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The development of *in vitro* rooting systems for cold-tolerant *Eucalyptus grandis* x *nitens* clones and the assessment of the hydraulic efficiency of roots produced by *in vitro* vs. cutting propagation

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The development of *in vitro* rooting systems for cold-tolerant *Eucalyptus grandis* x *nitens* clones and the assessment of the hydraulic efficiency of roots produced by *in vitro* vs. cutting propagation

by

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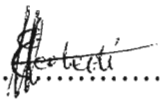
"This tree [*Eucalyptus*] grows faster than the cottonwood, taller than the redwood, straight as a fir. Its timber is as strong as oak, as elastic as hickory and as beautiful as maple and as enduring as cedar".

Pratt, 1910

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 1998 to December 1999, under the supervision of Dr. Paula Watt and Prof. Norman Pammenter.

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.



M.E.O. Mokotedi
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ABSTRACT

Hybrid clones of the fast-growing *Eucalyptus grandis* and cold-tolerant *E. nitens* (GN clones) have been identified by the South African Forestry Industry as being highly suitable for plantations in cold-dry marginal areas. However, one of the main problems regarding their propagation is the difficulty in rooting of cuttings, both *in vitro* and *ex vitro*. The aims of this investigation, therefore, were (1) to develop widely applicable and efficient *in vitro* rooting system(s) for these commercially important clones, and (2) to assess some physiological characteristics of the roots produced.

Adventitious shoots (15-20 mm in length) were obtained (10 shoots/explant) from axillary buds on Murashige and Skoog's (MS) medium containing 0.01 $mg.l^{-1}$ NAA, 0.01 $mg.l^{-1}$ IBA and 0.2 $g.l^{-1}$ FAP. The effect of various medium components, as well as modification of culture environment on *in vitro* rooting, were investigated. The highest rooting frequencies in clones GN121 (75%) and GN107 (65%) were achieved on ¼ MS with additional 0.22 $g.l^{-1}$ $CaCl_2.2H_2O$ and 0.18 $g.l^{-1}$ $MgSO_4.7H_2O$, 0.1 $mg.l^{-1}$ IBA, 0.1 $mg.l^{-1}$ biotin, 0.1 $mg.l^{-1}$ calcium pantothenate, 15 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Best culture conditions were an initial 72-hours dark incubation followed by a 16-hours day/8-hours night photoperiod at a PPFD of 37 $\mu mol.m^{-2}.s^{-1}$ and 23°C day/21°C night for seven days, after which the PPFD was increased to 66 $\mu mol.m^{-2}.s^{-1}$ at 27°C day/21°C night for 18 days.

Towards the development of a more widely applicable *in vitro* rooting protocol for GN clones, the use of *Agrobacterium rhizogenes* strains was investigated. Production of transgenic roots was observed on carrot discs and shoots from seedlings of *Eucalyptus grandis* and *E. nitens*, but not on shoots of GN clones. Therefore, a method needs to be established for the successful transfer and integration of the Ri plasmid of *Agrobacterium* into the hybrid plant genome for induction of transgenic roots.

The quality of roots produced *in vitro* and from cuttings was assessed by examination of root anatomy and hydraulic characteristics. Adventitious roots were prepared for measurement of hydraulic conductivity by detopping explants, then filtered, acidified distilled water was drawn through undisturbed potted root systems under partial vacuum, causing no damage to the roots. Initial studies showed that tissue culture-derived roots exhibited a higher specific root mass hydraulic conductivity than those derived from cuttings (6.46×10^{-6} vs. 3.06×10^{-6} $g.kPa^{-1}.s^{-1}.g^{-1}$ dry root), probably due to root architecture. Curves relating vulnerability to water potential were constructed and both types of roots showed vulnerability to cavitation at high water potentials. Differences were also observed in staining reactions (safranin and fastgreen) which might suggest differences in presence and level of secondary metabolites in these roots at the juvenile stage.

Applications of the developed protocols and future research strategies are discussed.

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LIST OF ABBREVIATIONS

Acetosyringone	3',5'-dimethoxy-4'-hydroxyacetophenone
ATP	adenosine triphosphate
BAP/BA	N^6-Benzylaminopurine/N^6-Benzyladenine
cm	centimeter
CO_2	carbon dioxide gas
Co.	company
°C	degrees centigrade
FAP	6-furfurylaminopurine (kinetin)
GC/MS	gas chromatography-mass spectrometry analysis
GN	*Eucalyptus grandis* x *nitens*
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
kPa	kilopascal (unit of pressure)
L	Luria broth
LB	Luria-Bertani broth
M	molar
mM	millimolar
mol	mole
MS	Murashige and Skoog (1962) medium
NAA	α-naphthalene acetic acid
NaOCl	sodium hypochlorite
pH	hydrogen ion concentration
PPFD	photosynthetic photon flux density
PCV	polyvinyl chloride
PVP	polyninylpyrrolidone
pRi	root inducing plasmid
rpm	revolutions per minute
T-DNA	transferred DNA
Tween-20	polyoxyethylene sorbitan monolaurate
µ	micro

$\mu mol.m^{-2}.s^{-1}$	micro moles per meter squared per second
vir	virulence
v/v	volume by volume
w/v	weight by volume
$W.m^{-2}$	watt per meter squared
%	percent/percentage

CHAPTER 1: GENERAL INTRODUCTION

1.1 Brief history and importance of *Eucalyptus*

The genus *Eucalyptus* (family Myrtaceae) was first discovered in Australia just over 200 years ago by Charles Louis L'Heritier de Brutelle (Turnbul, 1991). Later in the nineteenth century, seed dispersal by travelers, traders, gold miners, soldiers, priests and botanists spread eucalypts to many parts of the globe. Australia's soils are generally ancient, of Tertiary origin (about 50 million years), and many are rather nutrient poor, particularly phosphorus (Beadle, 1981). Most eucalypts are thus adapted to occasional droughts and soils low in phosphorus and other nutrients (Muller-Dombois, 1992). It has been reported recently that there are now over eight million hectares of eucalypt plantations established worldwide (Turnbull, 1991). Approximately one and a quarter million hectares (ha) are found in the southern hemisphere (Smith, 1996), and this is made up of 524 000 ha in South Africa, 240 000 ha in Argentina, 238 000 ha in Chile and 127 000 ha in Australia.

The South African government began developing plantations in the early 20th century to take the pressure off indigenous forests (Smith, 1996; Anon, 1998). In the 19th century, during the discovery of gold in the Witwatersrand, the forestry industry was mainly based on the harvesting of indigenous trees for timber production. Since the indigenous species generally grew too slowly for the increasing demand, gum trees (*Eucalyptus* species, especially *E. grandis*) with key characteristics were imported to South Africa from Australia. The development of plantations was uncontrolled by the government until 1972 when the Afforestation Permit System (APS) was introduced to regulate the planting of exotic trees and to reduce the area of land to be planted to trees so that water could be conserved (Anon, 1998). Land varies from flat to steep terrain and soil from

sand to loamy (Smith, 1996). A range of species is planted such as *Eucalyptus grandis, E. saligna, E. macarthurii, E. fastigata, E. diversicolor, E. paniculata, E. nitens,* and *E. dunnii* as well as several hybrids (Smith, 1996; Denison and Kietzka, 1993). In addition to pulp and paper production, South African companies (mainly Mondi Forests and Sappi) produce eucalypts for woodchip export and mining timber (Smith, 1996).

Eucalypts are regarded as "super trees" because they provide many useful products to mankind (Smith, 1996). Different species are used for a wide variety of purposes, including production of industrial charcoal, domestic and industrial energy, sawn timber, essential oils, honey, tannin, shade and shelter, and leaves are used as animal fodder (Hills and Brown, 1978; McComb and Bennett, 1986; Turnbull, 1991; Le Roux and van Staden, 1991b). Rural communities (e.g. in China, India and South Africa) are often forced by poverty and population pressure to destroy natural forests to survive. Hence, Turnbull (1991) and Denison and Kietzka (1993) suggested that eucalypt plantations would be competitive with wood from natural forests in the future and take pressure off this resource.

In Australia, eucalypt plantations have been used on a small scale for disposal of industrial and urban waste (Turnbull, 1991). According to that author, such plantations offer the possibility of producing cheap fuelwood as well as providing the solution to an urban environmental problem. In South Africa there is a large potential market for high grade *E. grandis* and *E. saligna* timber which dominate the hardwood sawn timber industry (Muller, 1988). However, hybrids (especially cold-tolerant clones) are expected to play a more significant role in clonal forestry (Denison and Kietzka, 1993). South Africa is arguably the world leader in small size log utilization and drying, but Australia and New Zealand which handle a more diverse range of species have much to contribute from processing (sawing and seasoning) research (Turnbul, 1991).

1.2 Importance of intensive hybrid forestry in South Africa

The areas most suitable for commercial forestry plantations in South Africa stretch across a narrow belt along the east coast because of good climate, rainfall and soils (Denison and Kietzka, 1993). However, such 'fertile' areas are also in demand for agricultural crops and conservation of indigenous flora. Therefore, forestry companies are not able to purchase 'fertile' land anymore, and the government has also imposed strict policies to control commercial forestry plantations in an effort to balance the hydrological circle (Denison and Kietzka, 1993; Smith, 1996). In order to remain productive and meet the required wood product demands, the forestry industry needs to maximize productivity from existing plantations by incorporating biotechnological methods (e.g. tissue culture) in breeding programmes and also use land in cold and dry marginal areas that was traditionally considered unsuitable for plantation forestry (Denison and Kietzka, 1993; Watt *et al.*, 1997). Most species of the genus *Eucalyptus* that possess rapid growth and good form characteristics are frost-sensitive (e.g. *E. grandis*, and *E. nova-anglica* - Mehra-Palta, 1982). A few, such as *Eucalyptus nitens* have attracted attention as fast-growing and productive species which, due to their inherent cold-tolerance, are suitable for temperate regions subject to frost attack (Bandyopadhyay *et al.*, 1999; Denison, 1999).

About ten years ago Mondi Forests started an intensive hybrid breeding programme with *E. grandis* and other species for planting in marginal areas and increasing productivity from existing plantations (Denison, 1999). The most common hybrid combinations for the more subtropical areas are *Eucalyptus grandis* crossed with either *E. camaldulensis* (GC), *E. urophylla* (GU), or *E. tereticornis* (GT) (Denison and Kietzka, 1993). For the temperate areas the hybrids produced are *E. grandis* x *E. nitens* (GN) and *E. grandis* x *E. macarthurii* (GM) (Denison and Kietzka, 1993). These hybrids outperform the pure species on marginal sites and are consistently more resistant to diseases, pests, cold, heat and drought (Denison and Kietzka, 1993). The

wood and growth properties of these hybrids are normally intermediate between the parent species, but superior growth to both parents is common (Denison and Kietzka, 1993). Further, the benefits of hybrid forestry include: (1) hybrid vigour (heterosis) (stronger in zones which are marginal for pure species), (2) disease resistance (*E. grandis* is resistant to many diseases, so are its hybrids), (3) wood properties (more homogenous wood density), (4) nursery efficiency (hybrids root more readily compared with pure species), and (5) adaptability (hybrids can tolerate limiting factors and withstand stress more readily) (Denison and Kietzka, 1993).

Several authors have warned that one must be careful in the assessment of hybrid vigour because it is affected by time and location (Zobel and Talbert, 1984; Martin, 1988; Denison and Kietzka, 1993). According to Denison and Kietzka (1993), many of the *E. grandis* hybrids express heretosis early in life, but do not maintain the good growth through to rotation (sprinters). Those authors suggested that decisions on the acceptance of hybrid combinations for operational programmes should not be made until at least half rotation age for the eucalypt hybrid. Presently, Mondi's commercial clonal programme is comprised of *E. grandis*, GC, GT, GU, GN, *E. nitens* hybrids and *E. dunnii* clones matched to site and product (Denison, 1999). Although cold-tolerant GN hybrids are difficult to root, they have shown superior pulp properties and yield (Denison, 1999), and research is currently ongoing to improve their rooting ability. Further, these GN hybrids may be the most desirable for planting in marginal areas as studies have indicated that they appear to be more water-use efficient than pure *E. grandis* and some hybrids such as GC (February *et al.*, 1995).

1.3 Propagation of *Eucalyptus* species and hybrids

1.3.1 *Vegetative propagation in clonal programmes*

In areas of natural forests (e.g. Australia) where trees are removed by selective felling, natural regeneration from seed occurs (McComb and Bennett, 1986). However, eucalypts are preferably propagated as rooted cuttings (McComb and Bennett, 1986) or scions are grafted onto seedling rootstocks of the same genotype (Gardner, 1998). Cuttings are normally obtained from clonal hedges every three to four weeks, treated with rooting powder and maintained under misthouse conditions. However, a significant drawback against cuttings is the physiological aging of parent plants from which cuttings are obtained (Biondi and Thorpe, 1981; Le Roux and van Staden, 1991b). Burdon (1988) notes that hedging appears to be effective technically, although few hopes are held for it halting maturation indefinitely. Some species of *Eucalyptus* such as *E. regnans* and *E. nitens* do not sprout readily at all, whereas others (e.g. *E. camuldulensis* and *E. deglupta*) sprout vigorously and root easily while other species sprout readily but root with difficulty (*E. globulus*) (Zobel, 1993). Paton *et al.* (1970) reported that cuttings from most mature eucalypts do not root, and suggested the presence of a rooting inhibitor that is expressed only in adult material. Zobel (1993) emphasized the fact that there is no one methodology of rooting that works for all *Eucalyptus* species and suggested that each organization must work out techniques suitable for clonal propagation for its own conditions.

Grafting is expensive but can be used to 'rejuvenate' shoots for subsequent use, either as cuttings or as explants for tissue culture (McComb and Bennett, 1986). At ICFR (Institute for Commercial Forest Research, Pietermaritzburg), researchers observed that late grafting (after early spring) may have disastrous effects on percentage of survival of grafts made (Gardner, 1998). For example, of the 600 grafts made for *E. dunni*, 56.5 % survived after six months compared with 9.5 % survival of 410 grafts made for *E.*

nitens and 32.5% of 320 grafts made for *E. smithii*. As with grafting, air layering is a labour-intensive process and eucalypts are very slow to root when layered (Hartney, 1980; McComb and Bennett, 1986).

Benefits of vegetative propagation include production of more uniform wood, selection of clones for desired wood, and the opportunity to match clones with site and silvicultural treatment (Zobel, 1993). Zobel (1993) further noted that eucalypt clones generally show a strong genotype x environment interactions when environments vary considerably. An example cited by that author referred to wind and rain damage of one of the best phenotypes which produced superior clonal planting for the first 1.5 years of its test. Every member of that particular clone had only two major roots that were approximately 180° apart. In the wet soil, the wind just pivoted the trees out of the ground. Such observations emphasize the need to study root characteristics (morphology, anatomy and physiology) in relation to methods of propagation and plantation sites. In some genera, rooted cuttings have root systems that are qualitatively different from those formed by seedlings. For example, roots of cuttings of *Bombacopsis quinata* may be shallow and fibrous, without formation of massive taproot generally formed by seedlings (Zobel, 1993). This limits the use of rooted cuttings to moister sites because they may be drought prone on the drier sites (Zobel, 1993). The pyramid shaped inserts that are normally used in *ex vitro* rooting of cuttings (e.g. at Mountain Home Laboratory, Mondi Forests) may further minimize plagiotrophic root growth and encourage gravitropism instead.

1.3.2 *Biotechnological approaches*

Biotechnology as a scientific discipline encompasses a large number of different techniques such as tissue culture, genome analysis, molecular markers, gene cloning and the genetic modification of organisms using combination of several methods (Schuch, 1991). Of these, relevant to the present study are tissue culture

(micropropagation) and genetic modification (i.e. production of chimeras) using *Agrobacterium rhizogenes*.

In vitro techniques such as micropropagation may be utilized in conjunction with a macropropagation production programme (rooting of cuttings) (Biondi and Thorpe, 1981; Yang *et al.*, 1995; Watt *et al.* 1997). Asexual multiplication of both hardwoods (eucalypts) and softwoods (pines) using tissue culture methods can be achieved by three approaches, namely (1) enhancing axillary bud breaking, (2) production of adventitious buds and (3) somatic embryogenesis (Thorpe *et al.*, 1991). The first two approaches lead to plantlet formation via organogenesis through the production of unipolar shoots, which must then be rooted in a multistaged process. In contrast, somatic embryogenesis leads to the formation of a bipolar embryo, through steps that are often similar to zygotic embryogenesis (Thorpe *et al.*, 1991). The potential for forming large numbers of plants *in vitro* increases in the above order, but unfortunately, so does the difficulty in producing plantlets (Thorpe *et al.*, 1991). Bornman[1] (pers.comm. 1998) expressed similar sentiments and emphasized that the main drawback in somatic embryogenesis is the maturation step. In many species, plantlets recovered from somatic embryos have lost clonal fidelity owing to induced mutation during tissue culture (Barwale and Widholm, 1987). For these reasons, the forestry industry has opted to invest funding into research to establish protocols using the direct organogenesis approach to clonally propagate selected superior *Eucalyptus* trees *in vitro*.

Tissue culture may be used for the production of stock and hedge material from which cuttings are harvested for plantation establishment. In this regard, Watt *et al.* (1995, 1997) reported that cuttings from micropropagated *Eucalyptus* species and hybrids exhibit higher percentages of rooting, and in some cases produce more shoots than conventional adult sources. However, with some plant species and hybrids the problem of poor rooting ability persists *in vitro*, especially with cold-tolerant *E. grandis* x *nitens*

[1] C. H. Bornman, Saxtorpsvaegen 233, S-261 94 Landskrona Sweden

clones. One of the major problems is the formation of large amounts of callus at the base of stems which may inhibit the development of roots. Hence, there is an urgent need for research to improve rooting as it is regarded as the rate-limiting step in micropropagation (Németh, 1986), and also because root quality directly affects the quality and size of the plants produced (Adendorff and Schön, 1991). Once rooting protocols are well established, it is expected that micropropagation will improve forestry yield by producing many plantlets per parent genotype in a shorter period, and also reduce the cost of micropropagules as direct planting stock (Haines, 1994).

Towards the development of more efficient *in vitro* rooting methods for micropropagated shoots of woody trees that do not root readily, research institutes are moving towards using wild-type strains of *Agrobacterium rhizogenes*. Most dicotyledonous plants have shown susceptibility to this gram negative soil pathogen, and studies have shown that at the site where the bacterium infects the plants, tissues are typically induced to produce a profusion of roots covered with root hairs ('hairy roots') (Riker *et al.*, 1930; Hildebrand *et al.*, 1934; van Wordragen *et al.*, 1991). The effect is caused by the transfer of a root-inducing plasmid (pRi) from the bacterium to the host (McAfee *et al.*, 1993). The bacterium inserts a part of its DNA (T-DNA) into the plant genome where the T-DNA becomes stabilized and expressed. Root promotion is due to just a few T-DNA loci (*rol* A, B and C, particularly *rol* B) (Rugini and Mariotti, 1991). The symptoms observed with *A. rhizogenes* are suggestive of auxin effects resulting from an increase in cellular auxin sensitivity rather than auxin production (McAffee *et al.*, 1993). However, not all dicotyledonous plants are susceptible to *A. rhizogenes* and recalcitrant species might be influenced by both the *Agrobacterium* strain and the cultivar used (Puonti-Kaerlas *et al.*, 1989). George (1996) concluded that root induction, which depends on the transformation by the *A. rhizogenes* plasmid or its genes, would be useful only if it were able to improve rooting and survival of difficult subjects.

Plantlet production via *in vitro* multiplication is a laborious and relatively expensive process compared to the production of rooted cuttings (Watt *et al.*, 1995). It has been estimated at Mondi Forests that under the present production regimes, the production cost for a plantlet produced via tissue culture is approximately three times that of a rooted cutting (Watt *et al.*, 1995). According to Kozai (1991), the high production costs in conventional micropropagation are mainly due to high labour costs, limited rates of plant growth during multiplication, poor rooting and low survival rates of the plantlets during acclimatization. Some authors (McComb and Bennett, 1986; Constantine, 1986) have suggested that the *in vitro* rooting stage should be omitted and shoots be treated as tender cuttings and rooted in the greenhouse as it has been done for some fruit trees and other woody plant cultures (Hutchinson, 1982; Watt *et al.*, 1998). According to Thorpe *et al.* (1991), the main advantages of using *ex vitro* conditions is that rooting and acclimatization can be carried out simultaneously, and callus rarely forms at the base of shoots thereby ensuring a continuous vascular connection between shoot and roots. This technique of rooting micropropagated shoots *ex vitro* is also used routinely at Mountain Home Laboratory (Mondi Forests, Hilton) for clones that root readily (i.e. 'good rooters') such as *E. grandis* but it is not feasible with many species and hybrids. Constantine (1986) suggested that another method of tackling the high cost of micropropagation is to replace labour with machines (automation).

Apart from the large investments required for the development and appropriate testing of most biotechnologies, large investments in many tree breeding programmes are required to reach appropriate levels of advancement, frequently with added levels of structural sophistication (Haines, 1994). Therefore, the high costs of the required research programmes argue strongly for collaboration, rather than competitive proprietary biotechnology.

1.4 Some ecological aspects of *Eucalyptus* plantations

The antagonistic approach towards eucalypts has taken a number of forms, and ranges from uninformed to serious scientific uncertainty about research results and their implications for land use options and policies (Zobel, 1993). The objection to *Eucalyptus* may have nothing to do with the properties of the tree and the way it is managed but may have something to do with traditional patterns of land-use and social customs (Adlard, 1987). Problems have arisen when eucalypts have been planted indiscriminately near villages, near stream sources or where the ground water table is near the surface. According to Adlard (1987), the few well-documented cases of the adverse effects of *Eucalyptus* plantations on soils, associated crops and water relations under specific conditions of those studies are often quoted out of context in support of quite independent political and psychological issues. For example, Armstrong *et al.* (1998) claimed that timber farming in South Africa affected all three components of biodiversity (composition, structure and function) by eliminating genes, populations and species, and changing communities (e.g. open grassy plains and hillsides to woodland).

Eucalypts, being gross users of ground water, have been pinpointed by conservationists as a primary cause of drought in many parts of South Africa (Smith, 1996). Once the roots reach the water table lack of stomatal control of transpiration in some species (e.g. *E. grandis*), even at high temperatures, can lead to excessive water use (Adlard, 1987). However, studies from Australia have shown that eucalypts use water conservatively (as opposed to coniferous forests) and that water use is generally consistent with stands of other tree species (Florence, 1983; Adlard, 1987). Therefore, it is the management's responsibility to examine the annual water use patterns of a species before it is selected for planting and make careful site selection and matching with original climate and soil type (Adlard, 1987).

Eucalyptus woodlot farming has been criticized as diverting potentially good agricultural land to tree crops resulting in less food production and requirements for labour, and to being allelopathic on some agricultural crops (Turnbull, 1991). Hence, the South African government has effectively banned new plantation establishment by making it more difficult for companies to obtain new permits to plant (Turnbull, 1991; Smith, 1996; Watt *et al.* 1997; Meadows, 1999). Other hardwoods such as *Gmelina*, *Acacia*, and *Populus* are preferred by conservationists (Zobel, 1988) because they are considered to be 'environmentally friendly'. Although these species are also important, they are not as economically viable as eucalypts to meet the increasing global wood demands because their growth properties are not as good as those of eucalypts.

1.5 Aims of this study

Biotechnological techniques such as micropropagation have a very significant role in the improvement of tree breeding in forestry. The demand by the forestry industry for improved productivity through these techniques has emphasized the need for a greater understanding of factors regulating *in vitro* plant development, especially the rooting stage which has proven difficult both *in vitro* and *ex vitro*. Further, very little is known about physiological properties (e.g. hydraulic conductivity) of roots produced *in vitro* which might be related to the survival, establishment and fast-growth of micropropagated trees.

