Quercus robur* (Puddephat *et al.*, 1999), and Das and Mitra (1990) achieved 60-80% rooting of *E. tereticornis*. In this study, 1mg/l IBA (½ MS) generally elicited a better rooting response from shoots than 0.1mg/l IBA (½ MS). However, observations by Lakshmi Sita (1986) showed that reducing the concentration of IBA actually led to enchanced rooting, and Isikawa (1987) achieved highly successful rooting of *Crytomeria japonica* using only 0.3mg/l IBA. Further, Jokinen and Tormala (1991) achieved best rooting of *Betula* clones on a medium containing 0.2mg/l IBA and Cid *et al.* (1999) use 0.5mg/l IBA to achieved maximal rooting in *E. grandis x urophylla*.

With reference to culture conditions, Mohamed and Vidaver (1988) stated that light and temperature affect rooting by controlling the activity of auxin in the rooting medium. In this study, the transfer of shoots from total darkness (72 hours) to low light intensity and temperature for 7 days then high light intensity and temperature for 21 days, was based on an optimised protocol devised by Mokotedi (1999) for two clones of *E. grandis x nitens* (GN). Although workers have rooted shoots under the same lighting and temperature conditions as used for preceding culture stages (Sankara Rao and Venkateswara, 1985; Despande *et al.*, 1999), most authors have adopted the approach of subjecting cultures to an initial period of complete darkness before transferring them to light conditions and a photoperiod. This dark treatment has ranged from 72 hours (Mascarenhas *et al.*, 1981: *Punica granatum*; Mederos Molina *et al.*, 1998: *Vitis vinifera*) up to 10 days (Trindade and Pais, 1997: *E. globulus*, 4 days; Warrag *et al.*, 1990: *E. grandis* hybrids: 5 days; Bolar, *et al.*, 1998: *Malus x domestica*, 7 days; Furze and

Cresswell, 1985: *E. grandis* and *E. nitens*, 7-10 days; Das and Mitra, 1990: *E. tereticornis*, 7-10 days).

3.3.5 Plantlet yields from established protocol for each hybrid

The feasibility of applying the results of this study commercially has to be based on the realised plantlet yield from the assessment of predicted plantlet yields from the protocols recommended for each hybrid (Figures 3.9, 3.10, 3.11). In this regard, a projection of plant yields for each hybrid was undertaken. Beginning with 100 nodal explants after surface sterilisation, the number of viable propagatory units at each stage of the culture process is presented for each clone. This is based on the percentage yields at each culture stage for each clone. Following rooting, the number of plants available for deployment is based on survival rates routinely achieved by workers in our laboratory (Mokotedi, *pers comm.*) and at Mondi Forests micropropagation laboratories (McAlister, *pers comm.*). This exercise gives an indication of potential yields from the application of these results, as well as stages of the protocol that could be improved, so that higher yields may be achieved.

Table 3.18 shows the potential yields for GN clones, based on 100 initial nodal explants following successful surface sterilisation (protocol 6, Chapter 2), and the non-clone specific protocol that was established for this hybrid (Figure 3.9). This approach would realise 168-667 successfully hardened-off plants (depending on clone) after approximately 6 months. Hence, with certain exceptions, GN clones can be propagated successfully in high numbers by using the established hybrid-specific protocol. One such exception is GN 1, for which a clone-specific protocol (involving using M1 at multiplication) appears to be necessary should higher plantlet yields (336 hardened-off plants) be required. In this regard, it may be more viable to bulk up cultures of this clone using the specific multiplication medium rather than the non-clone specific formulation. In addition, the low percentage rooting of GN 9 shoots in particular suggests that a higher-yielding rooting medium needs to be established for this clone.