The aims of this study were, therefore (1) to develop widely applicable *in vitro* rooting protocol(s) for two cold-tolerant clones of *Eucalyptus grandis* x *nitens* (GN), and (2) to assess the anatomy and hydraulic efficiency of roots produced by *in vitro* vs. cutting propagation. The first objective was to establish an efficient and reliable *in vitro* rooting protocol by optimizing medium and culture conditions. With the objective of developing a more widely applicable *in vitro* rooting protocol for GN clones, the use of wild-type strains of *Agrobacterium rhizogenes* was investigated. The second objective

was to assess the quality of roots produced *in vitro* and to compare it with that of roots produced *ex vitro* from cuttings. The strategy employed was to investigate the root anatomy of the two clones before and six months after hardening-off. The anatomy of roots from cuttings was compared with that of roots produced *in vitro* after six months. The physiological functioning of these roots was investigated by determining their hydraulic conductivity as a function of root dry mass and the vulnerability of these roots to cavitation.

CHAPTER 2: DEVELOPMENT OF *in vitro* ROOTING SYSTEMS FOR COLD-TOLERANT CLONES OF *Eucalyptus grandis* x *nitens*

2.1 LITERATURE REVIEW

2.1.1 *Micropropagation via axillary bud proliferation*

Micropropagation is the true-to-type propagation of a selected genotype using *in vitro* culture techniques (Nashar, 1989; Debergh and Read, 1991). Micropropagation involves at least three stages (a) establishment of cultures, (b) regeneration of plants and (c) transfer of plants from the test tube to soil (Bajaj, 1986). One of its main applications is to multiply trees that are old enough to have demonstrated their superior characteristics (Biondi and Thorpe, 1981). For most micropropagation work, the explant of choice is an apical or axillary bud (Biondi and Thorpe, 1981; Nashar, 1989; Watt *et al.*, 1996). The age of the stock plant, the physiological age of the explant and its developmental stage, as well as its size can determine the success of the procedure (George and Sherrington, 1984; Debergh and Read, 1991). In micropropagation of forest tree species, axillary buds are preferred for maintaining the selected genetic qualities, however, the most successful explants for initiation of cultures that root readily have been mature embryos or seedling parts (Biondi and Thorpe, 1981; Le Roux and van Staden, 1991b; Watt *et al.*, 1996). Thorpe *et al.* (1991) suggested that the reason for this choice of explants is that by the time some trees are old enough for evaluation, they are often recalcitrant in culture.

In many institutes practicing micropropagation, regular spray programmes with fungicides and insecticides are instituted to minimize subsequent culture contamination (Cohen, 1986; Le Roux and van Staden, 1991b). Bacterial and fungal infections are often exacerbated when the starting material for culture (i.e. the explant) is taken from

field-grown parent plants which carry endogenous (systemic) contaminants that cannot be eradicated by conventional surface sterilization (Warrag *et al.*, 1990; Watt *et al.*, 1996). Fortunately, *Eucalyptus* has the ability to coppice and sprout, thus producing juvenile material less exposed to micro-organisms (Hartney, 1980). To yield more hygienic explants, stock plants can be grown in the greenhouse (Debergh and Read, 1991). Sterilization of the initial explants is generally obtained by treatment with fungicides, alcohol and calcium or sodium hypochlorite solutions (Nashar, 1989; Warrag *et al.*, 1990) in the presence of Tween-20 to increase surface wetting (Gordon, 1991). Eucalypt tissue is often killed by sterilization solutions (time and exposure are species dependent) and explants tend to produce a brown exudate which prevent growth of axillary shoots that do survive (Cresswell and de Fossard, 1974; Das and Mitra, 1990; Watt *et al.*, 1996; Mokotedi, 1997). Some fungicides such as Benlate (at 0.5 and 1 $g.l^{-1}$) and Bravo (at 0.25 and 0.5 $g.l^{-1}$) have been found to be phytotoxic on *E. grandis* because they inhibited survival, multiplication and growth as well as rooting of seedlings (Watt *et al.*, 1996). Kelly (1993) suggested that Benlate cause various types of injury, ranging from stunted plant growth to leaf drop and small twisted leaves in ornamentals, vegetables and fruit crops. Further, Debergh and Read (1991) found that with *Cordyline* species, the sterilizing agents easily entered the stem through scars (stems defoliated to minimize contamination) on the leaf bases and the surrounding tissues, and in most cases the axillary buds were killed.

Under sterile culture conditions, rapid heterotrophic growth of bacteria and fungi is often observed when there is sugar in the medium (Kozai, 1991). Cytokinins used to induce proliferation of shoot cultures impede the growth of cryptic bacteria, which may only become virulent once plant material is moved to rooting media (George, 1996).

2.1.2 *Factors affecting* in vitro *rooting*

In addition to stable shoot multiplication, micropropagation requires also successful rooting of microcutings (Sanchez *et al.*, 1996). As previously mentioned, micropropagated shoots of some *Eucalyptus* species (e.g. *E. grandis* x *nitens*) do not root readily. In addition, preliminary studies in our laboratory have shown that different clones of the same hybrid required different conditions for rooting (Makwarela, 1996).

In vitro rooting protocols for most eucalypts are usually first established with seedling or juvenile material. However, according to McComb and Bennett (1986), such a strategy of using juvenile explants has not been entirely successful for the development of rooting techniques for shoots from adult material. Those authors explained that the problem could be difficult to alleviate by manipulations of the media as it may be a reflection of the time the material has been in culture, or the individual genotype. The most often manipulated parameters for improving *in vitro* rooting are growth regulators, light and temperature (Debergh and Read, 1991).

a) *Chemical factors*

Plant growth regulators: Root formation in eucalypts is generally inhibited by high concentration of cytokinins used to induce shoot multiplication or shoot formation (de Fossard *et al.*, 1978). Occasionally, sufficient cytokinin may be carried over from elongation stage to inhibit rooting (McComb and Bennett, 1986; Thorpe *et al.*, 1991; Blomstedt *et al.*, 1991; Bennett *et al.*, 1992, 1994; George, 1996). However, Curir *et al.* (1990) carried out a study on the physiological effects of cytokinins on *in vitro* rooting of *Eucalyptus gunnii* microcuttings, and found that kinetin (6-furfuryl aminopurine) and zeatin [6-(4-hydroxy-3-methylbut-2-enylamino) purine] induced a physiological state characterized by high sensitivity of microcuttings to the rooting stimulus exerted by IBA (indole-3-butyric acid), whereas BA (N^6-benzyladenine) did not have the same

effect (all concentrations at 0.5 $mg.l^{-1}$). The rooting response was correlated with the production of particular flavonoids (quercetin glucosides) under the influence of the cytokinin in the shoot multiplication medium. Working with clones of *Eucalyptus globulus, E. occidentalis and E. marginata*, Bennett *et al.* (1992) and Trindade and Pais (1997) (*E. globulus*) found that alternation of the use of BA with kinetin improved rooting up to 75% when kinetin was used in the last multiplication medium. It is possible that BA is more effective than kinetin in switching on endogenous cytokinin production (Vankova *et al.*, 1991) and this in turn may inhibit root initiation (Bollmark *et al.*, 1988).

It is well established that auxins are the main factors involved in the formation of roots (Németh, 1986; George, 1996). Indole-3-butyric acid (IBA) is the most commonly used auxin for rooting woody microcuttings because of its stability (Curir *et al.*, 1990; Riov, 1993) and has been effective with several eucalypt species (Bennett and McComb, 1982; Franclet and Boulay, 1982; McComb and Bennett, 1986; Burger, 1987; Le Roux and van Staden, 1991 a,b). However, excessive concentrations of IBA (depending on genotype) promotes callus formation at basal ends of shoots, and roots produced have an abnormal appearance and their average length, and subsequent shoot growth may be decreased (Warrag *et al.*, 1990; George, 1996). Le Roux (1990) employed 2 $mg.l^{-1}$ IBA to induce rooting in a cold-tolerant *E. grandis* x *macarthurii* clone and reported that plants with roots developed from callus at basal ends of shoots did not survive the hardening-off period. This suggests that vascular connections between the roots and the stem do not form if the proliferation of callus occurs before root initiation. Improvements in the frequency of rooting have also been reported when α-naphthalene acetic acid (NAA) was used instead of IBA (Rasmussen, 1991), but that growth regulator is not commonly used. That author observed that *in vitro* rooting of cold-tolerant clones of *E. nitens* increased as the level of NAA was reduced (from 9 $mg.l^{-1}$ to 4.5 $mg.l^{-1}$) in the ¼ Murashige and Skoog (¼ MS) medium (Murashige and Skoog,

1962). Veierskov and Andersen (1982) suggested that some of the effects of auxins on rooting ability of pea cuttings was caused by a mobilization of carbohydrates.

Although IBA has been identified by GC/MS (Gas Chromatography-Mass Spectrometry) analysis as an endogenous constituent of various plants (e.g. carrot, Epstein *et al.*, 1991; Epstein and Ludwig-Muller, 1993), it is still regarded as a synthetic auxin. Several studies have shown that synthetic IBA is rapidly metabolized into auxin conjugates (with aspartic acid to form indole-3-butyrylaspartic acid [IBAsp] or with glucose [GE] to form IBA-GE) which then act as rooting promoters (for example, in *Vigna radiata*, Riov, 1993; *Pyrus communis*, Baraldi *et al.*, 1993 and *Sequoia sempervirens*, Blaźkova *et al.*, 1997). Conjugates of IBA seem to be a better source of free auxin compared with IAA conjugates because they are more stable to metabolic degradation within the plant tissues (Riov, 1993). There is increasing evidence that indole-3-acetic acid aspartate (IAAsp), the major IAA conjugate (with aspartic acid) is subjected to oxidation to biologically inactive products such as oxoindole-3-acetylaspartic acid (Riov, 1993). The rooting activity of applied auxins depends on the release of free auxins from the endogenously formed conjugates following applied auxin application. Epstein *et al.* (1993) postulated that an easy-to-root cultivar of sweet cherry (*Prunus avium*), as opposed to the difficul-to-root cultivar, had the ability to hydrolyze the ester conjugate at appropriate time to release free IBA which promoted root initiation. Moreover, Baraldi *et al.* (1993) showed that only the easy-to-root cultivar of pear showed the ability to convert IBA into free IAA during the root induction period.

Gaseous environment: The available oxygen within cultured plant tissues is influenced by (1) the concentration of the gas in the ambient atmosphere; (2) its rate of diffusion into the culture vessel (influenced by the vessel shape and type of enclosure); and (3) its rate of diffusion into the cultured cells or tissues (George, 1993). Submerged tissues or organs such as the basal part of the stems in a static medium are poorly aerated. In

vessels sealed with Parafilm, the use of oxygen by the culture creates a local deficit which may not be immediately compensated because of the impedance to diffusion (George, 1993). Increasing incubation temperature of a culture decreases the amount of oxygen which can be dissolved in the medium (e.g. from 21 to 25°C, amount of oxygen is decreased by approximately 9% - George, 1993). Further, dissolved salts and non-electrolytes such as sucrose, diminish the solubility of gases. The amount of oxygen in the culture media will depend on the surface to volume ratio of the medium in the vessel and the concentration (partial pressure) in the immediate gas phase (George, 1993).

In vitro rooting of eucalypts and other plant species has generally been improved when large vessels (as opposed to tubes) were used in conventional and photoautotrophic micropropagtion (see Section 2.1.4 later) (Kozai, *et al.*, 1988, Kozai, 1991; Kirdmanee, 1999; Kirdmanee *et al.*, 1995; McClelland and Smith, 1990). One of the reasons given for that observation was that gas exchange (carbon dioxide and oxygen) was more efficient because of greater quantity of air and less ethylene was accumulated within culture vessels. Rooting in large vessels probably resembles *ex vitro* rooting because of better aeration of the medium.

b) *Biological factors*

Positional effect: Ammirato (1986) and Cohen (1986) pointed out that although cells within an organism may be considered to be of the same genotype, there are striking differences from cell to cell and from organ to organ within a plant in the ability to undergo morphogenesis. Further, the explant may retain a memory of its position on the parent plant that affects its growth. This memory may be stable over many subcultures and is one example of epigenetic change, i.e. a change in gene expression that can persist in vegetatively propagated progeny but is not transmitted through sexual reproduction (Cohen, 1986). The implication of the above statement is that the differences usually observed in the rooting ability of shoots regenerated from different

axillary buds of the same parent plant may be attributed to differences in physiological age and position of the axillary buds. Therefore, investigations may be required to establish best rooting 'lines' and these should be maintained (possibly) indefinitely on multiplication medium for subsequent rooting.

Browning: The cut surface of many explants start to discolour soon after excision and explants frequently continue to darken when they are introduced into the culture vessel, whereby they may also exude dark coloured substances (phenolics) into the medium. This type of 'blackening' or 'browning' is associated with wounding (Debergh and Read, 1991; George, 1993). Browning is often particularly apparent on a solid medium where exudates are trapped by the agar/Gelrite and become concentrated in the vicinity of the explant. Toxicity is thought to occur by phenols becoming reversibly attached to proteins by hydrogen bonding, and by their oxidation to highly active quinones which then become cyclic or polymerized, and/or oxidize proteins to form increasingly melanic compounds (Harms *et al.*, 1983), which are sometimes termed 'polyphenols' in the literature (George, 1993).

A special group of phenolics are auxin synergists and protectors (antioxidants inhibiting the oxidation of IAA catalyzed by peroxidants) (Debergh and Read, 1991; George, 1993). Browning which results from the oxidation of phenols (especially in light – Creasy, 1968) is therefore not necessarily detrimental to morphogenesis as substances formed by the wound reaction can promote rooting (George, 1993). Further, it has been suggested that rhizogenesis capacities induced by auxin (IBA) are modulated when phenolic compounds (e.g. phloroglucinol, myricetin, quercetin glucosides) are added in the medium (Zimmerman, 1984; Curir *et al.*, 1990; Jay-Allemand *et al.*, 1993).

Activated charcoal and polyvinylpyrrolidone (PVP) have been used in some studies to adsorb and/or inhibit the oxidation and polymerization of phenolics (Le Roux and van Staden, 1991 a,b; Jones and van Staden, 1994). However, George (1993) cautioned that

activated charcoal must be used with caution because it can also adsorb growth regulators and other essential components of the medium. Further, not all antioxidants are effective, some like ascorbic acid, are only suited for short period interventions and quickly become strong oxidants themselves (Debergh and Read, 1991). There are many reports of successful treatments with PVP, but it does not always stop blackening of tissue cultured plants (George, 1993). George and Sherrington (1984) suggested that cultures should be kept in the dark to prevent or reduce the activity of enzymes concerned with both the biosynthesis and oxidation of phenolics. However, their activity can also be reduced by lowering temperature (Cresswell and Nitsch, 1975).

Callus formation: Callus is usually formed at basal ends of shoots most notably when concentration of auxins (e.g. IBA, NAA) and nutrients are high in the medium (Mohammed and Vivader, 1988; Le Roux, 1990; Le Roux and van Staden, 1991 a,b; Mokotedi, 1997) or when cultures are left too long in the rooting medium (Thorpe *et al.*, 1991). If roots originate from these cells, the vascular connections between the root and shoot may be interrupted (Thorpe *et al.*, 1991; Le Roux and van Staden, 1991 a,b). Callus formation at basal ends of shoots prevents rooting in most cold-tolerant clones of *E. grandis* (Le Roux and van Staden, 1991), probably by limiting efficient absorption and movement of water and nutrients (Martin, 1985).

c) *Mineral composition of culture medium*

The medium of Murashige and Skoog (1962) (MS) is the most popular nutrient formulation in micropropagation because most cultures react to it favourably (George, 1996). However, Eliasson (1978) reported that applied nutrients had no or slight influence on the number of roots formed on cuttings of *Pisum sativum*. Furthermore, that author suggested that applied nutrients may not be needed during rooting because endogenous nutrients (products of photosynthesis) are basipetally transported from the shoot to the rooting zone. Further, Warrag *et al.* (1990) observed that the level of basal

minerals (¼, ½ and full strength MS nutrients) had little effect on the percentage of rooting of *Eucalyptus grandis* x *camaldulensis* and *E. grandis* x *robusta*, and suggested that the use of ¼ MS may be more economical. However, other studies have shown that a medium with a lower salt concentration than that used for axillary shoot induction is necessary for root initiation (Gasper and Coumans, 1987; Norton and Norton, 1989; Blomstedt *et al.,* 1991; Cheng *et al.*, 1992; George, 1996; Mokotedi, 1997). Similarly, Rasmussen (1991) reported that *E. nitens* and some of its tested hybrids rooted more efficiently (above 80%) when the concentration of MS nutrients was reduced to ¼ MS. Working with a rose cultivar, Rahman *et al.* (1992) suggested that the effect of the strength of basal nutrients on rooting depends on the type and concentration of auxins used, but such an effect has not been reported for *Eucalyptus*.

Nitrogen: One of the most important constituents of the medium in effecting morphogenesis is the source and concentration of nitrogen (Ammirato, 1986). For many species, the optimum nitrogen concentration for rooting has been found to be much lower than for adventitious shoot formation and growth (George, 1996). Hyndman *et al.* (1982) showed that the improved root initiation on rose shoots was particularly due to the provision of a total nitrogen concentration closer to the optimum of 7.5 mM (instead of 60 mM present in full MS). In the MS formulation, nitrogen is provided as ammonium and nitrate ions. Although the effect of the ionic form and concentration of nitrogen required for root development has been reported for several pine species (Kirby *et al.*, 1987), this information is not available for eucalypts.

Carbon source: Explants and plantlets *in vitro* have been considered to have little photosynthetic ability and require sugar (usually sucrose) as a carbon and energy source for their heterotrophic or mixotrophic growth (Kozai, 1991). Root formation is an energy-demanding process and carbohydrates must be provided through photosynthesis or form exogenously supplied sugars (Rahman *et al.*, 1992; George, 1996). If the combined source is too great, rhizogenesis may be inhibited (George, 1996). According

to Kozai (1991), growth and development of explants *in vitro* is probably improved with sugar supply at the very early stages of multiplication, even if the *in vitro* environment is properly controlled for promoting photosynthesis. Mohammed and Vivader, 1988) compiled studies which showed that decreased sucrose levels in the rooting medium helps conifer plantlets later during the transition from heterotrophism to autotrophism.

Calcium and boron: Many authors have stressed the importance of calcium ions (Ca^{2+}) for rooting of eucalypts. De Fossard *et al.* (1978) found high Ca^{2+} desirable for rooting *Eucalyptus* cuttings. McComb and Bennett (1982) also obtained better rooting of plants in this genus in ¼ MS macronutrients than in full-strength solution, provided that $CaCl_2$ was kept at ½MS level. Similar results were reported for *Pisum sativum* (Eliasson, 1978). As a cation, calcium helps balance anions within the plant, but it is not readily mobile. Many plant enzymes are also calcium-dependent and calcium is a cofactor in the enzymes responsible for the hydrolysis of ATP (adenosine triphosphate) (George, 1993). Calcium ions are actively removed from the protoplasm to prevent the precipitation of phosphates (and the disruption of phosphate-dependent metabolism) and interference with the function of Mg^{2+} (George, 1993).

Calcium deficiency in plants results in poor root growth and in the blackening and curling of the margins of apical leaves, often followed by a cessation of growth and death of the shoot tip (George, 1993). After death of the tip, shoots often produce lateral branches, and in extreme cases the tips of these will also die and branch again. As calcium is not remobilized within plant tissues, actively growing shoots need a constant supply of ions in the transpiration stream. An inadequate supply of calcium can result from limited uptake of the ion, and inadequate transport, the latter being caused by the absence of transpiration due to the high humidity in the culture vessel (McCown and Sellmer, 1987; George, 1993). A remedy can sometimes be obtained by reducing the culture temperature so that the rate of shoot growth matches calcium supply, using

culture vessels which promote better gas exchange (thereby increasing the transpiration and xylem transport), or by increasing the concentration of calcium in the medium (McCown and Sellmer, 1987). However, adding extra calcium ions to the medium is not always effective and can introduce undesirable anions. Chloride toxicity can result if too much calcium chloride is added to the medium (George, 1993). To solve this difficulty, McCown *et al.* (Zeldin and McCown, 1986; Russell and McCown, 1988) added 6 mM calcium gluconate to woody plant medium (WPM) to correct calcium ion deficiency, without altering the concentrations of the customary anions.

Myo-inositol: Vitamins such as myo-inositol are generally thought to be inessential additives to media in which shoots are to be rooted (George, 1996), but are sometimes added to rooting media of eucalypts (Le Roux and van Staden, 1991 a,b; Jones and van Staden, 1994). The myo-inositol molecule has six hydroxyl units, therefore it can react with up to six molecules forming various esters (George, 1993). It appears that inositol phosphates act as secondary messengers to the primary action of auxin in plants: phytic acid (inositol hexa-phosphate) is one of these (George, 1993). The addition of small amounts of myo-inositol is frequently found to stimulate cell division (Murashige, 1974). However, Blomstedt *et al.* (1991) reported that the presence of myo-inositol in the rooting medium lowered the rooting frequency of *Eucalyptus regnans* explants.

d) *Environmental factors*

In micropropagation systems, environmental factors such as light intensity and temperature are easy to control and standardize. These factors ultimately influence the physiological state of the explant and also responses in culture (Biondi and Thorpe, 1981). According to Mohammed and Vivader (1988), light intensity and temperature affect rooting possibly by controlling auxin activity.

Temperature: In their natural environment, plants usually experience temperatures that fluctuate widely, especially between day and night (George, 1993). In many experiments, this change in temperature is simulated by increasing temperature during light hours and decreasing it during the dark hours of the photoperiod. One of the advantages of alternating temperatures is that they assist the exchange of gases in culture vessels (Chalupa, 1987; George, 1993). Studies have shown that the optimum range for rooting most forest tree species is between 26 and 28°C (e.g. Mehra-Palta, 1982; Warrag *et al.*, 1990; George, 1996). According to studies compiled by George (1996), root induction *in vitro* is generally promoted by culturing shoots in relatively high temperatures and even shoots of species adapted to cool climates are induced to form roots most rapidly at temperatures several degrees above normal soil temperatures. Zimmerman (1984) reported that dark incubation of shoots of a difficult-to-root apple cultivar at 30°C for the first week improved rooting significantly. Therefore, the beneficial influence of higher temperatures on root initiation may be due to its effect on translocation of supportive factors (carbohydrates) and on the related increase in respiration (Ooishi *et al.*, 1978) and in catabolism of simple sugars stored in starch at lower temperatures (Veierskov and Andersen, 1982).

Light intensity and photoperiod: Although photosynthesis provides carbohydrates needed for root initiation and root growth, keeping micropropagated shoots (or just the bases of the shoots) in the darkness during the inductive phase, is generally favourable for rooting (George, 1996; Sanchez *et al.*, 1996). Photoperiod, light intensity and wavelength affect shoot growth and morphogenesis as well as rooting, in addition to having a role in photosynthesis (Cohen, 1986; Hughes, 1981; Thorpe *et al.*, 1991). A period of dark incubation is often required for rooting (Wang, 1992; George, 1996). Druart *et al.* (1982) suggested that the beneficial effect of darkness on rooting could be due to the dark induced decrease of peroxidase activity and an increase of endogenous phenols in shoots during root initiation. For *in vitro* rooting of eucalypts and other woody forest trees, a 16-hours light/8-hours dark photoperiod has been found to be

optimum (Franclet and Boulay, 1982; Zimmerman, 1984; Trindade and Pais, 1997; Le Roux and van Staden, 1991a,b; Wang, 1992).

Light, acting through the ubiquitous photomorphogenetic sensor pigment phytochrome, and hormones, are decisive development effectors directing growth and morphogenesis of the multicellular plant (Pfaff and Schopfer, 1980; Hughes, 1981). However, with *Sinapsis alba*, Pfaff and Schopfer (1980) showed that phytochrome does not operate through changes of the growth regulator (IAA, gibberellic acid, kinetin, abscicic acid and ethylene) levels. Depending on concentration, the application of those growth regulators was either ineffective or inhibitory in the rooting response when hypocotyl explants were incubated in the dark or far red light. Phytochrome (P) has been found to be most effectively mediated by red (P_r, 660 nm), far-red (P_{fr}, 730 nm) and blue (350 nm) lights as well as darkness, and may control developmental response by differential gene activation, differential gene repression or both (Hughes, 1981; Walker *et al.*, 1987). The major photosynthetic pigments, chlorophyll a (absorption peaks at 440 and 680 nm) and chlorophyll b (470 and 650 nm), absorb in both the red and blue portions of the spectrum, hence red and blue lights are necessary for photosynthesis (Hughes, 1981). Illumination of the cultures with red light at 36 $\mu mol.m^{-2}.s^{-1}$ was found to be as effective on promoting rooting as treatment with 1.0 $mg.l^{-1}$ NAA (pear cultivars - Bertazza *et al.*, 1995) or 0.3 $mg.l^{-1}$ IBA (wild cherry – Rossi *et al.*, 1993). White light (a mixture of wavelengths) has been found to be secondary to red and far red lights in promoting rooting (Rossi *et al.*, 1993; Bertazza *et al.*, 1995).

Short days and low irradiance generally promote root induction in a similar fashion to darkness. For instance, de Fossard *et al.* (1978) noted that a low light level (10 $\mu mol.m^{-2}.s^{-1}$) was conducive to rooting *Eucalyptus* in a similar way to darkness, whereas 300 $\mu mol.m^{-2}.s^{-1}$ was clearly inhibitory. In contrast, Jones and van Staden (1994) reported that shoots of clones of *E. grandis* x *urophylla* often became etiolated as a result of low light intensity (14 $\mu mol.m^{-2}.s^{-1}$). The effect of light intensity on *in*

vitro rooting of *E. grandis* hybrids therefore appears to be genotype specific. Light given to basal part of *Pisum sativum* (Eliasson, 1978) and *Quercus rubur* and *Q. rubra* (Sanchez *et al.*, 1996) cuttings was reported to have a strong inhibitory effect on rooting and negatively affected the number of roots formed.

2.1.3 Hardening-off of regenerated plants

The *in vitro* grown plants are characterized by an absence of epicuticular wax and a modified epidermis due to the water saturated atmosphere in the flask (Nashar, 1989). The plantlets must undergo a gradual transition (over 2 – 3 weeks) from constantly high humidity regime to one of varying and low humidity in the greenhouse, inside a 'close case' environment provided by polyethene tents or fogging (Biondi and Thorpe, 1981; Constantine, 1986; Nashar, 1989). Some shading is necessary to prevent leaf burn during the plantlet's transition from laboratory conditions to full sunlight. Soil should be sterilized but, even so, systemic fungicides may be needed to prevent fungal attack (Le Roux and van Staden, 1991b). The transfer of plantlets to nursery beds or the field is sometimes a problem because the vascular connection between shoot and root is incomplete, as is lack of root hairs in agar-grown roots (Biondi and Thorpe, 1981). Transfer success can be improved by developing rooting media that induce a minimum of callus before roots appear and by transferring from the culture media as soon as the roots emerge rather than wait for long roots to develop (McComb and Bennett, 1986).

2.1.4 Photoautorophic micropropagation

A most recent form of vegetative micopropagation is the so-called photoautotrophic micropropagation. All the carbon is derived from CO_2 (Kozai, 1991; Kozai *et al.*, 1988, 1999; Kirdmanee *et al.*, 1995; Zobayed *et al.*, 1999). Carbon dioxide inside the culture vessel can be increased easily using a gas permeable film as a closure, however, CO_2 enrichment for growth promotion is effective only under high light intensity (Kozai,

1991). Photosynthetic photon flux density (PPFD) above the vessel should be at least 100 $\mu mol.m^{-2}.s^{-1}$ and preferably 150 or more, compared with a PPFD of 30 – 50 $\mu mol.m^{-2}.s^{-1}$ normally applied in conventional micropropagation. Time periods of multiplication, rooting and acclimatization will be shortened with a high PPFD and CO_2 enrichment (Kozai, 1991). Eliasson (1978) suggested that mobilization of nutrients (carbohydrates and nitrogenous compounds from the leaves) within a cutting is enhanced under increased high irradiance, however, the presence of sucrose in the medium under high irradiance (40 $W.m^{-2}$) delayed root emergence and also reduced root length. Kirdmanee *et al.* (1995) obtained high (100%) *in vitro* rooting frequency on *Eucalyptus camuldulensis* shoots under photoautotrophic conditions.

When using a gas permeable film as closure, loss of water from the medium and/or plantlets *in vitro* to the room air and a resultant decrease in relative humidity in the vessel may occur (Kozai, 1991; George, 1993). Because of the air conditioner, the air in the culture room is almost always dehumified during the photoperiod. In circumstances where this water loss is not desirable for any reason, one can use an ultrasonic humidifier, which produces fog, with a humidistat to increase the humidity (Kozai, 1991). It should be noted, however, that desiccation of the head space *in vitro* may result in beneficial effects such as the development of a thicker cuticular layer, more normal stomatal functioning, greater nutrient uptake with greater water uptake, etc. Risks of growth of fungi on the surfaces made of inorganic matter in the culture room can be minimized if there is no condensation on the surfaces and the relative humidity in the room is maintained lower than 80% (Kozai, 1991). The environmental conditions created for the photoautotrophic treatment under forced ventilation also made possible the *in vitro* acclimatization of *Eucalyptus camaldulensis*. Thus, after exposing the plantlets to the *ex vitro* conditions, transpiration and the percent of water loss were reduced in comparison to those of the photomixotrophic plantlets (Zobayed *et al.*, 1999). In photoautotrophic micropropagation, the multiplication, rooting and

acclimatization can be conducted at the same time under aseptic or semi-septic conditions (Kozai, 1991; Kozai *et al.*, 1999).

2.1.5 Root induction with *Agrobacterium rhizogenes*

Many woody plants, economically important for timber and/or fruit production, are often difficult to root both in conventional and *in vitro* propagation. Recently, many attempts to overcome this problem have been carried out on fruit trees and woody species using *Agrobacterium rhizogenes* (Damiano and Monticelli, 1998). The hairy root syndrome, caused by *A. rhizogenes* on dicotyledonous plants, characteristically consists of an abundant proliferation of adventitious roots at the site of bacterial infection (Cardarelli *et al.*, 1987b). However, root formation is sometimes preceded by tumour-like outgrowth, while in some hosts (e.g. *Nicotiana glauca*) only undifferentiated tumours are observed upon inoculation with *A. rhizogenes* (White *et al.*, 1982; Spano *et al.*, 1985). Tepfer (1983) reported that in axenic, continuous culture, roots induced by *A. rhizogenes* differ morphologically and physiologically from normal roots: (1) they grow faster, (2) they are highly branched (reduced apical dominance), and (3) they are plagiotrophic, i.e. they tend to grow horizontally instead of downward. Rapid tip elongation combined with a high frequency of lateral root formation results in an elevated accumulation of root biomass in culture. The root systems of T-DNA containing plants, regenerated from transformed roots, exhibit the *rhizogenes* phenotype (Tepfer, 1983, 1984, 1990). The aerial parts of these plants are also modified: (1) the leaves are wrinkled, (2) apical dominance is reduced, i.e. they are highly branched, and (3) some biannual species (e.g. carrot) become annual (Tepfer, 1983).

The molecular biology of *Agrobacterium*-plant cell interactions was intensively studied throughout the 1980s with *A. tumefaciens*, which has been called a natural genetic engineer of plants (Walden, 1988). It is now well known how *A. tumefaciens* induces plant tumours by transferring a piece of its DNA (tumour-inducing or Ti DNA) into the

plant cell, where it is integrated and expressed in the host genome. However, very little is known about how its close relative, *A. rhizogenes*, induces the formation of hairy roots in dicotyledons (via the root inducing plasmid – pRi). Further, De Cleene and De Ley (1981) reported that under natural conditions, the host range of *A. rhizogenes* is much more limited than that of *A. tumefaciens*. Host range is broadly described in terms of the organisms on which a parasite can live, from which it obtains food (Grimsley, 1990). It has generally been assumed in the literature that the mechanism by which pRi is transferred into plant cells resembles that of the Ti plasmid of *A. tumefaciens* (Tepfer, 1983; Spáno *et al.*, 1985). The essential difference between the Ri and Ti T-DNAs lies in their effects on the plant cell. The genes contained by the transferred DNA (T-DNA) are thought to be controlled by plant regulatory sequences, and they encode proteins involved in the biosynthesis of plant growth factors (the oncogenes) (Tinland, 1996; Nilsson *et al.*, 1997) and bacterial nutrients (the opine synthesis genes) (Tepfer, 1983; Petit and Temper, 1985): the opine concept proposes that opines act as chemical mediators of parasitism (Petit and Tempe, 1985). In addition, wound sites are necessary for infection by *Agrobacteria* (Lippincott and Lippincott, 1969; Hooykaas, 1983; Winans, 1992).

Virulent *A. rhizogenes* contains a large plasmid, which has a virulence (*vir*) region homologous with the *vir* region of the Ti plasmid and also transfers T-DNA to the plant genome (Walden, 1988). The T-DNA does not encode any genes important for the transfer process – the *vir* genes are located elsewhere on the Ri/Ti plasmid (Tinland, 1996). The only *cis*-acting elements required during this step are two (imperfect) 25 base pair (bp) direct repeat sequences, the border sequences, which delimit the region of DNA to be excised from Ri/Ti plasmid (Wang *et al.* 1987; Tinland, 1996). It is possible to insert any DNA sequence to be introduced into plant cells between these two borders, because none of the genes carried by the T-DNA are required by for the transfer step (Tinland, 1996).

Petit *et al.* (1983) surveyed literature on all *A. rhizogenes* strains available and concluded that these belong to two main opine-types, the agropine-type and the mannopine-type, corresponding to two main opines (derivatives of sugars and amino acids) synthesized in hairy roots and catabolized by the inducing bacterium. In a sense, therefore, *Agrobacterium* can be said to use genetic engineering to transform the host plant into a producer of custom-made chemical food (Nilsson and Olsson, 1997). Veluthambi *et al.* (1989) alleged that opines (octopine and nopaline at 1 to 10 mM) added to the induction medium of *A. tumefaciens* (strain A348) enhanced its virulence two to 10-fold. In addition, another important opine function is to act as a signal molecule to stimulate the spread of the molecular disease determinant (the Ti/Ri plasmid) to other *Agrobacterium* cells by conjugation (Veluthambi *et al.*, 1989).

The agropine-type root inducing plasmid (pRi) transfers two separate T-DNA regions, left (T_L) and right (T_R) regions (based on their position on the conventional plasmid map), to the plant genome, suggesting that agropine type Ri plasmids from strains A4 and HR1 can induce root proliferation by two independent transformation mechanisms (Vilaine and Casse-Delbart, 1987). According to Vilaine and Casse-Delbart (1987), the intensity of the plant response to T_L-DNA or T_R-DNA transfer varies according to the plant species. On the other hand, the mannopine (e.g. 8196) and cucumopine (e.g. 2659) Ri plasmids (both lack auxin-synthesis genes) appear to have a single T-DNA region (Walden, 1988). Vilaine and Casse-Delbart (1987) hypothesized that the molecular mechanism of root proliferation induced by the T_L-DNA is probably equivalent to that of mannopine type Ri plasmid T-DNA. The copy number of T_L and T_R-DNA of the agropine type Ri plasmids is not always the same in the transformed tissue and whereas the presence of the T_L-DNA is essential for the hairy root phenotype, T_R is not, and may be absent altogether (Walden, 1988). However, neither of these regions alone provides a response as strong as that of the wild-type strain, suggesting that the two regions co-operate in the wild-type response (Vilaine and Casse-Delbart, 1987).

The T_L-DNA of the agropine type Ri plasmids has been sequenced and found to contain 18 open reading frames (ORFs) (Slightom *et al.*, 1986) of which ORFs 10, 11, 12 and 15 correspond to (root loci - *rol*) *rol* A, B, C and D loci which are important in affecting the virulence on different plants (White *et al.*, 1985). A detailed analysis of the functions of *rol* genes has been reviewed by Nilsson *et al.* (Nilsson and Olsson, 1997; Nilsson *et al.*, 1997). It should be noted, however, that of the four *rol* genes, only *rol*B has been shown to be able to induce hairy roots on its own (White *et al.*, 1985; Spena *et al*, 1987; Capone *et al.*, 1989; Nilsson and Olsson, 1997). However, Di Cola *et al.* (1996) argued that the presence and expression of *rol*B is not sufficient to induce rhizogenesis in transformed cells, and that a high rooting potential should be associated with the presence of pre-committed cells, whose potentiality could be amplified by *rol*B. T_R region contains an agropine synthase gene as well as genes which are homologous to the auxin biosynthetic genes of octopine T_L-DNA of *A. tumefaciens*, but these are not absolutely required for the maintenance of the hairy root phenotype (Walden, 1988). However, it is possible that the gene products of the Ri T_L-DNA make the transformed cell more responsive to auxins either synthesized by the plant itself or, in the case of the agropine type T-DNA, auxins encoded by the T_R-DNA. Hence T_R may be seen as an accessory DNA acting to extend the virulence of the agropine-type strains of *A. rhizogenes* on different host plants (Carderalli *et al.*, 1987 a,b).

2.1.5.1 *The mechanism of DNA transfer from* Agrobacterium *to the plant genome*

Transfer of DNA from the *Agrobacterium* to the plant cell requires the participation of both the plant cell and the bacterium, hence the developmental stage of the plant cell which is to be transformed is critical (Vilaine and Casse-Delbart, 1987; Walden, 1988). The mechanism of T-DNA transfer has been divided into several stages:

a) *Chemotaxis and plant cell conditioning*

Agrobacterium species are peritrichous motile organisms and there are several reports indicating that motility and chemotaxis play a role in the early events of the infection process (Winans, 1992). According to Walden (1988), it is now clear that wounded plant tissue releases several phenolic derivatives and one of them, viz., acetosyringone, acts at low concentrations as a chemical attractant to *Agrobacterium* (Stachel *et al.*, 1985; Spencer *et al.*, 1990). At the same time, nopaline-type *Agrobacterium* synthesize and secrete trans-zeatin which is thought to 'condition' plant cells to transformation, possibly by inducing cell division (Walden, 1988). Several bacterial chromosomal loci are involved in the plant-bacterial interaction and mutations in these genes are pleotrophic and result in the bacteria being unable to attach to the plant cell wall (Lippincott *et al.*, 1977; Walden, 1988).

b) *Induction of* vir *loci*

Expression of the *vir* genes appears to be under the control of at least two regulatory mechanisms: *vir*A and *vir*G are expressed constitutively at significant levels in the bacteria whereas *vir*C and *vir*D are expressed at very low basal levels in un-induced bacteria and appear to be controlled by chromosomal *ros* locus (Walden, 1988). When bacteria are exposed to wounded plant cells the expression of *vir* B, C, D, E and G is induced to high levels. The induction of the *vir* and *pin* (plant inducible) loci is mediated by the phenolic compounds acetosyringone and α-hydroxyacetosyringone which are released by wounded plant cells (Stachel *et al.*, 1985; Stachel *et al.*, 1986a). The *vir*A product is a protein associated with the bacterial membrane which is thought to act as a sensory molecule (hence influence host range specificity), sensing the presence of acetosyringone in the rhizosphere and in turn interacting with the *vir*G product which induces the transcription of the *vir* and *pin* loci (Walden, 1988; Spencer *et al.*, 1990; Tinland, 1996).

c) *Expression of the* vir *loci*

Following its interaction with acetosyringone-activated *vir*A product, the *vir*G protein induces the transcription of not only itself but also *vir* B, C, D, E and G (Stachel and Zambryski, 1986a,b; Tinland, 1996). The *vir* loci encode a large number of polypeptides some of which are absolutely essential for tumour/root formation (*vir* A, B, G and D) whereas others are not (*vir* C, E, F) (Walden, 1988).

d) *Production of T-DNA intermediates and transfer of DNA to the plant cell*

Induction of the *vir* loci results in the appearance of both site- and strand-specific nicks in the bottom strand of the 25 bp border sequences of the T-DNA and the appearance of a single-stranded DNA, the T-strand, that corresponds to the bottom strand of the T-DNA with its 5' and 3' ends mapping to the right and left borders, respectively (Walden, 1988). The nicks appear approximately 12 hours after the bacterial cells have been exposed to acetosyringone and occur between third or fourth base (± 1 or 2 bases) from left hand side of the 25 bp border repeats (Albright *et al.*, 1987; Wang *et al.*, 1987; Stachel *et al.*, 1986 a,b). It is known that *vir*D encodes site specific endonuclease that regulates the production of single-stranded T-strand which is thought to act as the T-DNA transfer intermediate (Walden, 1988). *Vir*D2 and *vir*D1 proteins excise the single-stranded T-DNA from the Ti/Ri plasmid (Tinland, 1996); this molecule is then exported to the plant cell through a channel, probably based on the *vir*B proteins (11 different *vir*B proteins produced by the *vir*B operon). This channel is also used for the export of *vir*E2 proteins (protects T-stranded T-DNA against nucleases in the plant cell) (Tinland, 1996). In plant cells, association of *vir*E2 with the single-stranded T-DNA-*vir*D2 molecule leads to the T-complex structure. The *vir*D2 and *vir*E2 proteins mediate the entry of T-DNA into the nucleus through the nuclear-pore complexes (Tinland, 1996). In the nucleus, the T-DNA can integrate randomly into the plant cell genome (Walden, 1988).

A single bacterium can transfer more than one copy of the T-DNA to the plant cell during one infection event (Walden, 1988). During infection the majority of the singly-transformed plant cells are infected by a single bacterium and many of these plant cells contain more than one T-DNA copy integrated into the nucleus suggesting that replication of the T-DNA takes place either within the bacterium or the plant cell prior to stable integration into the plant genome (Depicker *et al.*, 1985).

e) *Stabilization of the T-DNA within the nucleus*

At present, the only way to investigate the mechanism by which stabilization takes place is by analyzing the T-DNA in the transformed cell and make comparisons with untransformed control cells (Walden, 1988). Comparison of the plant sequence before and after insertion of the T-DNA has shown that complex rearrangements of the plant DNA, presumably during insertion of the T-DNA, can take place, including small deletions (< 100 nucleotides) and rearrangements (Gheysen *et al.*, 1987; Tinland, 1996). Because there appears to be no firm evidence for the involvement of *Agrobacterium* coded polypeptides in this process, it is considered that the plant's normal recombination and repair mechanism are responsible for T-DNA integration (Gheysen *et al.*, 1987; Tinland, 1996).

f) *Expression of the T-DNA and establishment of transformed phenotype*

The enzymes encoded by the Ti/Ri plasmid synthesize auxin and cytokinin which disrupt the hormonal balance of the cell and initiate disorganized growth and synthesis and selection of opines (bacterial source of carbon and nitrogen) (Walden, 1988). The neoplastic growth (tumours and/or hairy roots) of infected plants creates an ecological niche for the *Agrobacterium*. On the other hand, gene products of the T_L-DNA of the Ri plasmid appear to sensitize the transformed cell to the auxin whose synthesis can be directed by the T_R-DNA, if it is present, or those that are being synthesized by the plant

cell (Walden, 1988; Shen *et al.*, 1988). Epstein *et al.* (1991) provided evidence which showed that endogenous levels of IAA and IBA in carrot tissue increased following treatment of discs with wild-type *A. rhizogenes* (strain A4). Those authors concluded that increased level of endogenous auxins is essential for hairy root formation. Plant auxin can also trigger root differentiation of cells transformed by *Agrobacterium rhizogenes* T-DNA devoid of auxin genes (e.g. mannopine type T-DNA) (Cardarelli *et al.*, 1987a).

2.1.5.2 *Methods used in the transformation of woody trees*

For laboratory purposes, a standard method used in many studies to test the virulence (i.e. presence of functional Ri plasmid) is the carrot disc assay (Moore *et al.*, 1979; Pawlicki *et al.*, 1992). Once virulent strains are established, they are then used to transform the selected plant material, and routinely used strategies for delivering the Ri plasmid across the plant cell are summarized in Table 2.1. Following interactions with bacterial cells, inoculated explants are washed in a solution of an antibiotic, usually cefotaxime (250 - 500 $mg.l^{-1}$) to cure bacterial cells. That antibiotic is usually also incorporated into regeneration/co-cultivation media for a number of subcultures until colonies of *A. rhizogenes* are cured. Antibiotics act by binding to proteins in the bacterial periplasm, thereby interrupting the synthesis of peptidoglycan and provoke lysis of the cell wall of the bacteria (Nauerby *et al.*, 1997).

As previously mentioned, acetosyringone induces the virulence of *Agrobacterium* (Fenning *et al.*, 1996). Data of Veluthambi *et al.* (1989) and Godwin *et al.* (1991) suggested that the incubation of *A. tumefaciens* cells overnight or for 24 hours with acetosyringone (10 µM and 200 µM, respectively), and opines such as nopaline (30 mM) results in the increased transformation frequency of plant cells (e.g. *Nicotiana tabacum*, *Glycine max* and *Antirrhinum majus*). However, from the work of Godwin *et al.* (1991),

there was some evidence which suggested that acetosyringone may suppress virulence in some strain/plant species interactions.

Agroinfection, a term coined by Grimsley *et al* (1986, *loc.sit.* Grimsley, 1990) can be broadly defined as the introduction of plant infectious agents (such as viral or viroidal sequence) into plants via *Agrobacterium*. In the present study, however, this term has been used to refer to inoculation or infection of plant tissue with wild-type strains of *A. rhizogenes*.

Table 2.1: Production of chimerical woody trees by transformation of stem bases with wild-type strains of *A. rhizogenes*.

Plant species	Explant	*A. rhizogenes*[*]	Method	Results	Reference
Prunus amygdalus	Bare rootstock	232 (derivative of TR105)	Explants were cut to expose a fresh surface, then co-cultured with bacterial suspensions for 24 hours in darkness at 20 - 23°C. Plants were then covered with sandy loess soil.	Larger root number and mass. Improved shoot morphology.	Strobel and Nachmias, 1985
Olea europea	Bare rootstock	232 (derivative of TR105)	Explants co-cultured with bacterial suspensions for 28 hours at 22°C. Placed in loess soil, then transferred to the greenhouse at 21°C, 12-hours photoperiod, 270 $\mu mol.m^{-2}.s^{-1}$.	Secondary roots initiated by bacterium integrated into existing vascular system of older roots.	Strobel *et al.*, 1988
Eucalyptus grandis, E. dunnii and *E. nitens*	2 week-old seedlings	LBA 9402 (+++), R1601 (+) and TR8.3 (+)	Stem bases inoculated with 48-hours old cultures grown on LB plates, then inverted in tubes for seven days, thereafter normal position; 16-hours photoperiod, 26°C, 36 $\mu mol.m^{-2}.s^{-1}$.	Hairy roots. Plants appeared normal without *rhizogenes* phenotype.	MacRae, 1991
Populus tremula x *alba, Prunus avium* and *Juglans nigra* x *regia*	Microcuttings	A4	Explants infected by wounding with a trident forceps dipped into bacterial suspension before wounding.	Dense roots with hairy root phenotype on poplar. Small tumours on wild-cherry. 90% necrosis on walnut.	Brasileiro *et al.*, 1991

[*]Number of crosses indicates the degree of virulence

Table 2.1: continued...