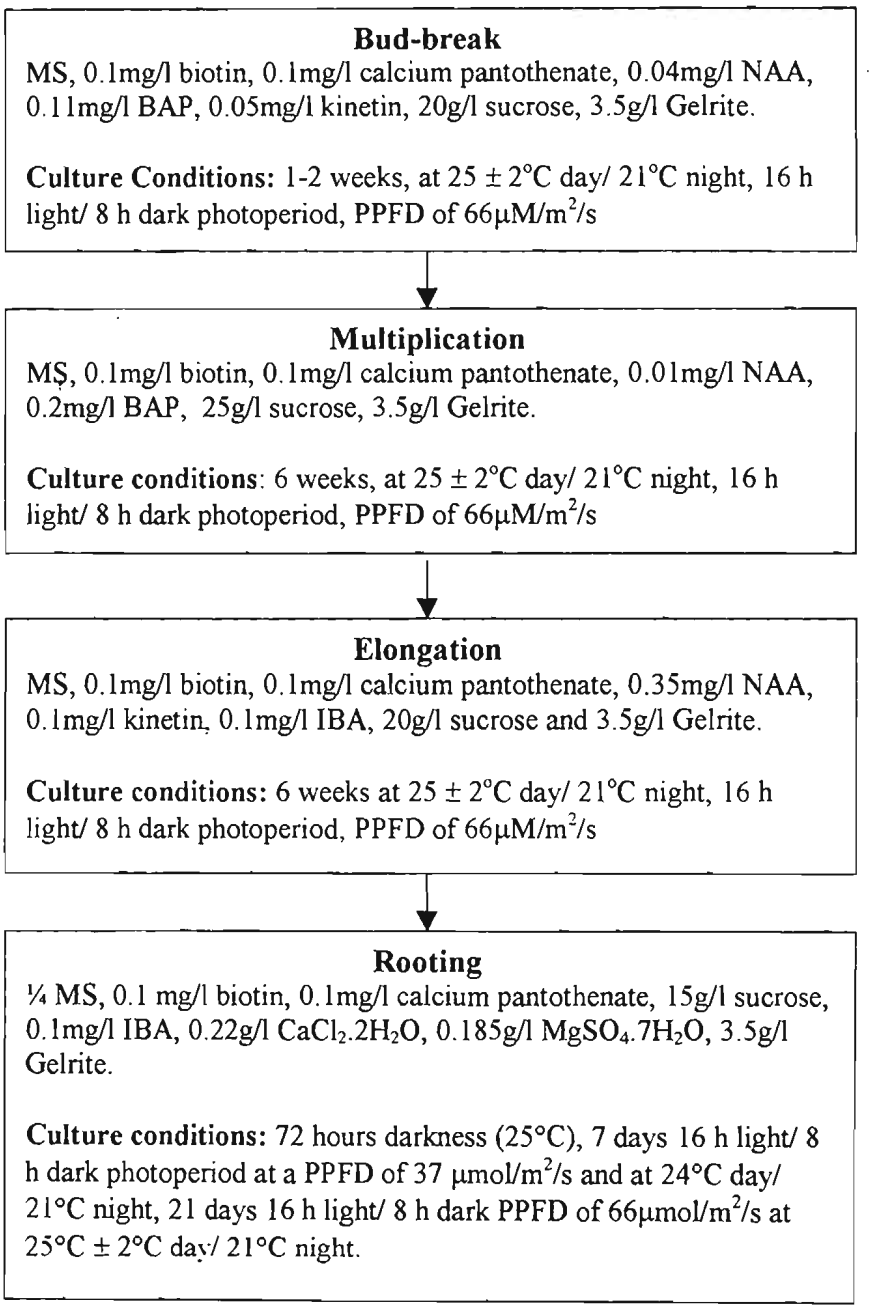

Figure 3.9. **Hybrid-specific protocol for axillary bud proliferation of five clones of *E. grandis* x *nitens* (GN).**