Plant species	Explant	*A. rhizogenes*[*]	Method	Results	Reference
E. globulus and *E. gunnii*	Seedlings, hypocotyls, cotyledons	8196 (++), 1855 (+), 2659 (+)	Explants co-cultured with 72-hours old bacterial cultures for 12 hours.	Depending on age: callus, bud regeneration or hairy roots.	Chriqui *et al.*, 1991
Pinus monticola P. banksiana, and *Larix laricina*	Shoots from mature embryos and seedlings	A4 (++) , R1000 (+)	Diagonal basal cut on elongated shoots, then inoculated with a loop scrapped across bacterial lawn. De-rooted six-week old seedlings wounded at the base with scalpel, co-cultured with 48- hours isolates, cultured on vermiculite. 16-hours photoperiod, 22°C, 120 µmol. $m^{-2}.s^{-1}$.	Hairy roots. Taller stems and greater root mass.	McAfee *et al.*, 1993
Pelargonium species.	Leaf discs, mirocuttings	A4 (+), HR1 (++), 8196 (+), A4RSI (+)	Each explant was cut and left in the bacterial suspension for 30 minutes. 28°C max/22°C min, 16-hours photoperiod, 40 µmol. $m^{-2}.s^{-1}$.	Hairy roots.	Pellegrineschi and Davolio-Mariani, 1996
Pinus nigra	3-8 week old de-rooted seedlings	8196 (++), 15834 (+), A4 (+)	Explants smeared with a scrap of freshly prepared bacterial culture. Kept on moist sterile filter paper for 12, 24 or 48 hours.	Adventitious root induction without hairy root phenotype. Improved growth.	Milhaljević *et al.*, 1996

[*]Number of crosses indicates the degree of virulence

Table 2.1: continued...

Plant species	Explant	*A. rhizogenes*[*]	Method	Results	Reference
Juglans nigra	Microcuttings	1855	Basal part immersed into bacterial suspension, gently shaken for 24 hours in the dark. 12-day maintenance in the dark, then light. 16-hours photoperiod, 45 $\mu mol.m^{-2}.s^{-1}$, 21± 1°C.	Callus formation, followed by root development.	Caboni *et al.*, 1996
Zizipus jujuba	Cuttings from mature trees	A4 (+), TR105 (++)	Vertical shallow slice across the base, then scrape against the actively growing bacterial colonies. Stuck in vermiculite and kept in the greenhouse under intermittent mist.	Hairy roots.	Hatta *et al.*, 1996
Eucalyptus grandis x *urophylla*	3-cm high de-rooted seedlings	A4 (++), R1601 (+), LBA9402 (+++), 8196 (+), 2659 (+)	Explants wounded at the base with a trident forceps dipped into the bacterial suspension. 23 ± 2°C, 16-hours photoperiod	Hairy roots. However, most plants developed only tumors.	Machado *et al.*, 1997
Prunus amygdalus and other fruit trees	Microcuttings	1855	Basal parts of explants dipped for 24 hours in darkness in 0.5 ml bacterial suspension. 16-hours photoperiod, 21± 2°C, 37 $mmol\ m^{-2}\ s^{-1}$	Both transgenic (6.8%) and non-trangenic (67%) roots. 26.2% showed both types.	Damiano and Monticelli, 1998

[*]Number of crosses indicates the degree of virulence

2.2 MATERIALS AND METHODS

2.2.1 Plant material and maintenance of parent plants

Cutting-derived potted plants of *Eucalyptus grandis* x *nitens* (clones GN121 and GN107) and seeds of *E. grandis* and *E. nitens* were obtained from Mountain Home Laboratory, Mondi Forests, Hilton (KwaZulu-Natal, South Africa). The seeds were wrapped tight in plastic bags and stored at 10°C. The potted plants 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 weekly. The fungicides were mixtures of 0.2% (w/v) mancozeb (Dithane; Efekto, South Africa) and 0.1% (v/v) chlorothalonil (Bravo; Shell, South Africa) (applied as a foliar spray), and 0.1% (w/v) prochloraz manganese chloride (Sporgon; Hoechst Schering AgrErvo, South Africa) and 0.125% (w/v) tebuconazole (Folicur; Bayer, South Africa) (applied as soil spray). The fertilizer were 0.25% (v/v) trace element solution (per liter: 18g Fe, 4g Cu, 2g Zn, 1g B and 0.4g Mo) (Trelmix; Hubers, South Africa) (applied as foliar spray) and 0.1% (w/v) Mondi orange 1N-2P-1K (Harvest Chemicals, South Africa) (applied as soil spray). These parent plants were cutback every three to four weeks to stimulate coppice growth.

2.2.2 Establishment of *in vitro* shoot cultures

a) *From nodal material*

Fresh sprouted lateral branches (10 - 20 cm in length) with preformed apical and axillary buds were harvested and surface sterilized in 0.2 $g.l^{-1}$ mercuric chloride plus one drop of Tween-20 for 10 minutes and rinsed several times in sterile distilled water. The material was then soaked for 10 minutes in 10 $g.l^{-1}$ calcium hypochlorite and thereafter rinsed several times in sterile distilled water. The sterile branches were trimmed into nodal sections (3 – 3.5 cm in length) each containing two leaves cut to a

third of their original length. Shoot proliferation was induced in multiplication medium comprised of Murashige and Skoog (MS) nutrients (Murashige and Skoog, 1962; Highveld Biological, South Africa), 0.01 $mg.l^{-1}$ α-naphthaleneacetic acid (NAA), 0.2 $mg.l^{-1}$ 6- benzylaminopurine (BAP), 0.1 $mg.l^{-1}$ biotin, 0.1 $mg.l^{-1}$ calcium pantothenate, 30 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite (Polychem, South Africa). As soon as the axillary buds opened (approximately five to ten days after culture initiation), they were excised from the nodal explants and transferred to fresh multiplication medium. After four weeks, they were subcultured for another month. Several batches were maintained in multiplication stage to bulk up material for subsequent rooting experiments. Shoots were elongated in MS nutrients, 0.01 $mg.l^{-1}$ NAA, 0.01 $mg.l^{-1}$ indole-3-butyric acid (IBA), 0.2 $mg.l^{-1}$ 6-furfurylaminopurine (FAP), 0.1 $mg.l^{-1}$ biotin, 0.1 $mg.l^{-1}$ calcium pantothenate, 25 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. All cultures were grown under a 16-hours photoperiod at a photosynthetic poton flux density (PPFD) of 66 $\mu mol.m^{-2}.s^{-1}$ provided by Biolux tubes (Osram L58W) (sideways and overhead lighting) and 27°C day/21°C night. For both shoot multiplication and elongation, four shoots or three small clumps of shoots were cultured in bottles (5 x 7.5 cm) containing 20 ml medium. After three to four weeks, 1.5 to 2 cm-long shoots were used for the rooting experiments and a single shoot was cultured per culture tube (2.5 x 10 cm) containing 10 ml of rooting medium. All media were adjusted to pH 5.8 with 1M NaOH and autoclaved for 20 minutes at 120°C and 121 kPa.

b) *From seed material*

Seeds were sterilized and germinated according to Thokoane (1998). They were surface sterilized for 15 minutes in 3.5% (v/v) sodium hypochlorite plus one drop of Tween-20. The husks were released when the beaker containing seeds was gently swirled and these husks were aspirated with a Pasteur pipette. After several rinses in sterile distilled water seeds were soaked in 4% (v/v) hydrogen peroxide for 10 minutes, rinsed several times in water and then immersed in 50 $mg.l^{-1}$ Gentamycin sulphate (Sigma Chemical

Company, USA) solution. Seeds were placed on MS nutrients (without hormones) fortified with 1 $g.l^{-1}$ casein hydrolysate, 1 $g.l^{-1}$ benomyl (Benlate; Efekto) 10 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite, incubated in the dark at room temperature for five days, and then transferred to a 16-hours photoperiod at 66 $\mu mol.m^{-2}.s^{-1}$ PPFD and 27°C day/21°C night. After 14 days, the seedlings were subcultured onto the same medium without casein hydrolysate and Benlate. Another week later, elongated seedlings (3 – 4 cm in length) were used to test the efficiency of the established *in vitro* rooting protocol and also the ability of wild-type strains of *A. rhizogenes* to induce transgenic roots.

2.2.3 Establishment of the *in vitro* rooting protocol

Initial studies were undertaken with clone GN121 and the best protocol was then applied to GN107 clone. The basic protocol involved placing shoots in modified ¼ MS nutrients (Ca^{2+} and Mg^{2+} as for ¾ MS), 0.01 $mg.l^{-1}$ IBA, 0.1 $mg.l^{-1}$ biotin, 0.1 $mg.l^{-1}$ calcium pantothenate, 15 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite (Mokotedi, 1997). The shoots were incubated in the dark for 72 hours (~25°C), after which they were transferred to a 16-hours photoperiod at 66 $\mu mol.m^{-2}.s^{-1}$ PPFD and 27°C day/21°C night. The rooting period, including the three days of dark incubation, lasted four weeks.

Tested alterations to this protocol involved exposing shoots to various media compositions and environmental conditions. These included: (1) elimination of the vitamin myo-inositol in the ¼ MS formulation, (2) the replacement of ammonium nitrate in the presence and absence of 1 $g.l^{-1}$ PVP (polyvinylpyrrolidone) in ¼ MS formulation with 0.15 $g.l^{-1}$ L-glutamine, the latter medium was then designated ¼ MSG; For subsequent experiments, the concentration of IBA was increased from 0.01 to 0.1 $mg.l^{-1}$ IBA, then (3) ¼ MS was modified to contain calcium (as $CaCl_2.2H_2O$) and magnesium (as $MgSO_4.7H_2O$) to ½ and ¾-strength of full MS by addition of 0.11 $g.l^{-1}$ and 0.22 $g.l^{-1}$ Ca, and 0.092 $g.l^{-1}$ and 0.18 $g.l^{-1}$ Mg; (4) rooting efficiency in tubes (2. 5 x 10 cm) was compared to rooting in small and larger culture bottles (5 x 7. 5 mm and 6 x

11 cm, respectively); (5) a step-wise increase in light intensity and temperature, following an initial 72-hours dark incubation period at room temperature from 37 $\mu mol.m^{-2}.s^{-1}$ PPFD (sideways lighting only) and 23°C day/21°C night for seven days to 66 $\mu mol.m^{-2}.s^{-1}$ PPFD (sideways and overhead lighting) and 27°C day/21°C night for 18 days.

To test the effect of temperature, shoot explants of GN107 and/or GN121 clones were incubated in the dark at 10°C or 30°C for 72 hours prior to a 16-hours photoperiod under conditions described in (5) above. Temperature was also kept constant at 27°C day/21°C night following a 72-hours dark incubation at 30°C. The effect of light intensity was tested by maintaining temperature constant at 27°C day/21°C night following dark incubation at room temperature (~25 ± 2°C). Light intensity was then increased from 37 to 66 $\mu mol.m^{-2}.s^{-1}$ PPFD; and finally (6) concentration of molybdenum (as $NaMo_4.2H_2O$) was increased to ½ and ¾ levels of full MS in modified ¼ MS by addition of 6.25×10^{-5} $g.l^{-1}$ and 1.25×10^{-4} $g.l^{-1}$ Mo.

2.2.4 Hardening-off of regenerated plantlets

Plantlets were transferred to sterile, moistened potting mixture of 1 river sand : 9 pine bark (v/v, pH 5.6 - 6.5) (Grovida Horticultural Products, South Africa), enveloped in transparent plastic bags and kept in the greenhouse. After one week, the humidity of the microclimate was reduced by punching holes on the bags, which were then removed completely a week later. The hardening-off period to greenhouse conditions lasted four weeks when plant survival was recorded. The plantlets were then maintained under normal greenhouse conditions (Section 2.2.1).

2.2.5 Growth and maintenance of *Agrobacterium rhizogenes*

Twelve wild-type strains of *A. rhizogenes* were tested, viz., LBA9402, A2183, R47, TR7, TR8.3, HR1, R1500, R1600, R1601, A4, TR101 and 8196; the last eleven strains were obtained from Ms. Thiru Naidoo (CSIR, Foodtek, Pretoria). Initially, the bacteria were grown in liquid YM broth containing 10 $g.l^{-1}$ yeast extract, 10 $g.l^{-1}$ mannitol, 0.65 $g.l^{-1}$ $K_2HPO_4.3H_2O$, 0.2 $g.l^{-1}$ $MgSO_4.7H_2O$ and 0.1 $g.l^{-1}$ NaCl. Growth was faster when bacteria were grown in high salt formulations of Luria-Bertani (LB) broth (5 $g.l^{-1}$ yeast extract, 10 $g.l^{-1}$ tryptone and 5 $g.l^{-1}$ NaCl) and Luria broth (L) (Kado and Liu, 1981) (5 $g.l^{-1}$ yeast extract, 10 $g.l^{-1}$ casein hydrolysate and 10 $g.l^{-1}$ NaCl) (Fig. 2.1). All media were supplemented with 50 $\mu g.ml^{-1}$ rifampicin (Sigma Chemical Co., St. Louis, USA) and adjusted to pH 7 with 1M NaOH. Cultures were grown at 28°C in a shaking incubator (G24 Environmental Incubator Shaker, New Brunwick Scientific Co., USA). When solid media were required, 15 $g.l^{-1}$ agar (Biolab Diagnostics, South Africa) was added.

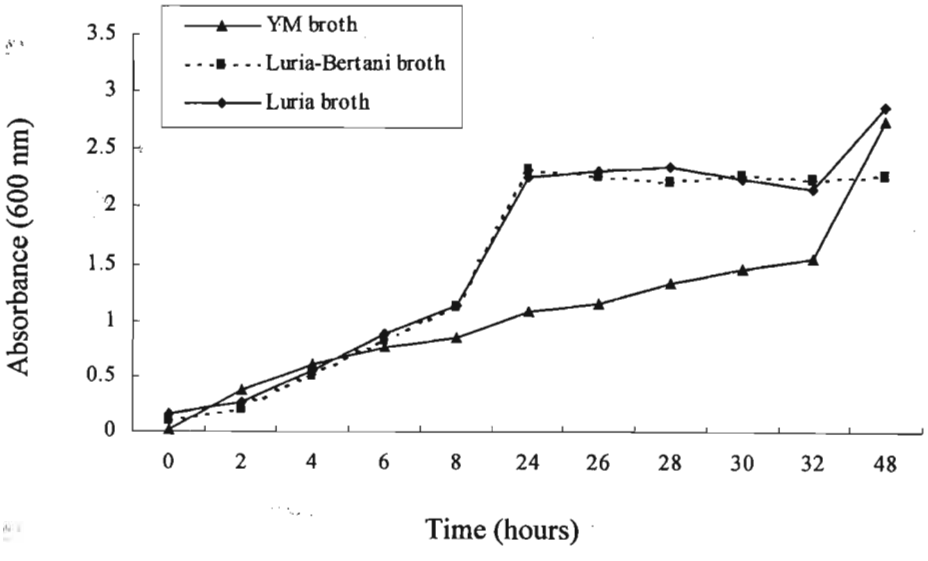

Fig. 2.1: Growth of *Agrobacterium rhizogenes* (strain LBA 9402) in media supplemented with 50 $\mu mol.m^{-2}.s^{-1}$ rifampicin.

Bacterial cultures were stored in three ways following an overnight growth in liquid YM broth plus 50 $\mu g.ml^{-1}$ rifampicin: (1) for short-term storage, cultures were streaked on 25 ml plates of YMA with 50 $\mu g.ml^{-1}$ rifampicin then stored at 8°C for four weeks; (2) for medium-term storage, stab cultures were prepared with 7 ml YMA plus 50 $\mu g.ml^{-1}$ rifampicin and stored at room temperature for a year; and (3) for long-term storage, liquid cultures were mixed with 40% (v/v) glycerol and frozen at –80°C.

Cell number

The bacterial cell numbers were determined according to Hope (1994). Briefly, bacterial cultures were grown overnight in LB broth plus 50 $\mu g.ml^{-1}$ rifampicin. The optical density (OD) was measured at 600 nm and serial dilutions were then performed with fresh LB broth plus 50 $\mu g.ml^{-1}$ rifampicin. Diluted cultures were grown overnight on LB plates at 28°C, resulting colonies were counted and the cell number per milliliter was then calculated (Fig. 2.2). The cell numbers for some strains were calculated as 4.8 x 10^{10} for TR8.3 strain (OD = 1.9), 2.8 x 10^{10} for R1601 strain (OD = 1.2), 2.5 x 10^{10} for HR1 strain (OD = 0.8), 1.8 x 10^{10} for R1600 strain (OD = 0.7) and 1.3 x 10^{10} for R47 strain (OD = 0.6).

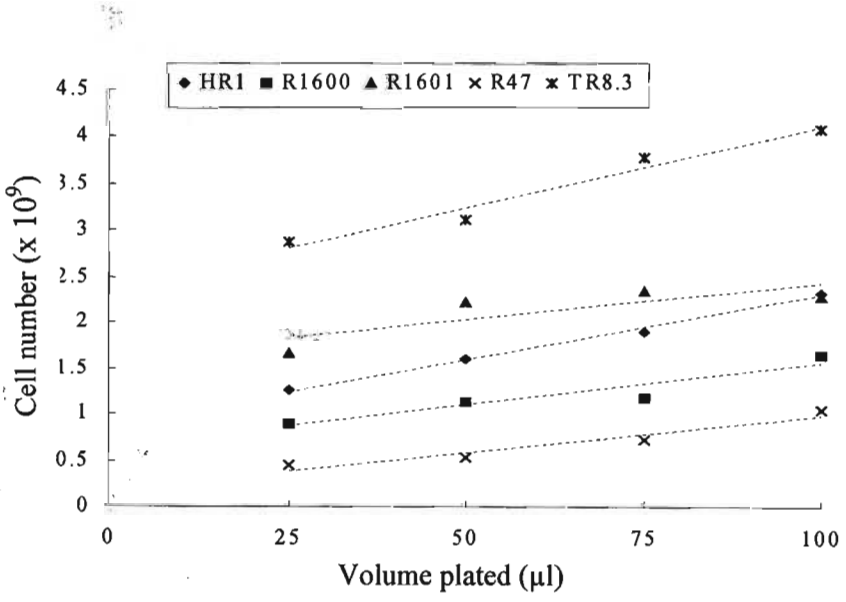

Fig. 2.2: The relationship between volume plated and cell number for *Agrobacterium rhizogenes* strains grown in LB broth.

2.2.6 Carrot disc assay

Fresh carrots were purchased from a local supermarket and the epidermal layer of roots was removed with a scalpel blade. Roots were then soaked for five minutes in 70% (v/v) ethanol, rinsed several times in sterile distilled water, thereafter immersed for 10 minutes in 0.2 $g.l^{-1}$ mercuric chloride plus one drop of Tween-20. After several rinses in sterile distilled water, carrot roots were submerged in 10 $g.l^{-1}$ calcium hypochlorite for 10 minutes then rinsed thoroughly in sterile distilled water. Sterile roots were sectioned into approximately 0.5 cm thick discs and these were used to test the root inducing ability of wild-type *A. rhizogenes* strains. Bacterial strains were grown overnight in liquid LB broth with 50 $\mu g.ml^{-1}$ rifampicin. Each carrot disc was inoculated with a drop of liquid culture (approx. 10^{10} $cells.ml^{-1}$). Eight discs from different roots were used per strain, four were inoculated on the apical side (facing the root tip) and four on the basal side (facing the shoot). The inoculated discs were incubated in the dark at room temperature in culture bottles (6 x 11 cm) containing 8 g vermiculite moistened with 60 ml MS nutrients plus 10 $g.l^{-1}$sucrose. Results were recorded after four weeks and the treatments were repeated three times.

2.2.7 Agroinfection of explants of *E.grandis* x *nitens* clones

2.2.7.1 *Localized inoculation method*

Protocol 1

Nodal cuttings of GN107 and GN121 clones were obtained from sterilized coppice (Section 2.2.2 (a)). Three days after sterilization, a fresh cut was made at the tip of the stem stump and leaves as well as part of the stem below the level of axillary buds were wounded with a razor blade. Five strains of *A. rhizogenes* (R47, HR1, R1600, R1601 and TR8.3) were selected on their ability to incite many transgenic roots on carrots discs. Overnight cultures (approx. 10^{10} $cells.ml^{-1}$) were centrifuged in Eppendorf tubes

at 8^0C for three minutes (6000 rpm, Sigma 201M), then resuspended in 2 ml LB broth without 50 $\mu g.ml^{-1}$ rifampicin. These were allowed to grow for an hour at 28°C. Thereafter, a flamed and cooled bacterial loop was dipped in liquid culture and used to inoculate the wounded areas. The inoculated and control explants (n = 12 per treatment) were cultured on ½ MS medium (without hormones), 15 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite (pH 5.8) in the dark at room temperature (~25 ± 2°C) for six weeks. The bacteria were not cured because there was no contact made with the culture medium.

In another treatment, lower leaves on stems of micropropagated shoots (1.5 to 2 cm in length) of clone GN121 were removed and a fresh cut was made across the bases of stems. A flamed and cooled bacterial loop was dipped in overnight cultures of strains mentioned above and used to inoculate the cut surfaces. Shoots were then placed up side down into tubes containing 10 ml of same medium used for nodal cuttings (MacRae, 1991). Tubes were not sealed with parafilm and were incubated in a ventilated oven at 28°C for 48 hours. Thereafter, agroinfected explants and controls (n = 12 per treatment) were soaked in a solution of 500 $mg.l^{-1}$ cefotaxime (Claforan; Roussel Laboratories, South Africa) for 45 minutes. Explants were blotted dry with sterile filter paper then cultured with their basal ends inserted in the medium containing 500 $mg.l^{-1}$ cefotaxime and transferred to a direct 16-hours photoperiod at 37 $\mu mol.m^{-2}.s^{-1}$ PPFD and 23°C day/21°C night.

Protocol 2

Leaves of two-week old sterilized coppice of clone GN121 were reduced from the apex to about two thirds of their original length. The five selected strains of *A. rhizogenes* (R47, HR1, R1600, R1601 and TR8.3) were grown overnight at 28°C on plates of LB plus 50 $\mu g.ml^{-1}$ rifampicin. Ten leaves were used per treatment and the freshly cut surface was scrapped across the bacterial lawn. Agroinfected leaves were then cultured with their basal ends in tubes containing 10 ml of medium (as for Protocol 1). Control

leaves were not inoculated with bacteria and all explants were incubated in the dark for four weeks.

2.2.7.2 *Leaf disc-liquid purge method*

Protocol 1

Expanded leaves were selected from micropropagated shoots of clone GN121 in elongation medium and sectioned into approximately 0.5 x 0.5 cm sections. Eighteen leaf pieces were prepared for each bacterial strain and six were cultured per Petri dish (9 cm in diameter) containing 25 ml of MS nutrients (with and without 0.1 $mg.l^{-1}$ IBA), 20 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite (pH 5.8), then incubated in the dark at room temperature for 48 hours. The explants were placed with their adaxial surfaces (i.e. upper leaf surfaces) on the medium. The five strains of *A. rhizogenes* were grown in 30 ml liquid LB broth and also in 30 ml liquid MS nutrients both supplemented with 50 $\mu g.ml^{-1}$ rifampicin for 48 hours (late log phase, approx. 10^{10} $cells.ml^{-1}$). Before co-culturing with leaf pieces, bacterial strains were centrifuged for five minutes at 8°C (3400 rpm, GPR Beckman GH 3.7 rotor) and resuspended in the fresh liquid MS or LB broth without 50 $\mu g.ml^{-1}$ rifampicin. Leaf discs were then submerged for two to five minutes in bacterial cultures (R47, HR1, R1600, R1601 and TR8.3) then blotted on sterile filter paper to remove unbound bacteria. They were then returned to the same Petri dishes in which they were pre-cultured, sealed with parafilm and incubated in the dark for 48 hours at 28°C. The leaf discs were subcultured to fresh medium with 500 $mg.l^{-1}$ cefotaxime every 2 – 3 days until the bacteria were cured after two weeks in the case of strains grown on LB broth. By the end of a six-week period, those grown in MS nutrients were still not cured.

In a modification of the above treatment, leaf pieces co-cultured with bacteria grown in MS nutrients were incubated in the dark for two weeks and thereafter transferred to a 16-hours photoperiod at 37 $\mu mol.m^{-2}.s^{-1}$ PPFD and 23°C day/21°C night for another two-week period.