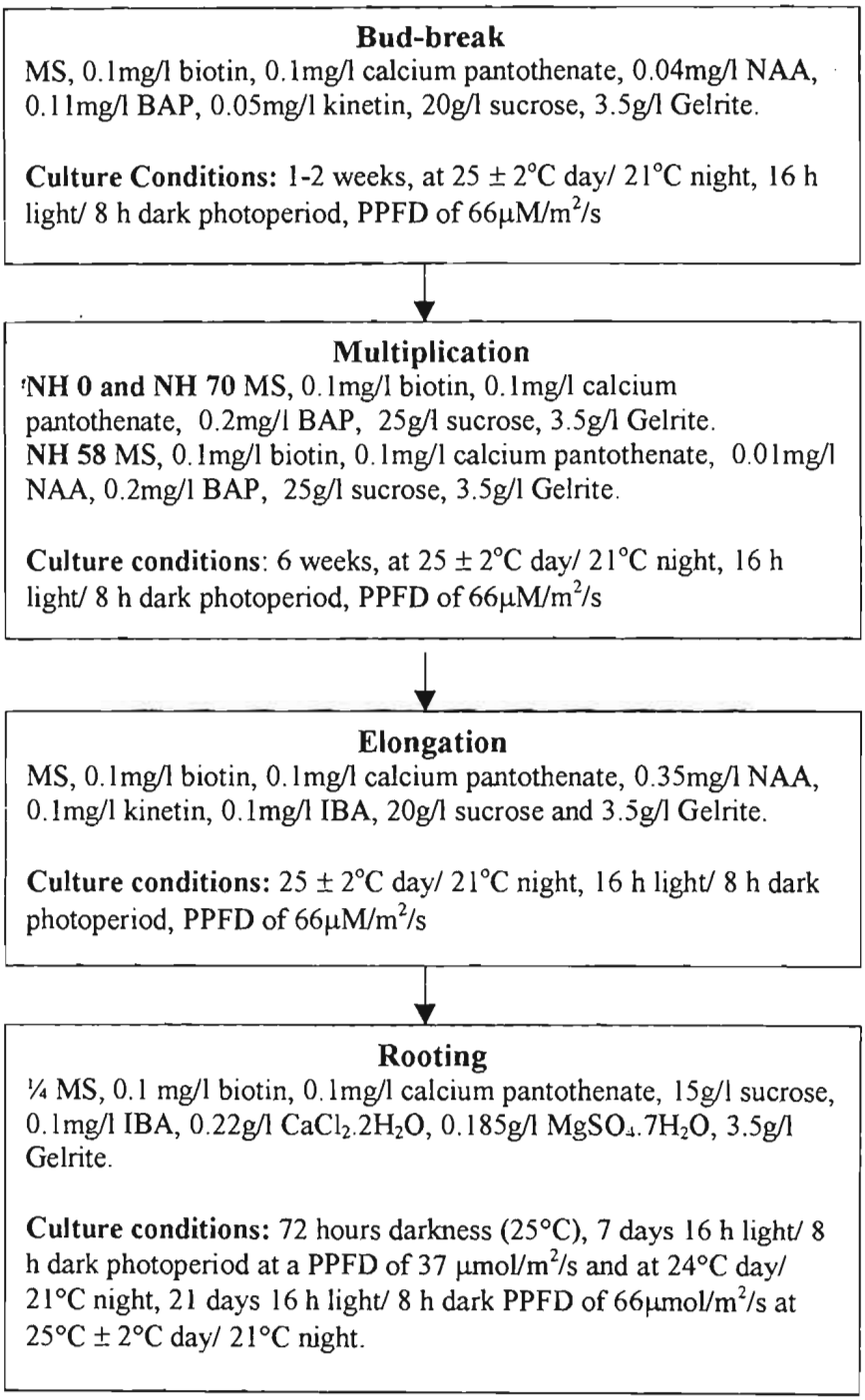

Figure 3.10. **Hybrid-specific protocol for axillary bud proliferation of three clones of *E. grandis* x *nitens* (NH).**

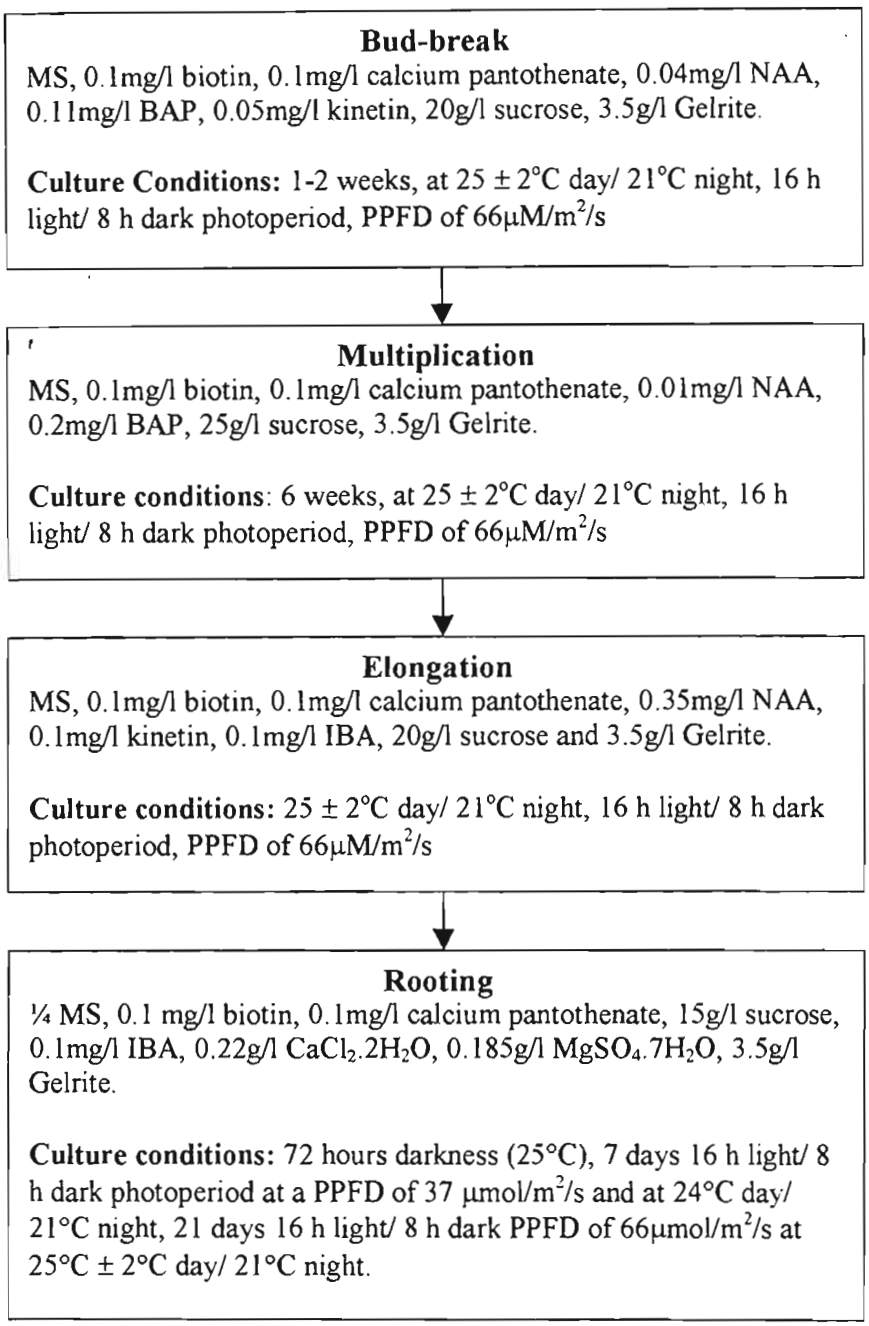

Figure 3.11. **Hybrid-specific protocol for axillary bud proliferation of four clones of *E. grandis* x *urophylla* (GU).**

Table 3.18. Yields of viable propagules per 100 nodal explants after each culture stage and hardening-off, for five clones of *E. grandis* x *nitens* (GN), based on the established hybrid-specific protocol (Figure 3.9).