Protocol 2

Leaf pieces or whole leaves (n = 10) from micropropagated shoots of clone GN121 were wounded with a razor blade on either the abaxial or adaxial surface, co-cultured overnight with five selected bacterial strains in LB without 50 $\mu g.ml^{-1}$ rifampicin for two to five minutes, thereafter incubated under conditions similar to those of the carrot disc assay (Section 2.2.6).

Protocol 3

Coppice leaves of GN121 and GN107 clones were sterilized as previously mentioned and sectioned into squares an hour before agroinfection to enable them to produce phenolics. The virulence of HR1, R1600 and R47 strains was induced with filter-sterilized acetosyringone (prepared in 50% (v/v) ethanol) at 0, 0.02 $g.l^{-1}$ (Fenning *et al.*, 1996), 0.2 $g.l^{-1}$ and 0.3 $g.l^{-1}$. The bacterial cultures were grown overnight in 80 ml of LB broth plus 50 $\mu g.ml^{-1}$ rifampicin, centrifuged, then resuspended in 80 ml of MS nutrients (without hormones) only. The suspension was split into four 20 ml aliquots to which acetosyringone was added and these were allowed to grow overnight at 28°C in a shaking incubator. Leaf pieces (n = 20) were co-cultured with bacteria (approx. 10^{10} $cells.ml^{-1}$) for 6, 12 and 24 hours in Petri dishes (9 cm in diameter) in the dark. At the end of each period, the explants were blotted on sterile filter paper, soaked for one hour in a 500 $mg.l^{-1}$ cefotaxime, thereafter split into two groups and cultured on ½ MS nutrients (with and without 0.1 $mg.l^{-1}$ IBA), 15 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Control explants were treated similarly but were not agroinfected.

In another treatment, 0.02 $mg.l^{-1}$ acetosyringone was incorporated into plates of LB broth plus 50 $\mu g.ml^{-1}$ rifampicin. Leaf pieces from coppice material of clone GN107 were scrapped across the overnight bacterial lawn of the three strains mentioned above and co-cultured for 6, 12 and 24 hours with bacteria on moist sterile filter paper in Petri dishes (9 cm in diameter). Thereafter, the explants were washed for an hour in a solution of 500 $mg.l^{-1}$ cefotaxime, blotted on sterile filter paper and cultured on ½ MS nutrients (without IBA), 15 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. They were incubated in the dark for three days then transferred to a 16-hours photoperiod at 37 $\mu mol.m^{-2}.s^{-1}$ PPFD and 23°C day/21°C night for six weeks.

2.2.8 Agroinfection of *E. grandis* and *E. nitens* seedlings

The method used was that of MacRae (1991). Roots of three-week-old seedlings of *E. grandis* and *E. nitens* (Section 2.2.2 (b)) were trimmed-off and basal ends of shoots (n = 20) were scrapped across bacterial lawns of R47, TR8.3, LBA9402, A4, HR1, R1600 and R1601 strains grown on plates of LB broth plus 50 $\mu mol.ml^{-1}$ rifampicin. Agroinfected seedlings were placed up side down into tubes containing ½ MS nutrients (without IBA), 15 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Explants were incubated in the dark at room temperature for 72 hours before a 16-hours photoperiod at 66 $\mu mol.m^{-2}.s^{-1}$ PPFD and 27°C day/21°C night for six weeks. Control shoots were treated similarly but were not agroinfected. Shoots were re-orientated into the medium after three to five days when the initial signs of root formation were observed. Results were recorded after two weeks and roots were excised and cultured in liquid MS nutrients (without hormones) supplemented with 15 $g.l^{-1}$ sucrose.

2.2.9 Data analyses and Photography

In vitro rooting experiments were repeated three times with 20 - 25 samples each. Experiments aimed at screening wild-type strains of *A. rhizogenes* for root inducing

ability where repeated two to three times and the number of replicates has been specified per treatment. Data were analyzed statistically using a one-way analysis of variance (ANOVA) and differences were contrasted using Duncan's multiple range test. Photographs of rooted shoots and transformed carrot discs were recorded with a Nikon FM2 camera fitted with a 60 mm Mikro Nikkor macro lens. Leaf explants used in transformation experiments were screened with a Wild M3 stereomicroscope and recorded using a Wild Photoautomat MPS 55 system.

2.3 RESULTS

2.3.1 Shoot production

Multiple shoots were regenerated *in vitro* from axillary buds. Buds were excised from parent nodal cuttings as soon as they broke (approximately 5 - 10 days after culture initiation) and transferred to fresh multiplication medium (Fig. 2.3). An average of 10 shoots per bud explant was obtained in four weeks and these were subcultured monthly to bulk up material for rooting experiments. Contamination resulted in the loss of less than 10% of nodal explants as well as micropropagated shoots. Shoots were elongated for three to four weeks before rooting trials were initiated. Explants in both multiplication and elongation media produced large amounts of callus at basal ends which was trimmed off at each subculture stage.

At the early stages of this study, shoot explants were lost as a result of browning which occurred most notably when elongated shoots (1.5 – 2 cm in length) were transferred to rooting media (30 –60%). In the multiplication and elongation stages, shoot cultures that produced large amounts of callus at basal ends encountered browning and die-back phenomenon (5 to 10%), which started at the apical bud and proceeded basipetally. Initially, those phenomena in the rooting stage were ascribed to nutrient limitation in the modified rooting media, but they persisted during experiments with various concentrations of macro- and micronutrients. Observations suggested that the method used to transfer those 'fragile' micropropagated shoots possibly played an important role in initiating browning and die-back. Explants died-back from the shoot tip because they were either held with forceps that were still too hot during the transfer process or their stems were held too tight. Some of the explants produced roots and then died-back (Fig. 2.4). Hence, browning accompanied by die-back might have affected the initial percentages of rooting. Those phenomenon were minimized by removing callus at basal

ends of shoots during each subculture stage and also by allowing forceps to cool-off sufficiently before transfer of explants.

Fig. 2.3: Initial stages on the micropropagation of cold-tolerant *Eucalyptus grandis* x *nitens* clones. 1 = nodal explants, 2 = shoot buds after being excised from parent plants, 3 = shoot multiplication, and 4 = shoot elongation. Bar = 1.7 cm.

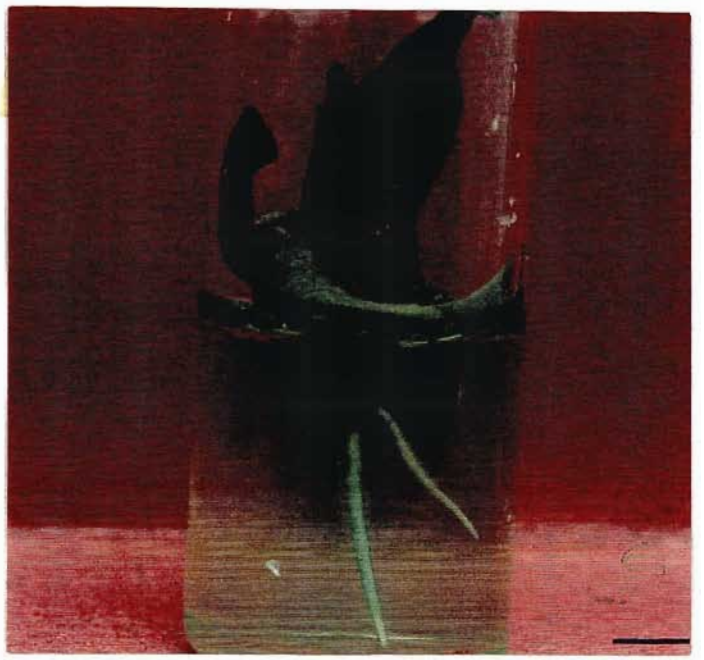

Fig. 2.4: Browning response and die-back of a GN121 shoot explant after two weeks in rooting medium. Bar = 5.8 cm.

2.3.2 Establishment of an *in vitro* rooting protocol

2.3.2.1 *Preliminary investigations with clone GN121*

In our laboratory, selected clones of *Eucalyptus grandis* x *nitens* were regenerated successfully via axillary buds up to the stage of shoot production by another worker. As mentioned previously, those initial studies showed that different clones required different rooting media and culture conditions for root initiation (Makwarela, 1996). In the present study aimed at the establishment of a basic rooting protocol, media were formulated based on information in the literature and on the initial investigations conducted with clone GN121 by Mokotedi (1997). Parameters investigated were concentration and mode of IBA application, concentration and formulation of nutrients, and the type of a supporting agent as well as presence of additives such as activated charcoal. Results of these preliminary investigations are presented here only as a brief summary.

Activated charcoal did not have a significant effect on rooting frequency but shoots appeared healthier and continued to elongate. No rooting was observed when vermiculite was used together with Gelrite for supporting shoots. A chronic application of IBA at 0.01, 0.1, 1, 2 and 20 $mg.l^{-1}$ (24-hours and 72-hours pulses) across different concentrations of MS medium (¼, ⅓, ½ and full MS) produced callus at basal ends of shoots, from which in some cases, roots developed (Fig. 2.5). Increasing the concentrations of calcium and magnesium to ¾-strength of full MS in ¼ MS medium did not improve rooting frequency significantly, but prevented the formation of large amounts of callus at shoot bases.

The observations mentioned above led to the formulation of an initial rooting medium that formed the basis of all subsequent investigations (¼ MS nutrients with additional

0.22 g.l^{-1} $CaCl_2.2H_2O$ and 0.185 g.l^{-1} $MgSO_4.7H_2O$, 0.01 mg.l^{-1} IBA, 0.1 mg.l^{-1} biotin, 0.1 mg.l^{-1} calcium pantothenate, 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite.

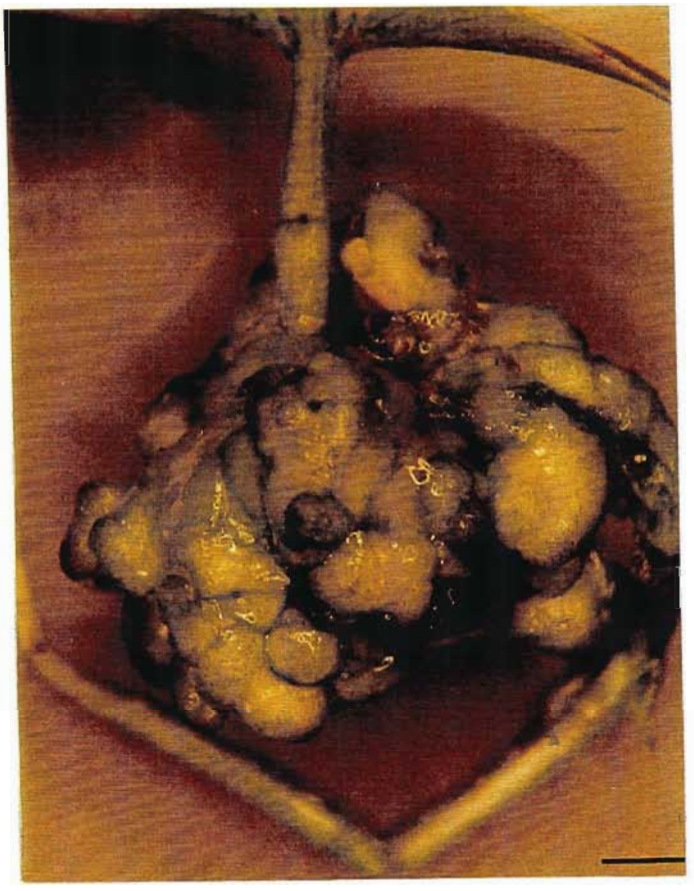

Fig. 2.5: Callus formation and adventitious rooting on micropropagated shoots of clone GN121 cultured on MS nutrients (¼, ⅓, ½ and full MS) supplemented with 0.1 mg.l^{-1} IBA. Bar = 0.1 cm.

2.3.2.2 *Optimization of the rooting protocol*

2.3.2.2.1 *Studies with clone GN121*

a) *Effect of myo-inositol*

The first step towards modification of the basic protocol involved elimination of the vitamin myo-inositol from the ¼ MS formulation. In the literature, low-rooting

percentages of hardwoods has generally been attributed by some authors to presence of vitamins, especially myo-inositol. However, elimination of myo-inositol in the present study lowered the previously observed percentage of rooting from 64% to 30% and shoots produced fewer, shorter roots (Table 2.2). Further, the percentage of browned shoots also increased in the absence of myo-inositol, but phenolics were not exuded into the rooting media.

Table 2.2: Effect of eliminating myo-inositol on *in vitro* rooting of clone GN121. Medium components were modified ¼ MS nutrients (Ca^{+2} and Mg^{2+} as for ¾ MS), 0.01 $mg.l^{-1}$ IBA, 0.1 $mg.l^{-1}$ biotin, 0.1 $mg.l^{-1}$ calcium pantothenate, 1 $g.l^{-1}$ PVP, 15 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Shoots were rooted under 66 $\mu mol.m^{-2}.s^{-1}$ PPFD and 27°C day/21°C night following an initial 72-hours dark incubation period.

Medium modifications	Rooting[z] (%)	Average root number per shoot	Average root length (mm)	Browned non-rooted shoots (%)
None	64.0 ± 0.5 a	2.0 ± 1.2 a	34.0 ± 25.1 a	0.0 ± 0.0 a
No inositol	30.0 ± 0.5 b	1.0 ± 0.9 b	31.0 ± 27.5 a	28.0 ± 0.3 b

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of one (control) and three replication(s). Results were recorded after 28 days. n = 20 -25.

b) *Effect of L-glutamine and the antioxidant PVP*

Ammonium nitrate is one of the important nitrogen sources for explants in the MS medium. In this study, NH_4NO_3 was replaced with 0.15 $g.l^{-1}$ L-glutamine in the modified ¼ MS medium (Ca^{2+} and Mg^{2+} as for ¾ MS) and this medium was designated ¼ MSG. Rooting in ¼ MSG medium occurred after approximately ten days (compared to 2 - 3 weeks in modified ¼ MS) but rooting frequency was still lower compared to the

basic protocol (57% vs. 64%) (Table 2.3), hence this medium was not used anymore. Further, most roots lacked well-developed lateral roots and about 5% of those rooted experienced browning (Fig. 2.4). In an effort to minimize browning, PVP (polyvinylpyrrolidone) was incorporated in the ¼ MSG medium. The presence of PVP lowered percentage of rooting further and resulted in increased browning of shoots in concert with the exudation of phenolics into the rooting medium (Table 2.3) (Fig. 2.6). Large amounts of callus were not produced at basal ends of shoots in all treatments.

Table 2.3: Effect of replacing ammonium nitrate with L-glutamine on *in vitro* rooting of clone GN121 in the presence and absence of 1 g.l^{-1} PVP. Other medium components were modified ¼ MS nutrients (Ca^{+2} and Mg^{2+} as for ¾ MS), 0.01 mg.l^{-1} IBA, 0.1 mg.l^{-1} biotin, 0.1 mg.l^{-1} calcium pantothenate, 1 g.l^{-1} PVP, 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite. Shoots were rooted under 66 µmol.m^{-2}.s^{-1} PPFD and 27°C day/21°C night following an initial 72-hours dark incubation period.

Medium modifications	Rooting[z] (%)	Average root number per shoot	Average root length (mm)	Browned non-rooted shoots (%)	Phenolic exudation (%)
None	64.0 ± 0.5 b	2.0 ± 1.2 a	34.0 ± 25.1 a	0.0 ± 0.0 a	0.0 ± 0.0 a
¼ MSG (no PVP)	57.0 ± 0.5 ab	3.0 ± 1.6 a	75.0 ± 54.1 b	37.0 ± 0.5 b	0.0 ± 0.0 a
¼ MSG (plus PVP)	50.0 ± 0.5 a	3.0 ± 1.5 a	75.0 ± 56.3 b	40.0 ± 0.5 b	45.0 ± 0.5 b

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values present means ± one standard deviation of one (control) and three replication (s).Results were recorded after 28 days. n = 20 -25.

Fig. 2.6: Production of phenolic exudates by rooted shoots on ¼ MSG medium supplemented with 1 g.l^{-1} PVP. Bar = 0.66 cm.

c) *Effect of additional calcium and magnesium*

The effect of increasing the concentrations of calcium (as $CaCl_2.2H_2O$) and magnesium (as $MgSO_4.7H_2O$) in ¼ MS nutrients to ½ and ¾-strength of full MS level was re-investigated (Section 2.3.2.1), but the concentration of IBA was increased from 0.01 mg.l^{-1} to 0.1 mg.l^{-1} IBA in an effort to improve rooting frequency. The presence of both additional calcium and magnesium at ¾-strength of full MS had no significant effect on percentage of rooting for clone GN121 (53% vs. 52% of control) (Table 2.4), but prevented callus formation at the bases of stems. Rooting frequency was lowered when those nutrients were furnished individually at ½ and ¾-strength in the medium. Large amounts of callus (that could inhibit rooting) were only visible at basal ends of control explants.

Table 2.4: Effect of additional calcium and magnesium on *in vitro* rooting of clone GN121 on ¼ MS medium supplemented with 0.1 mg.l^{-1} IBA. Other medium components were 0.1 mg.l^{-1} biotin, 0.1 mg.l^{-1}calcium pantothenate, 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite. Shoots were rooted under 66 µmol.m^{-2}.s^{-1} PPFD and 27°C day/21°C night following an initial 72-hours dark incubation period.

Medium modifications	Rooting[z] (%)	Average no. roots per shoot	Average root length (mm)	Browned non-rooted shoots (%)	Shoots callusing (%)
None	52.0 ± 0.5 b	2.0 ± 1.1 b	34.0 ± 22.4 a	0.0 ± 0.0 a	48.0 ± 0.5 a
¾ Ca and Mg	53.0 ± 0.5 b	1.0 ± 0.8 ab	27.0 ± 19.8 a	40.0 ± 0.5 b	0.0 ± 0.0 b
½ Ca	35.0 ± 0.5 ab	2.0 ± 1.9 ab	41.0 ± 25.0 a	30.0 ± 0.5 ab	0.0 ± 0.0 b
¾ Ca	25.0 ± 0.4 a	3.0 ± 1.2 ab	52.0 ± 48.5 a	38.0 ± 0.5 ab	0.0 ± 0.0 b
½ Mg	15.0 ± 0.4 a	2.0 ± 1.5 a	49.0 ± 16.8 a	42.0 ± 0.5 b	0.0 ± 0.0 b
¾ Mg	17.0 ± 0.4 a	2.0 ± 1.6 ab	34.0 ± 48.5 a	25.0 ± 0.4 ab	0.0 ± 0.0 b

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of three replications. Results were recorded after 28 days. n = 20.

d) *Effect of vessel size*

Rooting was attempted in small (5 x 7.5 cm) and larger culture bottles (6 x 11 cm) and the rooting frequency was then compared with that observed in tubes (2.5 x 10 cm). Percentage of rooting was significantly higher in tubes than in culture bottles (53% vs. 22 and 11% in small and larger vessels, respectively) (Table 2.5). The percentage of shoot browning also increased with the increasing size of the vessel. Browning was

attributed to vessel size because shoots were cautiously handled. Callus proliferation was not observed at basal ends of shoots in any of the treatments.

Table 2.5: Effect of vessel size on *in vitro* rooting of clone GN121 on modified ¼ MS nutrients (Ca^{+2} and Mg^{+2} as for ¾ MS) supplemented with 0.1 mg.l^{-1} IBA, 0.1 mg.l^{-1} biotin, 0.1 mg.l^{-1}calcium pantothenate, 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite. Shoots were rooted under 66 µmol.m^{-2}.s^{-1} PPFD and 27°C day/21°C night following an initial 72-hours dark incubation period.

Vessel size	Rooting[z] (%)	Average no. roots per shoot	Average root length (mm)	Browned non-rooted shoots (%)
Tubes (25 x 100 mm)	53.0 ± 0.5 a	1.0 ± 0.8 a	27.0 ± 19.8 a	40.0 ± 0.5 a
Culture bottles (50 x 75 mm)	22.0 ± 0.4 b	1.0 ± 0.5 a	49.0 ± 28.6 b	60.0 ± 0.5 a
Culture bottles (60 x 110 mm)	11.0 ± 0.3 b	1.0 ± 0.4 a	31.0 ± 21.1 a	78.9 ± 0.4 b

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of three replications. Results were recorded after 28 days. n = 30 - 60.

e) *Effect of light intensity and temperature*

In all investigations discussed, the light intensity and temperature culture regime was 66 µmol.m^{-2}.s^{-1} PPFD and 27°C day/23°C night. The study whereby ¼ MS medium was modified to contain Ca^{2+} and Mg^{2+} to ½ and ¾-strength of full MS (Table 2.4) was repeated with a different light and temperature culture regime. After the initial 72-hours dark incubation period, the cultures were placed under a 16-hours photoperiod at 37

$\mu mol.m^{-2}.s^{-1}$ PPFD and 23°C day/21°C night for seven days before being exposed to 66 $\mu mol.m^{-2}.s^{-1}$ and 27°C day/21°C night for 18 days. This step-wise increase in light intensity and temperature resulted in 75% rooting frequency (Table 2.6). Callus was not visible at basal ends of shoots and roots were long and thick with well-developed lateral roots (Fig. 2.7). At the end of *in vitro* rooting period, plantlets were hardened-off to greenhouse conditions (Section 2.2.4) and data were recorded after 28 days (Table 2.9).

Fig. 2.7: Adventitious root induction on micropropagated GN121 clone cultured for 28 days on modified ¼ MS nutrients (Ca^{2+} and Mg^{2+} as for ¾ MS) supplemented with 0.1 $mg.l^{-1}$ IBA and incubated under optimized culture conditions.
Bar = 5 cm.

Table 2.6: Effect of a step-wise increase in light intensity and temperature on *in vitro* rooting of clone GN121. Medium components were ¼ MS nutrients supplemented with 0.1 mg.l^{-1} IBA, 0.1 mg.l^{-1} biotin, 0.1 mg.l^{-1} calcium pantothenate, 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite. Light intensity and temperature were increased from 37 µmol.m^{-2}.s^{-1} PPFD and 23°C day/21°C night after seven days, to 66 µmol.m^{-2}.s^{-1} PPFD and 27°C day/21°C night for 18 days following an initial 72-hours dark incubation period.

Medium modifications	Rooting[z] (%)	Average no. Roots per shoot	Average root length (mm)	Browned non-rooted shoots (%)
None	53.0 ± 0.5 b	1.0 ± 0.8 a	27.0 ± 19.8 a	40.0 ± 0.5 ab
¾ Ca and Mg	75.0 ± 0.4 c	2.0 ± 1.6 a	49.0 ± 32.7 b	25.0 ± 0.4 a
½ Ca	66.0 ± 0.5 bc	2.0 ± 1.3 ab	36.0 ± 29.4 ab	30.0 ± 0.4 a
¾ Ca	40.0 ± 0.5 ab	1.0 ± 1.1 a	29.5 ± 26.4 a	35.0 ± 0.5 ab
½ Mg	28.0 ± 0.4 a	3.0 ± 0.8 b	82.0 ± 37.6 c	20.0 ± 0.4a
¾ Mg	35.0 ± 0.4 ab	2.0 ± 0.9 ab	47.6 ± 38.4 b	53.0 ± 0.5 b

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of three replications. Results were recorded after 28 days. n = 20.