Clone	Number of propagules after				
	Bud-break	Multiplication[x]	Elongation	Rooting	Hardening-off[y]
GN 1	90	360	296	187	168
GN 9	90	990	877	497	447
GN 15	87	1131	989	593	533
GN 108	88	968	796	742	667
GN 121	90	720	622	539	485

[x]= based on yields achieved after 6 weeks (1 subculture)

[y]= based on 90% hardening-off success

Table 3.19 shows the potential yields for NH clones using the established non-clone specific protocol, starting with 100 explants (Figure 3.10). By using this approach, a yield of 35-854 successfully hardened-off plantlets (depending on clone) would be possible after 6 months. Of the three NH clones for which yield predictions have been made, NH 58 exhibited a poor response to the bud-break (33%), multiplication (132 shoots/bud) and rooting media (39 rooted plantlets) (Table 3.19). The use of a clone-specific multiplication medium M2 (0.2,g/l BAP, 0.01mg/l NAA, 25g/l sucrose) for NH 58 may achieve slightly higher shoot yields (297 shoots/bud). However, this is offset by the low yields in subsequent culture stages (elongation and rooting) and would eventually result in only 79 plantlets after hardening-off. Thus, more suitable protocols need to be established for NH 58, to ensure maximum productivity. With regards to NH 69, the poor results achieved for bud-break and multiplication (Tables 3.7, 3.10), precluded any further studies on elongation and rooting and therefore, a yield prediction could not be carried out for this clone. More suitable protocols for NH 69 need to be devised to ensure the economic viability of micropropagating this clone on a commercial scale.

Table 3.19. Yields of viable propagules per 100 nodal explants after each culture stage and hardening-off, for three clones of *E. grandis* x *nitens* (NH), based on the established hybrid-specific protocol (Figure 3.10).

Clone	Number of propagules after				
	Bud-break	Multiplication[x]	Elongation	Rooting	Hardening-off[y]
NH 0	75	1575	1296	949	854
NH 58	33	132	106	39	35
NH 70	20	400	320	245	220

[x] = based on yields after 6 weeks (1 subculture)

[y]= based on 90% hardening-off success

Similarly, with five GU clones, (Figure 3.11), the hybrid-specific approach (starting with 100 nodal explants) would yield 54-349 hardened-off plantlets after 6 months (Table 3.20). Based on the predicted yields, the protocol can be used successfully for the maintenance of stock cultures, when high yields are not the primary objective. However, if the achievement of maximum yields is necessary, a more suitable multiplication medium needs to be employed for all the clones. For example, one could use medium M4 (0.1mg/l BAP, 0.01mg/l NAA, 0.2mg/l kinetin, 25g/l sucrose) which gave higher shoot yields after 6 weeks in multiplication, than M2 (0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose). Furthermore, the elongation of all four clones has to be improved to provide suitable shoots in sufficient numbers for rooting. With reference to rooting, GU 21 in particular, performed very poorly on the common rooting medium and a more appropriate medium must be devised in order to make micropropagation a viable option for propagating this clone.

Table 3.20. Yields of viable propagules per 100 nodal explants after each culture stage, and hardening-off, for four clones of *E. grandis* x *urophylla* (GU), based on the established hybrid-specific protocol for this hybrid (Figure 3.11).

Clone	Number of propagules after				
	Bud-break	Multiplication[x]	Elongation	Rooting	Hardening-off[y]
GU 21	91	182	128	60	54
GU 151	80	400	304	182	163
GU 244	91	273	207	158	142
GU 297	86	516	402	388	349

[x] = based on yields after 6 weeks (1 subculture)

[y]= based on 90% hardening-off success

In summary, non-genotype specific protocols for axillary bud proliferation were successfully established for GN and GU clones. For NH clones further research is necessary to establish media that can be used successfully for all the clones.