The effect of each temperature and light intensity on *in vitro* rooting was investigated separately by keeping one of the parameters constant. No rooting was observed when shoot explants of clone GN121 were incubated in the dark at 10°C for 72 hours prior to a 16-hours photoperiod under optimized culture conditions (data not shown). A 72-hours dark incubation period at 30°C lowered rooting frequency of clone GN121 from 75% frequency to 30% rooting frequency (an average of one root per shoot)

(data not shown). Leaves of all explants were yellowish-green and they were browned around the edges, probably by the heat. A similar response was observed when temperature was maintained constant at 27°C day/23°C night following dark incubation at 30°C. An increase in only the light intensity from 37 $\mu mol.m^{-2}.s^{-1}$ PPFD to 66 $\mu mol.m^{-2}.s^{-1}$ (constant temperature at 27°C day/23°C night) resulted in only 50% rooting frequency (an average of two roots per shoot) (data not shown) and none of the shoots were browned as previously observed with temperature studies.

f) *Effect of molybdenum*

Molybdenum is one of the micronutrients whose limiting effect if sometimes associated with browning and/or shoot-tip necrosis (Abrie,[2] pers.comm. 1998). In this study, this micronutrient was furnished as sodium molybdate and its ability to reduce browning/die-back and also improve rooting was tested by increasing its concentration to ½ and ¾-strength of normal MS. Percentage of rooting was insignificantly increased from 75% to 80% for clone GN121 when the concentration of molybdenum was increased to ½ MS level (Table 2.7). However, callus proliferation also increased from zero to 40% (½ Mo) and 65% (¾ Mo) and all rooted shoots had produced callus as shown in Fig. 2.5. Further, shoot browning also increased from 25% to 40% (Table 2.7) and molybdenum at ¾ MS level lowered the rooting frequency significantly.

[2] M. Abrie, Agrocultural Research Council-Roodeplaat, Pretoria 0001, South Africa

Table 2.7: Effect of increasing the concentration of molybdenum in modified ¼ MS nutrients (Ca^{+2} and Mg^{+2} as for ¾ MS) on *in vitro* rooting of clone GN121. Other medium components were 0.1mg.l^{-1} IBA, 0.1 mg.l^{-1} biotin, 0.1 mg.l^{-1}calcium pantothenate, 15 g.l^{-1} sucrose and 4.g.l^{-1} Gelrite. Light intensity and temperature were increased from 37 µmol.m^{-2}.s^{-1} PPFD and 23°C day/21°C night after seven days, to 66 µmol.m^{-2}.$s^{-1}$and 27°C day/21°C night for 18 days following an initial 72-hours dark incubation period.

Medium modifications	Rooting[z] (%)	Average no. roots per shoot	Average root length (mm)	Browned non-rooted shoots (%)	Non-rooted shoots callusing (%)
None	75.0 ± 0.4 b[z]	2.0 ± 1.6 ab	49.0 ± 32.7 a	25.0 ± 0.4 a	0.0 ± 0.0 a
½ $NaMo_4.2H_2O$	80.0 ± 0.4 b	3.0 ± 1.7 b	84.0 ± 53.2 b	25.0 ± 0.4 a	40.0 ± 0.5 b
¾ $NaMo_4.2H_2O$	45.0 ± 0.5 a	2.0 ± 1.1 a	38.0 ± 24.6 a	40.0 ± 0.5 a	65.0 ± 0.5 c

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of three replications. Results were recorded after 28 days. n = 20.

g) *Established rooting protocol for clone GN121*

The modified ¼ MS nutrients (Ca^{2+} and Mg^{2+} as for ¾ MS) without additional molybdenum was deemed the best medium for *in vitro* rooting of cold-tolerant GN121 clone because it resulted in 75% rooting frequency and produced no callus at basal ends of shoots. Therefore, the best protocol established comprised of modified ¼ MS nutrients (Ca^{2+} and Mg^{2+} as for ¾ MS) supplemented with 0.1 mg.l^{-1} IBA, 0.1 mg.l^{-1} biotin, 0.1 mg.l^{-1} calcium pantothenate, 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite. Best culture conditions were an initial 72-hours dark incubation period (~25°C) followed by a step-wise increase in light intensity and temperature under a 16-hours

photoperiod from 37 μmol.m^{-2}.s^{-1} PPFD and 23°C day/21°C night after seven days, to 66 μmol.m^{-2}.s^{-1} PPFD and 27°C day/21°C night for 18 days.

2.3.2.2.2. *Selected studies on clone GN107*

Effect of additional calcium and magnesium and the culture environment

The *in vitro* rooting ability of clone GN107 was also tested under increased concentrations of calcium and magnesium (Section 2.3.2.2.1(c)) and similar responses to clone GN121 were observed. Under a 66 μmol.m^{-2}.s^{-1} PPFD and 27°C day/21°C, the highest rooting frequency (30%) occurred when both calcium and magnesium concentrations were raised to ¾ MS (Table 2.8). The treatments were repeated with a temperature and culture regime previously found most suitable for GN121 (Table 2.6). After the initial 72-hours dark incubation period, the cultures were placed under a 16-hours photoperiod at 37 μmol.m^{-2}.s^{-1} PPFD and 23°C day/21°C night for seven days, then transferred to 66 μmol.m^{-2}.s^{-1} and 27°C day/21°C night for 18 days. As for clone GN121, this step-wise increase in light intensity and temperature produced a positive result (65% rooting) (Table 2.8), and callus was not visible at basal ends of shoots.

Although GN121 and GN107 clones were rooted under similar conditions, differences were observed in rooting frequency (75% vs. 65%) (Tables 2.6 and 2.8). This may be attributed to clonal variations. Therefore, it is suggested that the efficiency of the established protocol should be tested on other cold-tolerant GN clones.

Table 2.8: Effect of additional calcium and magnesium on *in vitro* rooting of clone GN107 on ¼ MS medium supplemented with 0.1 mg.l^{-1} IBA. Other medium components were 0.1 mg.l^{-1} biotin, 0.1 mg.l^{-1}calcium pantothenate, 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite. Shoots were rooted under two light and temperature regimes following an initial 72-hours dark incubation period.

Medium modifications	Rooting[z] (%)	Average no. roots per shoot	Average root length (mm)	Browned non-rooted shoots (%)
66 µmol.m^{-2}.s^{-1} PPFD and 27°C day/21°C night for 25 days[y]				
¾ Ca and Mg	30.0 ± 0.4 a	2.0 ± 0.6 a	27.0 ± 21.1 ab	20.0 ± 0.5 a
37 µmol.m^{-2}.s^{-1} and 23°C day/21°C night for seven days[x]*, thereafter standard conditions for 18 days*				
¾ Ca and Mg	65.0 ± 0.5 b	2.0 ± 1.4 b	28.0 ± 24.4 b	30.0 ± 0.4 a
½ Ca	15.0 ± 0.4 a	1.0 ± 0.5 a	20.0 ± 13.5 a	35.0 ± 0.4 a
¾ Ca	30.0 ± 0.4 a	1.0 ± 0.5 a	25.0 ± 10.0 ab	75.0 ± 0.4 b
½ Mg	15.0 ± 0.4 a	1.0 ± 0.0 a	30.0 ± 35.0 a	30.0 ± 0.4 a
¾ Mg	40.0 ± 0.5 ab	1.0 ± 0.0 a	20.0 ± 21.4 ab	65.0 ± 0.4 b

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of two replications. Results were recorded after 28 days. n = 20.

[y] Standard environmental conditions.

[x] Modified environmental conditions.

2.3.2.3 *Hardening-off of regenerated plants*

At the end of rooting period, the medium was gently rinsed from the roots of plantlets produced under improved light and temperature culture regime (Table 2.6 and 2.8). After determination of root number and length, plantlets were transferred to moist, autoclaved potting mixture of 1 river sand : 9 pine bark (v/v), then sealed in transparent plastic bags to maintain a high humidity condition. The humidity of the microclimate was gradually reduced by punching holes on the bags after one week and another week later, bags were removed completely. The number of plantlets that survived was recorded at the end of hardening-off period (28 days) (Table 2.9) (Fig. 2.8) and plantlets were subjected to normal greenhouse conditions (Section 2.2.1).

Table 2.9: Survival rates of regenerated plantlets of clones GN121 and GN107 after 28 days of hardening-off to greenhouse conditions. The potting mixture was sterilized in 250 cm^3 pots and one plant was cultured per pot.

Modifications of ¼ MS rooting medium	GN121 clone % survival[z]	GN107 clone % survival[z]
¾ Ca and Mg	78.0 ± 0.4 a	58.0 ± 0.4 a
½ Ca	87.0 ± 0.4 a	67.0 ± 0.3 a
¾ Ca	71.0 ± 0.3 a	0.0 ± 0.0 b
½ Mg	60.0 ± 0.4 a	50.0 ± 0.2 a
¾ Mg	88.8 ± 0.4 a	67.0 ± 0.3 a

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of three replications. Results were recorded after 28 days. n = 20.

Fig. 2.8: Hardened-off plantlets of *E. grandis* x *nitens* rooted with the optimized protocol. Results were recorded 28 days after hardening-off period. Bar = 1.8 cm.

2.3.2.4 In vitro *rooting of seedlings of* E. grandis *and* E. nitens

The optimized *in vitro* rooting protocol (Tables 2.6 and 2.8) was further tested with three-week-old shoots from seedlings of *E. grandis* and *E. nitens*. The objective was to ascertain if the parent genotypes affect *in vitro* rooting of GN hybrid clones. There was no clear indication of such a parental effect as there was no significant difference in the rooting frequency between the two species (Table 2.10). Over 90% of seedlings produced roots (Fig. 2.9), but significant differences were observed with respect to the average root numbers and length. Regenerated plantlets were hardened as previously mentioned (Section 2.3.2.3).

Table 2.10: Effect of the optimized *in vitro* rooting protocol on the rooting ability of seedling shoots of *E. grandis* and *E. nitens*. Medium components and culture conditions were as shown in Table 2.6.

Source of seedling shoots	Rooting[z] (%)	Average no. roots per shoot	Average root length (mm)
E. grandis	97.0 ± 0.2 a	4.0 ± 1.2 a	91.5 ± 38.7 a
E. nitens	93.0 ± 0.2 a	3.0 ± 1.0 b	63.96 ± 34.9 b

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of three replications. Results were recorded after 28 days. n = 20.

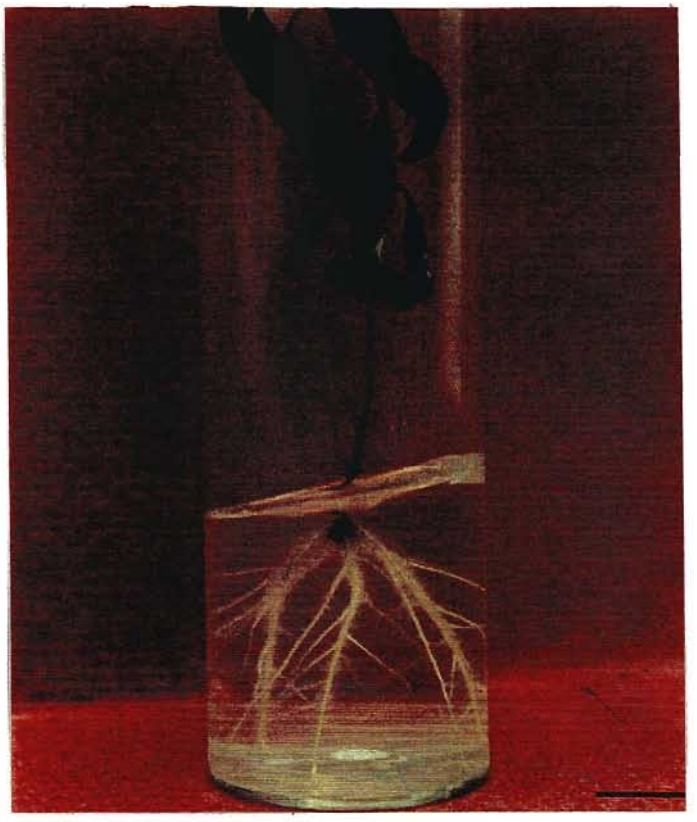

Fig. 2.9: *In vitro* rooting of seedlings of *Eucalyptus grandis* and *E. nitens* on optimized protocol. Bar = 6.8 cm.

2.3.3 Towards the development of a non genotype-specific *in vitro* rooting protocol

A literature survey revealed that rooting and subsequent survival of many woody tree species recalcitrant to rooting by conventional techniques (e.g. apple cultivars, pines, etc.) was improved by inoculation with wild-type strains of *Agrobaterium rhizogenes*. This bacterium in known to affect many dicotyledonous plants, producing transgenic roots and/or tumours/callus. The purpose of this investigation was to establish if wild-type strains of *A. rhizogenes* were suitable for inducing roots on two cold-tolerant GN clones.

2.3.3.1 *Selection of* A. rhizogenes *strains*

The carrot disc assay was conducted to test the virulence or root-inducing ability of twelve wild-type strains of *A. rhizogenes*, viz., LBA9402, R1500, R1600, R1601, HR1, TR7, R47, TR8.3, A4, 8196, A2183 and TR101. Fresh, sterilized carrots were sectioned into approximately 0.5 cm-thick discs and agroinfected with one drop (approx. 10^{10} cells.ml^{-1}) of overnight bacterial culture on either the apical side (facing the root tip) (four discs) or basal side (facing the shoot) (four discs). After four weeks of dark incubation at room temperature, only nine of the twelve strains had produced transgenic roots on the apical side of carrot discs (Table 2.11). Only HR1 and R1601 strains induced roots on both apical and basal surfaces of carrot discs (data not shown), and TR7, A2183 and TR101 produced only tumours/callus on both sides (Fig. 2.10). In some cases, the initial response preceding root production was tumour/callus formation that raised the surface of the pericycle (Fig. 2.10). The HR1 strain produced the highest number of roots, which was assessed by the size of root mass, whereas the LBA9402 strain produced the least number of roots.

Table 2.11: Induction of transgenic roots on apical side of carrot discs by wild-type strains of *A. rhizogenes* after four weeks of dark incubation. Agroinfected discs were placed on 8g vermiculite moistened with 60 ml MS nutrients supplemented with 10 $g.l^{-1}$ sucrose. Strains TR7, TR101 and A2183 produced only tumuors/callus on either the apical or basal side of the carrot discs.

Wild-type strain	Rooting (%)[z]	Wild-type strain	Rooting (%)[z]
Control	0.0 ± 0.0 a	R1500	33.0 ± 0.4 ab
HR1	92.0 ± 0.2 d	TR8.3	33.0 ± 0.4 ab
R1600	83.0 ± 0.4 cd	LBA9402	8.0 ± 0.2 a
R47	83.0 ± 0.4 cd	A4	33.0 ± 0.4 ab
R1601	50.0 ± 0.5 bc	8196	25.0 ± 0.4 ab

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of three replications. n = 20.

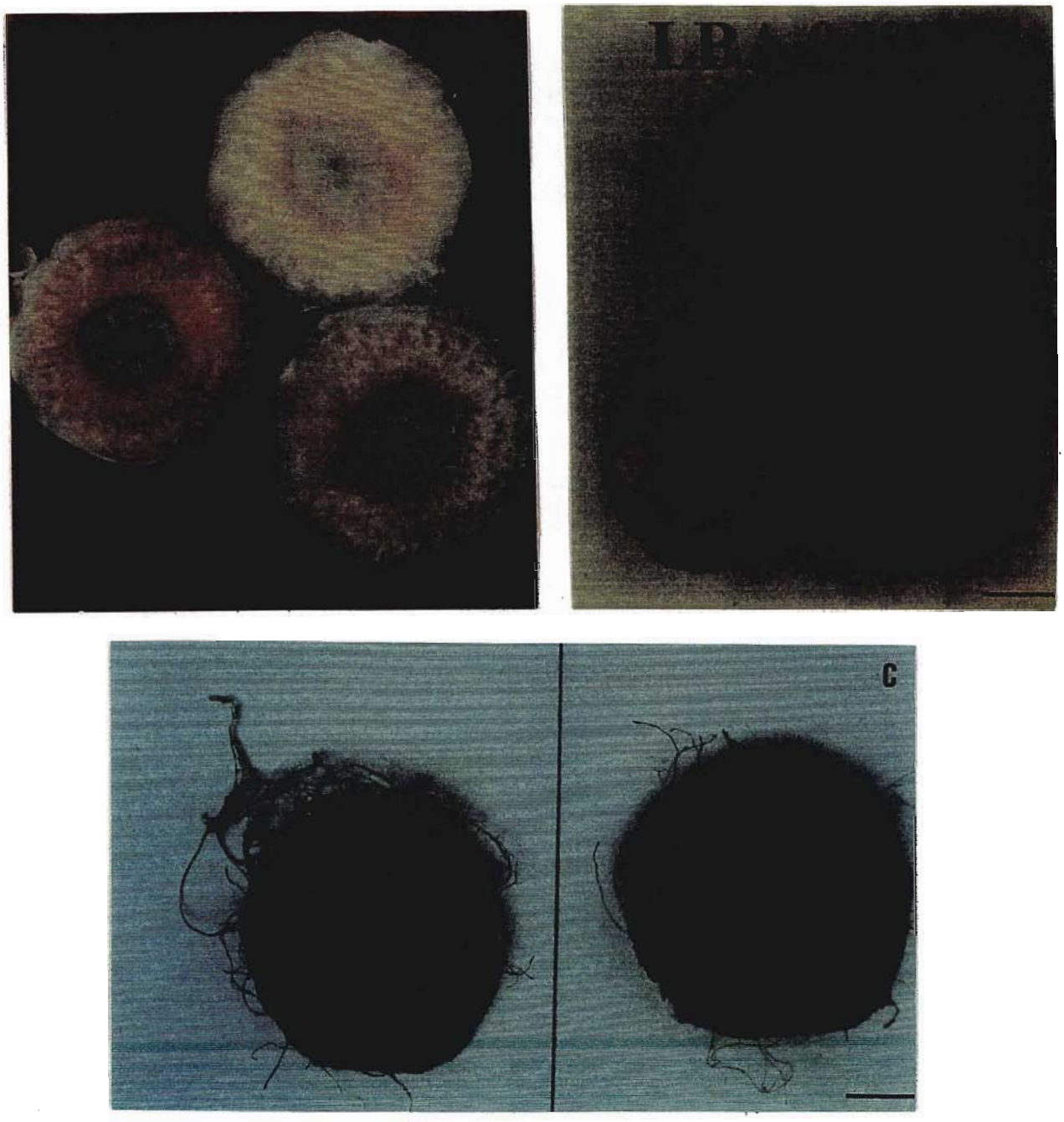

Fig. 2.10: Effect of wild-type strains of *A. rhizogenes* on carrot discs after four weeks on vermiculite moistened with MS nutrients (without hormones) supplemented with 10 g.l^{-1} sucrose. A = control discs, B = production of tumours/callus and roots and C = production of transgenic roots. Bar = 0.6 cm.

2.3.3.2 *Effect of* A. rhizogenes *on explants of* E. grandis *x* nitens

Based on the results of the carrot disc assay, the following strains were selected for subsequent experiments based on their ability to incite many transgenic roots, viz., HR1, R1600, R1601, R47 and TR8.3 (Table. 2.11). Studies focused on various explants of GN clones such as leaf and stem explants to ascertain the virulence of the selected strains.

a) *Localized inoculation method*

The surfaces of leaves and stem of nodal cuttings (GN107 and GN121) were wounded with a razor blade (three scratches) three days after sterilization. A flamed and cooled bacterial loop was dipped into overnight bacterial liquid culture (approx. 10^{10} cells.ml^{-1}) and used to inoculate wounded areas. Of the five selected strains, only the HR1 strain produced tumours/callus at all four inoculated areas (Fig. 2.11), but no rooting occurred in six weeks. Bud-break took longer, probably because of lack of hormones in the support medium and continuous dark culture conditions. On the controls and other remaining four tested strains, the wounded areas were browned and neither rooting nor tumour/callus proliferation was observed (Fig. 2.11).

Rooting did also not occur on leaves of clone GN121 obtained from fresh coppice and reduced to two thirds of their original length, then scrapped across the bacterial lawns of the five selected strains. Inoculated explants and about 50% of controls were browned from the cut-end up to the surface of the supporting medium (Fig. 2.12).

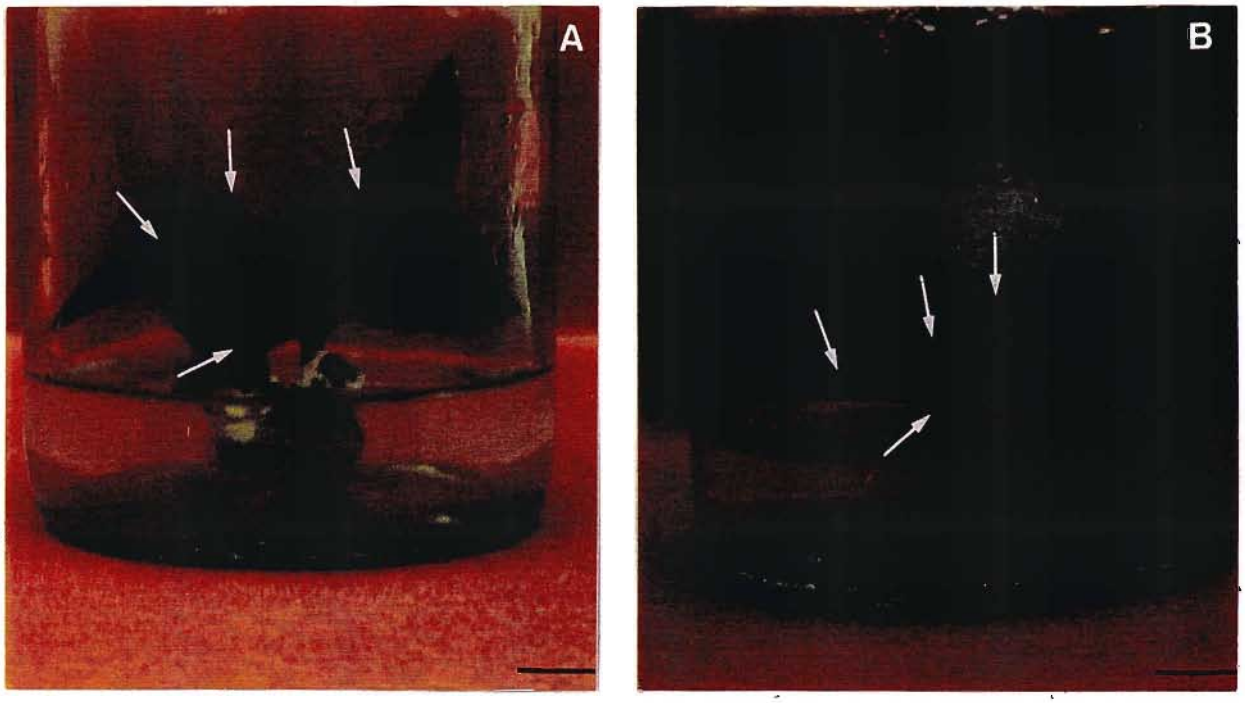

Fig. 2.11: Effect of HR1 strain on nodal explants of clones GN121 and GN107 cultured on ½ MS nutrients (without hormones), 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite after six weeks in the dark. Arrows indicate inoculated areas and plant response. A = control, B = agroinfected. Bar = 0.7 cm.

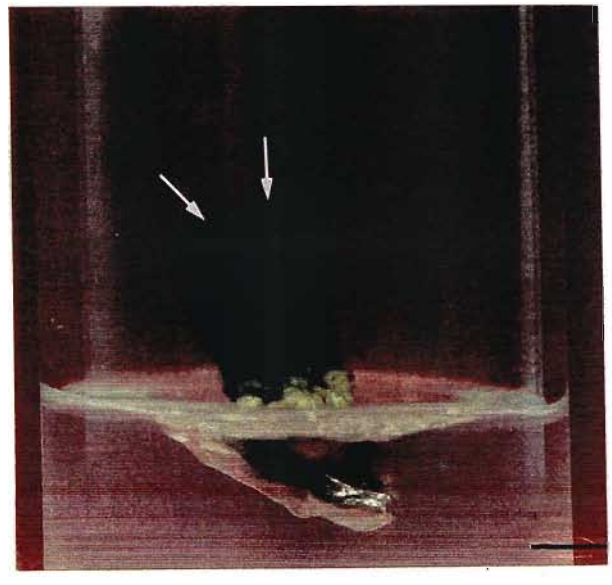

Fig. 2.12: Browning observed on coppice leaf explants of *E. grandis* x *nitens* clones agroinfected with selected strains of *A. rhizogenes* and cultured on ½ MS nutrients (without hormones), 15 g.l^{-1} sucrose and 4 g.l^{-1} Gelrite after four weeks in the dark. Arrows indicate the remains of bacterial colonies on the leaf explant. Bar = 3.5 cm.

In another treatment where micropropagated shoots were used, the HR1 strain also produced tumours/callus at basal ends of shoots of clone GN121 (results not shown). Shoot bases were agroinfected with a bacterial loop dipped in liquid overnight cultures of the five strains mentioned previously (Section 2.2.7). Agroinfected shoots and controls were placed up side down in tubes containing medium and incubated in a ventilated oven at 28°C for 48 hours. Those were culture conditions optimal for bacterial growth on solid LB or YMA media. However, after four weeks under a 16-hours photoperiod at 37 $\mu mol.m^{-2}.s^{-1}$ and 23°C day/21°C night, rooting was not observed at basal ends of shoots.

a) *Leaf disc - liquid purge method*

Expanded leaves selected from micropropagated shoots of clone GN121 were sectioned into approximately 0.5 x 0.5 cm sections and precultured on their adaxial surfaces on MS nutrients (with and also without 0.1 $mg.l^{-1}$ IBA). After 48 hours, explants had exuded phenolics into the medium and were then cocultured for two to five minutes with five selected strains of *A. rhizogenes* (HR1, R1600, R1601, R47 and TR8.3) grown either in LB broth or MS nutrients (without hormones). Explants were washed in 500 $mg.l^{-1}$ cefotaxime solution and subcultured regularly (every two to three days) to a medium with the same antibiotic. No rooting occurred on explants cultured on medium with or without 0.1 $mg.l^{-1}$ IBA. However, in both media the inoculated explants were curled and had produced callus from the midribs. The HR1 strain produced large amounts of tumours/callus and a more complex curling of leaf explants (Fig. 2.13). The choice of coculture medium (LB broth or MS nutrients) had no significant effect on formation of tumours/callus but leaf curling was more enhanced on explants inoculated with strains grown on the LB broth. Further, bacterial strains grown on MS nutrients were difficult to cure compared to those grown on LB broth which were cured within two to three subcultures. Pink stained substances (probably anthocyanins) were associated with tumours/callus produced by HR1 strain grown on MS nutrients.

Transfer of inoculated explants and controls on MS medium with and without 0.1 $mg.l^{-1}$ IBA at 37 $\mu mol.m^{-2}.s^{-1}$ and 23°C day/21°C night did not promote the initiation and developments of roots.

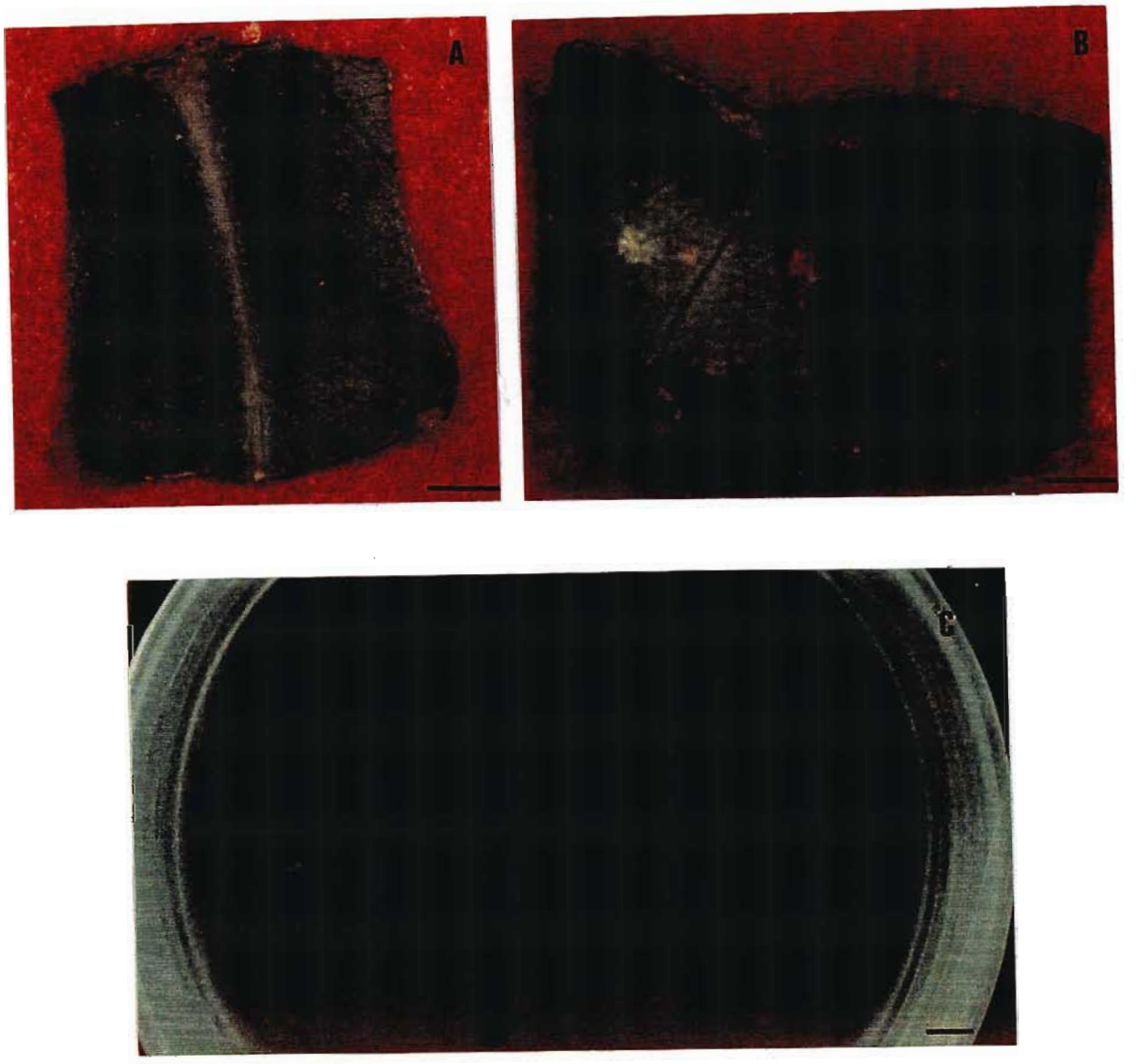

Fig. 2.13: The response of micropropagated leaf explants of clone GN121 to agroinfection with five selected strains of *A. rhizogenes*. Explants were cultured on MS nutrients (with and without 0.1 $mg.l^{-1}$ IBA), 20 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. A = control explant, B = explant inoculated with either R1600, R1601, R47 or TR8.3, C = explants inoculated with HR1 strain. Results were recorded after six weeks. Bar = 200 µm.

In another investigation, leaf pieces or whole leaves obtained from micropropagated shoots of GN121 clone were wounded with a razor blade, cocultured with five selected bacterial strains and cultured on vermiculite moistened with MS nutrients (without hormones) supplemented with 15 $g.l^{-1}$ sucrose. These were the culture conditions optimal for the carrot disc assay; however, by the end of a four-week period, all explants had turned brown and died (data not shown).

In an attempt to make HR1, R47 and R1600 strains more virulent and improve chemotaxis between bacterial and plant tissue, various concentrations of acetosyringone were tested. Bacterial cultures were grown first in LB broth then resuspended in MS nutrients (without hormones) supplemented with 0, 0.02, 0.2 and 0.3 $g.l^{-1}$ acetosyringone. Leaf explants from fresh coppice of both GN clones were cocultured for 6, 12 and 24 hours with bacterial cultures. No rooting occurred after six weeks and the number of explants lost to browning increased with an increase in coculture time and concentration of acetosyringone used. All explants submerged in culture medium containing 0.2 and 0.3 $g.l^{-1}$ acetosyringone died. Rooting did not occur also when coppice leaf explants were scrapped with the cut surface across the overnight bacterial lawns of the above strains grown on media with 0.2 $g.l^{-1}$ acetosyringone. Transferring explants to a 16-hours photoperiod at 37 $\mu mol.m^{-2}.s^{-1}$ and 23°C day/21°C night did not promote rooting.

In conclusion, the absence of transgenic roots on tested GN clones suggested that the root inducing plasmid (pRi) was either not efficiently transferred across the plant genome, or if transferred, it was not properly integrated. The production of tumours/callus in the presence of HR1 strain and absence of plant growth regulators in the induction medium suggested that GN clones could be susceptible to some bacterial strains, even though roots were not produced within four to six weeks. The tumours produced were not analyzed for the presence of opines which would confirm transformation. However, root production on carrot discs provided evidence that most

of the bacterial strains tested were virulent (i.e. a functional plasmid was present and transferred across the carrot genome). Therefore, a method for the transfer and successful integration of the Ri plasmid into the GN genome still needs to be established.

2.3.3.3 *Effect of* A. rhizogenes *on seedlings of* E. grandis *and* E. nitens

Since no transgenic roots were produced on explants of the two tested GN clones, the question arose as to the susceptibility of each of the two parent species to *A. rhizogenes*. Consequently, roots of three-week-old seedlings were trimmed-off and bases of shoots scrapped across overnight bacterial lawns of the following wild-type strains, viz., R47, TR8.3, LBA9402, A4, HR1, R1600 and R1601. Agroinfected plants and controls were placed up side down in culture tubes and rooting occurred within four days. All roots, including those produced by control explants appeared 'hairy' before stem bases were inserted into the medium. However, on explants inoculated with agrobacterial strains roots were produced higher on the stem at all points where contact was made with bacteria, whereas on control explant roots were produced only from the bases of stems (Fig. 2.14). Roots produced on inoculated explants maintained the 'hairy root' phenotype after bases of stems were inserted into the medium, but as for the controls, the hairy phenotype appeared to have been limited only to the initial stages before explants were re-orientated into the medium. Control roots did not grow as fast nor branch as profusely as those from inoculated explants. Differences in percentages of rooting were not significant within the two species, although more *E. grandis* shoots rooted under the influence of bacteria than those of *E. nitens* (Table 2.12). Significant differences were observed in the average number of roots produced per shoot explant for both species and bacterial cultures resulted in the production of fast-growing, long roots (Fig. 2.14). Roots were excised and cultured in liquid MS nutrients (without hormones) supplemented with 15 $g.l^{-1}$ sucrose for 28 days. Only roots from inoculated

shoots (i.e. transformed hairy roots) showed the ability to grow on hormone-free medium (Fig. 2.15).

Fig. 2.14: Effect of wild-type strains of *A. rhizogenes* on rooting ability of shoot explants of *E. grandis* and *E. nitens* obtained from three-week old seedlings. A= hairy root induction B = root morphology after 14 days, control explant is shown on the left. Bar = 0.6 cm.

Table 2.12: *In vitro* root induction on three-week-old seedlings of *E. grandis* and *E. nitens* by wild-type strains of *A. rhizogenes*. Medium components were ½ MS nutrients (without hormones), 15 $g.l^{-1}$ sucrose and 4 $g.l^{-1}$ Gelrite. Shoots were rooted under 66 $\mu mol.m^{-2}.s^{-1}$ and 27°C day/21°C night following an initial 72-hours dark incubation period.

Wild-type strain	*E. grandis* Rooting[z] (%)	*E. grandis* Average no. Roots per shoot	*E. nitens* Rooting[z] (%)	*E. nitens* Average no. roots per shoot
Control	74.0 ± 0.4 a	2.0 ± 1.2 a	52.0 ± 0.5 a	2.0 ± 0.8 ab
R47	78.0 ± 0.4 a	4.0 ± 2.4 ab	56.0 ± 0.5 a	2.0 ± 1.0 abc
TR8.3	70.0 ± 0.4 a	3.0 ± 1.6 ab	70.0 ± 0.4 a	2.0 ± 0.9 abc
LBA9402	68.0 ± 0.4 a	4.0 ± 2.3 ab	68.0 ± 0.4 a	2.0 ± 1.0 c
A4	70.0 ± 0.4 a	4.0 ± 2.7 ab	54.0 ± 0.5 a	3.0 ± 1.1 bc
HR1	80.0 ± 0.4 a	4.0 ± 2.5 ab	50.0 ± 0.5 a	2.0 ± 0.9 a
R1601	70.0 ± 0.4 a	5.0 ± 3.6 b	54.0 ± 0.5 a	1.0 ± 0.7 a
R1600	86.0 ± 0.4 a	4.0 ± 2.4 b	66.0 ± 0.4 a	2.0 ± 1.4 b

[z]Mean separation within columns by Duncan's multiple range test, $P \leq 0.05$. Values represent means ± one standard deviation of three replications. Results were recorded after 14 days. n = 20.

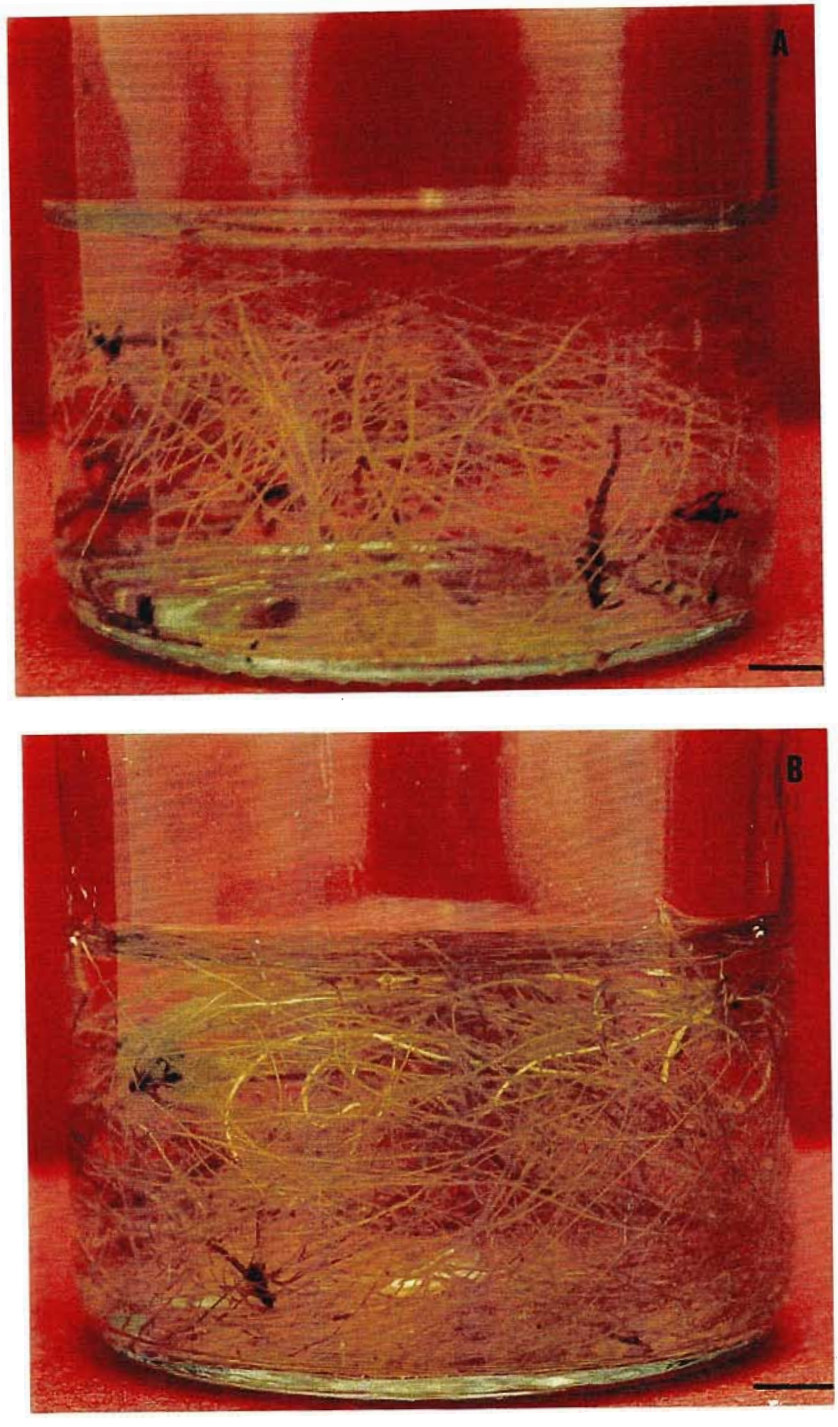

Fig. 2.15: Growth of transgenic roots of *E. grandis* on hormone-free MS nutrients supplemented with 15 g.l^{-1} sucrose. Roots were excised from three-week old shoots from seedling after 14 days. A= roots at day zero and B = roots after 28 days of growth in the dark Bar = 0.5 cm.

2.4 DISCUSSION

2.4.1 Establishment of shoot cultures

Sterile shoot cultures were successfully generated from axillary buds and few explants were lost because of contamination (< 10%), indicating that endogenous contamination (usually experienced with woody species, e.g. Warrag *et al.*, 1990; Le Roux and van Staden, 1991b) was not a problem. An average of ten shoots per bud explant was obtained in four weeks and these were routinely subcultured to bulk up material for subsequent rooting. Similar shoot yields (per bud explant) were obtained by Gupta *et al.* (1981) (5 - 10 shoots with *Eucalyptus citriodora*), Bennett *et al.* (1992, 1994) (11 shoots with *E. globulus*) and Yang *et al.* (1995) (10 shoots with *E. grandis* x *urophylla*).

Several studies have demonstrated that cytokinins used in multiplication and elongation media could affect subsequent rooting (Curir *et al.* 1990; Bennett *et al.*, 1992, 1994). Although the effect of those growth regulators was not investigated in the present study, it is possible that alternating BAP (in multiplication medium) with FAP (kinetin) (in elongation medium) affected the rooting ability of shoots, as reported for several eucalypts by Curir *et al.* (1990), Bennett *et al.* (1992, 1994) and Jones and van Staden (1994). In studies by Bennett *et al.* (1992, 1994), most clones of *E. globulus* and other eucalypts showed higher shoot survival in rooting medium when shoots were taken from the multiplication/elongation medium in which kinetin was used. Those taken from a BAP-containing medium experienced leaf reddening and abscission (Bennett *et al.*, 1992).

2.4.2 Parameters that affected rooting of GN clones

According to Srikandarajah *et al.* (1990), the physiology of adventitious root formation is still not clearly understood. Reports on difficult-to-root woody species have

concentrated on factors such as temperature (Zimmerman, 1984; Warrag *et al.*, 1990), light intensity and wavelength (Pfaff and Schofer, 1980; Hughes, 1981; Wang, 1992; Bressan *et al*, 1992), growth regulators (Nemeth, 1986; Curir *et al.*, 1990), activated charcoal (Blomstedt *et al.,* 1991; Sanchez *et al.*, 1996), support medium (Le Roux, 1990), number of subcultures in multiplication medium (Warrag *et al.*, 1990; Trindade and Pais, 1997) and phloroglucinol (Zimmerman, 1984). In this study, the most important factors that influenced rooting were light intensity and temperature (Tables 2.6 and 2.8, Fig. 2.7). However, the mineral composition of the medium (e.g. additional calcium and magnesium) also played a positively significant role (Table 2.4).

Much research on the effect of environmental factors such as light intensity and temperature has been conducted on fruit trees. Of the environmental factors, light has generally been accepted as being the most important as it regulates photomorphogenic processes in tissue culture through the phytochrome system (Wang, 1992; Rossi *et al.* 1993; Bertazza *et al.*, 1995). The temperature effects are normally eliminated by maintaining the same temperature under different light intensities (Le Roux, 1990; Wang, 1992). In addition to photomorphogenic functions, light also has the photosynthetic function on rooting of cuttings *in vitro*, especially under high intensity (Hughes, 1981; Walker *et al.*, 1987; Kozai, 1991).

The study whereby ¼ MS medium was modified to contain calcium and magnesium ions at ¾-strength of full MS was repeated with a different light and temperature culture regime (Tables 2.6 and 2.8). After the initial 72-hours dark incubation period, the cultures were placed under a 16-hours photoperiod at 37 $\mu mol.m^{-2}.s^{-1}$ PPFD and 23°C day/21°C night for 7 days before being exposed to 66 $\mu mol.m^{-2}.s^{-1}$ PPFD and 27°C day/21°C night for 18 days. This step-wise increase in light intensity and temperature resulted in 75% (GN121) and 65% (GN107) rooting frequency and callus was not visible at basal ends of shoots (Tables 2.6 and 2.8). The roots exhibited the desirable morphology of long and thick roots (Fig. 2.7) with well-developed lateral roots that

could improve survival in *ex vitro* conditions. These results were invariably consistent with those of several authors, where rooting was promoted when shoots were exposed initially to low and then high irradiance levels (Bressan *et al.*, 1982; Baadsmand and Andersen, 1984; Zimmerman, 1984). According to Wang (1992) the positive effect of increasing irradiance from 10 to 80 $\mu mol.m^{-2}.s^{-1}$ PPFD on rooting may be as a result of the uptake, transport and accumulation of applied auxins in cuttings. Eliezer and Morris (1980) suggested that high light intensity increase the velocity of auxin transport in intact pear plants. A condition of high light intensity was also reported to produce high carbohydrate availability relative to nitrogen (Hyndman *et al.*, 1982).

Since in this study low levels of sucrose were supplied in the rooting medium (15 $g.l^{-1}$) under increased irradiance, it is suggested that rooting occurred under photoautomixotrophic conditions because tubes were loosely capped with plastic lids and were not sealed with parafilm. Hence, CO_2 concentration inside the tubes could have been similar to the ambient level (± 350 ppm) (Kozai, 1991). In contrast, Rossi *et al.* (1993) as well as Bertazza *et al.* (1995) concluded that rooting differences found under different light regimes were unlikely related to differences in photosynthetic carbon fixation since under their experimental conditions there was an exogenous carbohydrate supply (20 $g.l^{-1}$ sucrose), low photon fluxes (maximum of 36 $\mu mol.m^{-2}.s^{-1}$) and the lack of CO_2 enrichment. Further, the 500 ml and 100 ml glass jars used for rooting by those authors, respectively, were wrapped in plastic foil, hence there was little, if any, air exchange. Therefore, in those studies, rooting response was attributed to the control of phytochrome because a linear relationship was determined between the two. Light quality has also been found to affect the action of auxins in regulating rooting (Rossi *et al.*, 1993).

High light irradiance (up to 280 $\mu mol.m^{-2}.s^{-1}$) improved percentage of rooting of the difficult-to-root clone of *Sequoia sempervirens* obtained from trees several hundred years old (Walker *et al.*, 1987), but it was reported inhibitory for cold-tolerant

E. grandis x *macarthurii* (Le Roux, 1990) and other woody species (Rugini *et al.* 1988). Le Roux (1990) observed that under high light intensity (40 to 60 $\mu mol.m^{-2}.s^{-1}$ PPFD), *in vitro* rooting of an *E. grandis* x *macarthurii* clone was inhibited by leaf senescence brought about by the production of white sugary callus on the surface of leaves. In contrast, Jones and van Staden (1994) reported that shoots of *E. grandis* x *urophylla* clones often became etiolated as a result of low light intensity (14 $\mu mol.m^{-2}.s^{-1}$ PPFD). The effect of light on *in vitro* rooting of *E. grandis* hybrids therefore appears to be genotype specific.