3.3.6 Conclusion

As discussed earlier (section 3.3.5), for clones of GN and GU, the use of a common hybrid-specific protocol for micropropagation is a viable alternative to using specific media for each clone. However, further research is necessary to determine similar high-yielding, hybrid-specific protocols for NH clones. For a few exceptions, *e.g.* GN 1, NH 58, GU 21, a specific medium for certain culture stages is necessary to realise higher yields than were achieved using the common protocol. The non-specific protocols are detailed in Figures 3.9, 3.10 and 3.11.

The non-clone specific approach can be employed successfully for GN clones. The exceptions are GN 1, which would require a specific multiplication medium and GN 9, which requires a more suitable rooting medium.

The responses of NH clones were highly genotype specific, for all culture stages, and the practise of using clone-specific micropropagation protocols for these clones will have to continue, at least until a suitable non-specific protocol can be determined.

Referring to GU clones, it is suggested that the non-specific multiplication medium is generally suitable for the maintenance of stock cultures. However, should high shoot yields be the priority, a more suitable hybrid-specific multiplication medium needs to be determined. Moreover, GU 21 responded poorly to the rooting media, and further research needs to be conducted to establish a more suitable rooting medium for this clone.

In conclusion, the findings of this study hold several implications for commercial production via micropropagation of the hybrid clones under study. Depending on the time available for production of viable plants, the manager has to decide whether the priority is maximum possible output of plants or not, and based on this a decision has to be made regarding whether or not the hybrid-specific protocols devised in this study, should be implemented. When dealing with GN and GU clones, and the achievement of the highest possible yields is not a priority, the general non-specific protocols can be used, since this saves time and is cost-effective. To realise maximum yields at certain culture stages, a specific protocol has to be implemented for particular clones, *e.g.* GN1. At present, the requirement for clone-specific protocols is mandatory for *E. grandis* x *nitens* (NH) due to the genotypic differences between clones.

Chapter 4:
Concluding Remarks and Future Research Strategies

4.1 Establishment and application of clone-unspecific sterilisation and micropropagation protocols

The genus *Eucalyptus*, in particular, *E. grandis* is a fundamental part of the South African forestry industry. Hybrids of *E. grandis* with other *Eucalyptus* species have also come to play a key role in commercial forestry enterprises, since they allow for the utilisation of marginal sites that would prove unsuitable for pure species and further, hybridisation can achieve improved wood quality and traits. In terms of the propagation of commercially important species, vegetative propagation by cuttings is the most commonly-used method for mass-production. However, because of certain shortfalls related to the high cost of *in vitro* methods, these are generally used only to supplement existing clonal programmes in commercial forestry companies. The somewhat limited application of techniques is mainly due to the fact that genotypic variation among clones requires the optimisation and implementation of specific media and protocols for each clone, which is time-consuming and expensive.

This study aimed to determine hybrid-specific rather than clone-specific protocols for axillary bud proliferation for clones of three commercially important *Eucalyptus* hybrids, *viz. E. grandis* x *nitens* (GN), *E. grandis* x *nitens* (NH) and *E. grandis* x *urophylla* (GU). This was accomplished by addressing each stage in the *in vitro* culture process separately, beginning with Stage I, surface sterilisation of nodal explants.

Preliminary investigations indicated that exposure time of the explant material to the sterilant solutions was a key factor in the management of necrosis levels. Further, investigations with mercuric chloride showed that this sterilant contributed to the high levels of necrosis experienced in several clones, and should thus be eliminated from the sterilisation protocol. This was accomplished in the optimised sterilisation method for all clones (Table 2.8, Section 2.3.3). The results showed that the protocol was successful in

reducing contamination (≤ 11%) and necrosis (≤ 22%) to levels that are considered acceptable by several authors (Ikemori, 1987; Le Roux and van Staden, 1991b; Jones and van Staden, 1994; Beck *et al.*, 1998). Although the protocol did work well for all the tested clones, it may be possible to reduce necrosis levels further in certain clones (e.g. NH 70). However, because only a few clones of each hybrid were tested, further studies should be conducted on other clones to determine whether this protocol can be more widely used. In terms of the commercial applicability of this common sterilisation technique, the use of this single method for a range of different genotypes is cost-effective, since it saves on chemicals, time and labour expended on the research and subsequent implementation and management of specific sterilisation methods for each clone. Furthermore, the elimination of mercuric chloride, a known toxic heavy metal compound, from the final protocol, makes for a safer working environment for workers and a more environmentally-friendly protocol, an important consideration for industries.

With regards to the stages of the actual tissue culture process, non-genotype specific protocols for axillary bud proliferation were established for GN and GU clones, albeit resulting in low yields. Non-clone specific protocols for NH clones could not be established for certain culture stages, due to the high degree of genotypic differences among clones. The established protocols and yields for clones of these hybrids, are presented in Figures 3.9, 3.10, 3.11 and Tables 3.19, 3.20 and 3.21 (Section 3.3.5). For clones of the NH hybrid, a single high-yielding multiplication medium could not be determined, due to the high clonal specificity in responses to the different media. Furthermore, the commercial viability of propagating NH 69 via axillary bud-proliferation needs to be re-assessed, due to the difficulties experienced in introducing into culture and bulking up this clone *in vitro*, both in this study and in independent investigations by other workers (McAlister, *pers comm.*). Unless a more suitable, high-yielding protocol can be established for this clone, it may be necessary to propagate this clone wholly by other vegetative methods such as stem cuttings.

Although a non-clone specific protocol was established for two of the three hybrids (GN and GU), it is recognised that for certain clones, it may be necessary to employ specific

protocols at certain stages to ensure maximum yields. For example, it is possible to achieve higher shoot multiplication yields for GN 1 by using multiplication medium M1 (0.2mg/l BAP, 20g/l sucrose) rather than the non-specific multiplication medium M2 (0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose) and rooting for GN 9 must be improved.

As with the sterilisation protocol, it cannot be assumed that the micropropagation protocols would be efficacious for all other genotypes of the GN and GU hybrids. Thus, more genotypes of these hybrids should undergo testing using the methods described. Finally, as discussed in Chapter 3 (section 3.3.5), the non-clone-specific micropropagation protocol devised for each hybrid can be used to achieve moderate to high plantlet yields for most of the tested GN clones (4.5-6.7 plants/explant), NH 0 (8.6 plants/explant) and NH 70 (2.2 plants/explant), as well as for GU 151 (1.6 plants/explant), GU 244 (1.4 plants/explant) and GU 297 (3.5 plants/explant). However, clone-specific protocols are still necessary to achieve higher plant yields for such clones as GN 1 (1.7 plants/explant), NH 58 (0.35 plants/explant) and GU 21 (0.54 plants/explant).

The achievements of this work and recommended future research strategies are summarised in Table 4.1.

4.2 Proposed future research strategies

As previously discussed, the hybrid-specific micropropagation protocols established in this study did not achieve high yields in all the tested clones at various culture stages. Hence, more suitable media and methods need to be determined for the clones that performed poorly on the established media. Also, the established protocols in this study need to be tested on more clones of the same hybrids, to determine their wide range applicability in a commercial setting. The possibility of applying these techniques to a wider range of clones and hybrids would prove more cost-effective and labour-saving than simply utilising them for a limited number of clones.

Table 4.1 Summary of investigations successfully completed during this study and areas of proposed future research.

Investigation	Result
Establishment of non-specific sterilisation Method	*Achieved for all clones* **GN, NH and GU**: (15 min. fungicide wash [1g/l Benlate, 1g/l boric acid, 0.5 ml/l Bravo], 3 min. calcium hypochlorite (10g/l)
Establishment of non-clone specific Micropropagation protocols	
Stages:	
a) Bud-break [x,y]	*Achieved, protocol for GN and GU* **GN**: 0.11mg/l BAP, 0.04mg/l NAA, 0.05mg/l kinetin, 20g/l sucrose. **GU**: 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose.
b) Multiplication [x,y]	*Achieved for GN and GU* **GN and GU**: 0.2mg/l BAP, 0.01mg/l NAA, 25g/l sucrose
c) Elongation [x,y]	*Achieved for all clones* **GN, NH and GU**: 0.35mg/l NAA, 0.1mg/l kinetin, 0.1mg/l IBA, 20g/l sucrose
d) Rooting [x,z]	*Achieved for GN 1, GN 15, GN 108, GN121, NH0, NH70, GU 151, GU244 and GU297* **GN, NH and GU**: ¼ MS, 0.1mg/l IBA, 0.22g/l $CaCl_2.2H_2O$, 0.185g/l $MgSO_4.7H_2O$, 15g/l sucrose.

[x] Unless otherwise stated, all media contained MS, 0.1mg/l biotin, 0.1mg/l calcium pantothenate and 3.5g/l Gelrite.

[y] Cultures conditions: 25 ± 2°C day/ 21°C night, 16 h light/ 8 h dark photoperiod (PPFD of 66μM/m^2/s)

[z] Cultures conditions: 72 hours darkness (25°C), and at 24°C day/ 21°C night, 7 days 16 h light/ 8 h dark photoperiod (PPFD of 37 μmol/m^2/s), 21 days 16 h light/ 8 h dark (PPFD of 66μmol/m^2/s) at 25°C ± 2°C day/ 21°C night.

In addition, the possibility of maximising yields by employing various strategies should be explored further. In this regard, other culture systems, such as temporary immersion systems (McAlister *pers comm.*; Alvard *et al.*, 1993; Teisson and Alvard, 1995; Nepovim and Vanek, 1998; Nixon *et al.*, 2000) have met with considerable interest and success at Mountain Home Laboratories (McAlister, *pers comm.*), and this can be explored more thoroughly with reference to a wide range of genotypes. Recently, at the Mondi Forests Mountain Home Laboratory, Nixon *et al.* (2000) showed that a temporary immersion system (RITA) tested on two *Eucalyptus* clones, yielded higher multiplication rates in a shorter time than semi-solid media. In addition, the RITA system reduced hyperhydricity and callus formation and the plantlets generated were darker green, larger and had a greater leaf area than plantlets on semi-solid media. Furthermore, the plantlets generated from the RITA system also exhibited a higher rooting efficiency and higher survival in the greenhouse than those obtained with semi-solid media (Nixon *et al.*, 2000). These observations confirm the recommendation by Teisson and Alvard (1995), who advocated the use of liquid temporary immersion systems for propagation on the basis that labour is reduced, changing of media is facilitated and the subculture time for explants in shorter. Moreover, because gelling agents need not be added to the culture media, the cost associated with including this component in the medium is removed. Additional advantages of using such a system include the fact that anti-microbial agents can be added to the liquid medium and the air-flow through the system is better than in semi-solid media, thus contributing to improved cuticle development and, hence, *ex vitro* survival of the plant. Further, Alvard *et al.* (1993) suggested that the medium for the immersion system could be simply sterilised by ultra-filtration rather than by autoclaving.

In summary, further studies will focus on achieving a common, non-clone specific protocol for NH clones, as well as improving yields already achieved for tested GN and GU clones. Other clones of the tested hybrids will also be subjected to the tested non-specific protocols. Strategies of improving yields and the quality and survival of plantlets, (*e.g.* temporary immersion systems) will also be investigated.

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