Temperature has been shown to exert a strong influence on rooting during dark incubation for apple cultivars (Zimmerman, 1984), pear rootstock (Wang, 1992), rose cultivars (Rahman *et al.*, 1992) and some other fruit trees Rugini *et al.* (1988). Rahman *et al.* (1992) reported that increasing temperature from 24 to 28°C under a 16-hours photoperiod gave a significantly higher number of roots with maximum shoot length increment. As observed in the present investigation, temperatures at and above 30°C were reported to lower rooting frequency (Zimmerman, 1984; Wang, 1992). However, temperature was initially increased simultaneously with light intensity (Tables 2.6 and 2.8), and its beneficial effect was probably on the translocation of supportive factors (carbohydrates) and related increase in respiration (Ooishi *et al.*, 1978). As suggested by Veierskov and Andersen (1992) for IAA on pea cuttings, it is assumed in the present study that IBA at lower temperatures (23°C day/ 21°C night) affected the accumulation of carbohydrates at the base of shoots, which were then used in root formation later when the temperature was increased (27°C day/ 21°C night) (Tables 2.6 and 2.8). Using infrared thermometry, Baadsmand and Andersen (1984) estimated leaf temperature of *Pisum sativum* at 38 $W.m^{-2}$ to be 33°C, while the air temperature was unchanged at 27°C. According to those authors, it was therefore possible that the effect of irradiance during auxin transport was temperature-mediated. Those authors further suggested that it was the temperature in the plant tissue that determined the transport velocity of auxins and not the ambient temperature. In addition, George (1996) pointed out that

temperature inside the culture is usually higher that outside because of the greenhouse effect. In their conclusion, Baadsmand and Andersen (1984) stated that the effect of high irradiance level (38 $W.m^{-2}$) in conjunction with increased tissue temperature determined the strength of the polarity of auxin transport in the pea cuttings. The observations made in the present study strongly support the above argument, because rooting was significantly improved when both light intensity and temperature where increased simultaneously (Tables 2.6 and 2.8). Increasing either light intensity or temperature reduced the percentage of rooting from 75% to 50% and 30%, respectively. It is suggested therefore, that increasing light intensity accounts for the accumulation of IBA (or its conjugate, IBAap) at the base of stems, whereas an increase in temperature improves IBA uptake as well as the velocity of carbohydrates and organic nitrogenous compounds towards the rooting zone.

Additional calcium and magnesium ions were shown to prevent callus formation at bases of shoots (Section 2.3.2.1). When those macronutrients were added separately at ½ and ¾-strength of MS level in the ¼ MS medium, they had a negative effect on rooting (Table 2.4). Although the highest rooting frequency (53%) was observed when Ca^{2+} and Mg^{2+} were maintained both at ¾-strength of normal MS, differences with the control explants were not significant (53% vs. 52%) (Table 2.4). Murashige and Skoog (1962) reported that in tobacco the requirements for calcium increased as those of magnesium increased. The ineffectiveness of calcium and magnesium alone in promoting root formation may have been due to lack of absorption and/or assimilation of either ion in the other's absence. Calcium ions may also act as chemical messengers; a temporary increase in calcium ions to 1 or 10 μM (approx. 6.6 μM in the present study) does not significantly alter the ionic environment with the cell, but is yet sufficient to trigger fundamental cell processes such as polarized growth, response to gravity and plant growth substances, cytoplasmic streaming, and mitosis (Ferguson and Drøbak, 1988; Poovaiah, 1988). Calcium has been suggested to prevent leakage of auxin

protectors from the tissue into the nutrient medium (Stonier, 1971 cited by Németh, 1986).

As previously mentioned, IBA is the most commonly used auxin for rooting of woody macro- and microcuttings. For continual maintenance on IBA, the concentration normally used for rooting *Eucalyptus* species and hybrids ranges from 1 – 2 $mg.l^{-1}$ (Le Roux and van Staden, 1991b). However, an increase in callus proliferation at bases of stems was observed previously with an increase in IBA concentration from 0.01 to 2.5 $mg.l^{-1}$ (72-hour pulses) and 20 $mg.l^{-1}$ (24-hour pulse) (Section 2.3.2.1). When callus proliferation proceeds root development, vascular connections between the roots and the stem may be interrupted, interfering with the transport of water and nutrients to the shoot (Martin, 1985). Further, essential nutrients that may be required by the shoot to synthesize rooting compounds could be directed towards nourishment of the undifferentiated cells.

In the present study, a chronic application of 0.1 $mg.l^{-1}$ IBA did not have any significant effect on rooting of clone GN121. However, rooting frequency was lowered slightly from 64% to 53% (Tables 2.2 and 2.4). Callus was not formed at bases of stems, probably due to additional Ca^{2+} and Mg^{2+} in the rooting medium. Studies have shown that IBA is rapidly conjugated to IBA aspartate (IBAsp) which actually acts as a rooting promoter (Riov, 1993; Epstein *et al.*, 1993; Baraldi *et al.*, 1993). Hence, increasing IBA concentration within limits could possibly increase the concentration and availability of IBAsp. However, Epstein *et al.* (1993) have shown that there are differences between cultivars of sweet cherry in their ability to hydrolyze the ester conjugate at appropriate time to release free IBA which may promote rooting. It may therefore be argued that easy-to-root eucalypts such as *E. grandis* possess the hydrolytic ability in contrast to difficult-to-root cold-tolerant hybrids such as *E. grandis* x *nitens*. Alternatively, such differences may also exist

between clones, resulting in the observed variation in rooting frequency between the clone GN121 (75% rooting) and clone GN107 (65% rooting).

The high variability in rooting percentages (shown by standard deviations) observed within the two tested clones could also be an indication of the physiological age difference of the axillary buds from which shoots were regenerated. Ammirato (1986) suggested that cells or organs on the same parent plant do not the same ability to undergo morphogenesis, therefore, it is possible that shoots regenerated from buds higher on the stem were physiologically more juvenile than those lower down, giving them the ability to root readily.

Other tested parameters discussed from here onwards had a negative effect on rooting frequency of GN121 and GN107 clones. The first parameter investigated was the use of the antioxidant PVP (polyvinylpyrrolidone) in an effort to minimize browning and shoot die-back and also to improve rooting. That antioxidant has been used successfully at 1 $g.l^{-1}$ in rooting media to minimize exudation of phenolics and to improve rooting frequency of clones of *E. grandis* x *macarthurii*, *E. macarthurii* as well as *E. grandis* x *urophylla* (Le Roux, 1990; Le Roux and van Staden, 1991a; Jones and van Staden, 1994). Le Roux (1990) suggested that the absence of PVP reduced rooting of *E. macarthurii* shoots and that in its presence, the antioxidant would bind auxin in root initiation media as well as inhibitory compounds released by the shoots. In the present study, however, the presence of PVP in the modified ¼ MSG medium lowered rooting frequency and stimulated the release and oxidation of phenolics (Table 2.3) (Fig. 2.6). George (1993) pointed out that although PVP has been used successfully in many other plant species, it does not always stop exudation of phenolics. Observations made in the present study lead to the suggestion that PVP might have interfered with normal activity of endogenous protectors of phenolics and promoted exudation of those substances whose oxidation was precipitated by light (Creasy, 1968).

Glutamine is synthesized from ammonium ions and may be regarded as a precursor of other amino acids in the metabolic pathway of nitrogen. By replacing ammonium nitrate with L-glutamine in the medium, a readily assimilated and energetically inexpensive nitrogen source that may be required in root initiation is made readily available (Kirby *et al.*, 1987). This may explain how rooting happened quicker in the presence of L-glutamine (¼ MSG medium) instead of ammonium nitrate, even though rooting frequency was lower than in the latter (50% vs. 64%) (Tables 2.3). Glutamine has also been shown to be a requirement for cell division in pines (Kirby *et al.* 1987). Working with a rose cultivar, Hyndman *et al.* (1982) reported that reducing the total nitrogen concentration in the medium to 7 mM (instead of 60 mM present in full MS) improved rooting frequency up to 75%. A similar response was observed in the present study for cold-tolerant clones of *E. grandis* x *nitens* when total nitrogen was reduced to about 15 mM (75% [GN121] and 65% [GN107] rooting frequency) under improved culture conditions (Table 2.6 and 2.8). Hyndman *et al.* (1982) added that neither nitrate (as $NaNO_3$) nor ammonium (as $(NH_4)_2SO_4$) alone had the effect on rooting that both had together in the ratio of the MS salt formulation, and that the ionic form of nitrogen in the medium appeared to have a significant effect on root initiation.

Hyndman *et al.* (1982) proposed that the presence of both nitrate and ammonium at ratios of MS formulation is important for maintaining the desired pH of the medium, because pH is increased by the former and lowered by the latter. Hence, the absence of ammonium nitrate in the present study could have made the medium more alkaline (and probably toxic), thus inhibiting some metabolic processes and the availability of some nutrients required for root initiation. In some cases, roots produced in ¼ MSG medium (i.e. ¼ MS in which NH_4NO_3 had been replaced with L-glutamine) lacked well-developed lateral roots (Fig. 2.4), a disadvantage not shown in normal ¼ MS. It is therefore possible that in the absence of ammonium ions, a situation of nitrogen deficiency occurred in ¼ MSG medium, resulting in the formation of undesired root morphology. However, Srikanthrajah *et al.* (1990) observed up to 100% rooting

frequency in difficult-to-root apple cultivars when NH_4NO_3 was completely omitted in the ¼ MS medium and KNO_3 was provided at full strength. Finally, since shoots grown *in vitro* are not photosynthetically efficient under heterotrophic or mixotrophic conditions (Kozai, 1991), it is possible that leaves were not synthesizing organic nitrogen at a rate that matched morphogenesis and growth, therefore, leaves and growing shoot tip might have acted as strong sinks for the supplied organic nitrogen source (L-glutamine) (as described by Altman and Wareing, 1975).

Svenson and Davies, jr (1995) found that the concentrations of molybdenum, iron and copper in the basal portions of unrooted stem cuttings of Poinsettia increased during the root initiation stage, suggesting that concentrations of those micronutrients may be important for early root primordia formation or other concurrent growth processes. Those authors suggested that accumulation of iron and molybdenum at root initiation site may help support nitrogenase and nitrate reductase activity on subsequent root growth and development. As an essential micronutrient, molybdenum is a component of the MS medium and its effect on rooting was tested at ½ and ¾-strength of normal MS in the modified ¼ MS medium (Ca^{2+} and Mg^{2+} as for ¾ MS). Although additional molybdenum slightly improved rooting frequency (from 75% to 80%) of clone GN121 (Table 2.7), the improvement was accompanied by callus proliferation at shoot bases (as shown in Figure 2.5). This should be avoided as it may reduce survival yield of micropropagated plants (Le Roux and van Staden, 1991 a,b). For these reasons, subsequent studies on clone GN107 omitted the use of molybdenum.

In the present study, rooting frequency was found to be greater in tubes than in bottles (Table 2.5). It was expected that rooting would be promoted better in larger vessels where oxygen content is higher than in tubes. However, that was not the case and browning accompanied by shoot die-back, was greater in bottles than in tubes and it probably had a significant effect on percentage of rooting (Table 2.5). Carter and Slee

(1991) observed high rooting percentage of *E. grandis* on a mixture of peat, perlite and sand, compared to perlite or sand alone and suggested that the relatively high moisture content may be more important than air content for maximum root formation on cuttings. In terms of *in vitro* rooting of GN clones, the implication of the above study is that relative humidity may be important in keeping shoots from desiccating in order to produce nutrients required for root initiation in response to culture stimuli. Further, *in vitro* shoot cultures have been reported to have little (if any) cuticle (Nashar, 1989) compared with their *ex vitro* counterparts. This may further explain the observed increase in browning with an increase in culture vessel size (Table 2.5). Shoots may have dehydrated because the humidity of the microclimate was probably reduced by high Gelrite (4 $g.l^{-1}$) concentration in the medium. Less water was available to create high humidity since little or no condensation was observed on the sides of the larger vessels or on the surface of the medium. Usually, about 2 $g.l^{-1}$ Gelrite is used to solidy media for rooting of eucalypts, however, explants encountered hyperhydric transformation due to excess humidity (Le Roux and van Staden, 1991a; Jones and van Staden, 1994).

2.4.3 Genotypic responses

Although the culture conditions were the same, the percentage of rooting of clone GN107 was lower than that of GN121 (65% vs. 75%). This may be accounted for by the fact that clonal variation does occur. Some authors have reported similar variations in rooting efficiencies among *Eucalyptus nitens* clones and suggested that the effect of provenance on rooting ability of *in vitro*-produced shoots and macrocuttings was due to genetic differences (Willyams *et al.* (1992) and Tibbits *et al.* (1997). Nevertheless, the environmental or culture conditions appear to determine the limits within which rooting is achieved.

Three-week-old shoots derived from seedlings of pure *E. grandis* and *E. nitens* were rooted with the optimized protocol. The results showed that both pure species rooted efficiently (over 90%) (Table 2.10) (Fig. 2.9) with the optimized protocol, with no significant differences in rooting frequency between them. The question remains as to the reasons for the lower rooting rates of the hybrid clones.

Willyams *et al.* (1992) observed that with *E. nitens* rooting recalcitrance increased with age of shoots derived from seedlings. For example, rooting frequency of shoots after one week, four weeks and eight weeks after germination was 93%, 80% and 43%. Furthermore, *E. nitens* has generally been reported to root with difficulty both *in vitro* and *ex vitro* although 'good' rooting clones have been found in certain families (Rasmussen, 1991; Willyams *et al.*, 1992; Tibbits *et al.*, 1997). In contrast, it is also agreed that *E. grandis* shoots root with relative ease (Willyams *et al.*, 1992). Therefore, it is tempting to suggest that GN clones are recalcitrant to rooting because of some *E. nitens* parent genotypes. It is therefore suggested that before crosses are made for creating hybrids, the *in vitro* rooting ability of seedlings from parent genotypes must be screened under different culture conditions so that rooting ability is not 'lost', or lowered by the poor rooting parent.

The frequency of the protocol established in this study for two GN clones requires to be tested further on other cold-tolerant *E. grandis* clones. Adendorff and Schön (1991) suggested that the propagation of a clone with a poor root strike (percentage of successfully rooted cuttings) and poor root quality (structure and size of a root system) is uneconomical, irrespective of the yield potential of the clone.

2.4.4 Production of plantlets

At the end of *in vitro* rooting period, regenerated plantlets were hardened-off to greenhouse conditions and data were recorded after 28 days. The GN121 clone

rooted with the optimized protocol (Table 2.6) exhibited higher survival yields (78%) after hardening-off compared to GN107 clone (58%) (Table 2.9). Warrag *et al.* (1990) also observed clonal variations with regard to survival of regenerated *E. grandis* hybrids after hardening-off. The observed hardening-off success in this study could have been affected by factors such as roots being injured during the transfer process from tubes or by leaf-burn during the initial stages of hardening-off. Many authors recommended that plantlets should be transferred to *ex vitro* conditions as soon as a few roots are produced and variability between clones (with respect to rooting frequency and survival after hardening-off) should be considered (Durand-Cresswell *et al.*, 1982; McComb and Bennett, 1986; Warrag *et al.*, 1990; Thorpe *et al.*, 1991). Jones and van Staden (1994) reported in their study that the survival of plantlets of *E. grandis* x *urophylla* plantlets could have been reduced by callus formed at the base of stems, which inhibited normal water transport to the shoot.

Although some of the factors that may affect *ex vitro* establishment of micropropagules have been established (Section 2.1.3), few studies (about 10% of those reviewed in this study, e.g. Yang *et al.*, 1995) have reported on survival of plantlets after hardening-off; only the number of plantlets rooted *in vitro* is reported (e.g. Bennett *et al.*, 1992, 1994). This is unfortunate as the success of a micropropagation protocol should be judged on the number of plants that can be transferred to the field. Such protocols may not be of much benefit to the forestry industry which is more interested in the number of plantlets (trees) suitable for plantations. This could probably explain why micropropagation as a means of improving forestry productivity has been criticized and branded a waste of resources (Wilson, 1998).

In summary, multiple shoots of cold-tolerant *E. grandis* x *nitens* clones may be cultured successfully *in vitro*, although the rooting frequency is clone dependent. In a research environment, an average of ten shoots per bud explant was produced in four weeks and

these were subcultured once to produce 10 more shoots each. Therefore, in theory, about one million shoots may be generated from one axillary bud after seven subcultures. Of these, 75% (GN121) and 65% (GN107) can be rooted successfully *in vitro*. With the observed hardening-off success, this translates to about 585, 000 and 377, 000 plants, respectively, within one year. If the successes of the research environment can be transferred to the commercial laboratory, some of these plants can then be used to establish clonal hedges and others for afforestation in dry, cold marginal areas.

2.4.5 Towards an establishment of a non genotype-specific *in vitro* rooting protocol

The production of cold-tolerant clones of *E. grandis* x *nitens* on a commercial scale has been hampered by their poor rooting ability. Although considerable success was achieved in Chapter 2, clonal variations to the established protocol were observed (Tables 2.6 and 2.8). In order to circumvent genotype-related difficulties in the rooting ability of the two GN clones (GN121 and GN107), the objective of the present investigation was to establish a more general, non genotype-specific protocol involving transformation of micropropagated shoots with wild-type strains of *Agrobacterium rhizogenes*.

Chimeras produced with *A. rhizogenes* have been shown to have normal shoot growth, better stand establishment and showed increased biomass production in both the root and aerial parts when compared with control plants (e.g. *Prunus amygdalus* and *Olea europea*, Strobel *et al.* [Strobel and Nachmias, 1985; Strobel *et al.*, 1988], *Populus deltoides* x *nigra*, Charest *et al.*, 1992; and *Pinus nigra*, Milhajevic *et al.*, 1996). Since only the basal cells of the shoot are transformed, aberrant morphology of the plants is usually avoided (Sutter and Luza, 1993). Unfortunately, if roots result from transformed tissue, the improved rooting will not be carried to the later vegetative generations

produced by stem cuttings (Hatta *et al.*, 1996). Transformed roots can either be functionally and anatomically similar to normal untransformed roots (e.g. in *Daucus carrota*, Moore *et al.*, 1979) or abnormal (e.g. in *Malus pumila*, Sutter and Luza (1993)).

In the present study, virulent strains of *A. rhizogenes* from a series of 12 (mostly agropine) wild-type strains were selected using the carrot disc assay (Table 2.11). Only the HR1 and R1600 agropine-type strains produced roots on both sides of the carrot discs (Fig. 2.10) and appeared to be the most virulent strains (Table 2.11). Similar observations were reported previously for several agropine-type strains (e.g. Bercetche *et al.*, 1987) and factors affecting root induction on carrot discs by *A. rhizogenes* were discussed by Whiteman Runs Him *et al.* (1988). In this study, mannopine strains (such as TR7, 8196) produced either tumours/callus only on both apical and basal sides of carrot discs, or produced mostly tumours/callus and few roots on apical side only (Table 2.11). However, the observations mentioned above could have been affected by factors such as the genotype of the carrots and the pH of the medium (Whiteman Runs Him *et al.*, 1988) and are in agreement with what was previously reported by Vilaine and Casse-Delbart (1987). Those authors reported that on carrot discs (0.5 cm in thickness), wild-type agropine strains (A4 and HR1) were virulent on both apical (facing the root tip) and basal (facing the shoot) surfaces, while the root inducing ability of mannopine strain TR7 was limited to the apical surface. Similar observations were recorded by Cardarelli *et al.* (1987a) who suggested that the basal side of carrot discs was auxin-depleted and mannopine-type strains such as 8196 depend on plant auxin for the triggering of neoplastic growth.

Huang *et al.* (1991) suggested that compatibility between *A. rhizogenes* and host plants, sensitivity of the plant tissues to the T-DNA, phytohormone production, and juvenility of the host tissue are important factors in inoculation success and hairy root production. In this study, explants of GN121 and GN107 clones were obtained from greenhouse

plants and *in vitro* shoots, and were treated with five wild type strains of *A. rhizogenes* selected from the results of carrot disc assay. Leaf explants appeared to be susceptible to all five strains (HR1, R47, R1600, R1601 and TR8.3) (Fig. 2.13) and their most common response was to curl and produce tumours/callus from the cut-end of midrib (Fig. 2.13). The curling leaf response was similar to that observed on leaf discs of *Nicotiana glauca* inoculated with *A. tumefaciens* (strain LBA1010 (p35SGUS-INT)) (Mozo and Hooykaas, 1992). Leaf curling and tumour/callus production was more pronounced on explants inoculated with HR1 strain (Fig. 2.13) which also appeared to be more virulent on carrot discs (Table 2.11). On nodal cuttings, only tumours/calli were produced by the HR1 strain at all inoculated points (Fig. 2.11). Similar observations were reported by Charest *et al.* (1992) with strains A4 and 8196 on microcuttings of *Populus deltoides* x *nigra* and *P. nigra* x *maximowiczii*. Those authors concluded that production of tumours instead of roots by A4 and 8196 strains could indicate that in poplar there is a potential effect of T-DNA hormonal genes on endogenous hormonal balance. Machado *et al.* (1997) attributed tumour/callus formation on *Eucalyptus grandis* x *urophylla* seedling explants treated with agropine strains (A4, R1601, LBA9402) to increased levels of cytokinin at the site of inoculation; only the cucumopine strain (2659) produced dense hairy roots. Van Wordragen *et al.* (1992) reported that hairy roots were later produced from tumours/callus produced by LBA9402 on explants of *Denranthema grandiflora.*

Because transgenic roots were not obtained on explants of GN clones, the next step was to ascertain if both parent genotypes were susceptible to transformation by *A. rhizogenes*. Three-week-old de-rooted seedlings of *E. grandis* and *E. nitens* were agroinfected at stem bases (according to MacRae, 1991) (Section 2.2.8). Both genotypes appeared to be susceptible to infection but responded differently in terms of rooting frequency, although there were no significant differences with the controls (Table 2.12). In addition, roots produced on control and inoculated explants appeared hairy before explants were inserted into the medium (Section 2.3.4.3). Therefore, it is

suggested that although *A. rhizogenes* is capable of producing transgenic roots (usually confirmed by opine assay), the 'hairiness' of those roots could be attributed to the fact that roots were exposed to air and produced many secondary roots to increase surface area for absorption of moisture/water. The obvious sign of the transformed nature of those roots in this study was their fast growth, profuse branching and ability to grow in hormone-free medium (Figs. 2.14 and 2.15).

A variety of factors appear to affect transformation frequency of those species that have been successfully transformed with *A. rhizogenes*. In the study of MacRae (1991), seedlings of *Eucalyptus grandis, E. dunnii* and *E. nitens* were inoculated with wild-type strains of *A. rhizogenes* and strain LBA9402 was found to be most virulent (80% rooting) (Table 2.1). In this study, however, that strain was not very efficient on carrot discs, explants of GN clones and seedlings of *E. grandis* and *E. nitens* (Table 2.11, Fig. 2.10). MacRae (1991) also observed variations in the response of *Eucalyptus* species to bacterial strains and found that percentage of rooting was dependent on both species and the bacterium. Nester *et al.* (1984) suggested that different individuals of a single species can give different responses to a particular bacterial strain, and even different organs of a single plant can give different responses. Therefore, it is possible that results obtained in this study were different from those of McRae (1991) because seeds from different parent plants were used.

MacRae (1991) reported that transformed roots displayed the typical hairy root phenotype and grew rapidly in hormone-free medium (see also Machado *et al.*, 1997). Such high density of *E. grandis* transformed roots was not observed in this study (Fig. 2.15). This could be because of both transgenic and normal roots being produced as reported by Hatta *et al.* (1996) and Damiano and Monticelli (1998) for several fruit trees.

With seedlings of *Pinus nigra*, Milhajević *et al.* (1996) reported that the frequency of root formation was related to the age of seedlings inoculated. Those authors observed best results (75% rooting frequency) with the explants of eight week-old seedlings, compared to 2.7% and 0% for six and four-week-old seedlings. Successful transfomation or production of chimeras with eucalypts has generally been achieved when very young material from seedlings was used, as shown by MacRae (1991) and Chriqui *et al.* (1991) (Table 2.1). Chriqui *et al.* (1991) have shown that differentiated organs from mature plants as well as micropropagated explants of *Eucalyptus globulus* and *E. gunnii* never reacted whatever the strain (8196, 2659, 1855) used. Only young seedlings or organs excised from these seedlings were susceptible and gave rise to rhizogenic symptoms, the optimal responses being observed on 24 day-old plantlets. Those authors associated the loss of susceptibility of differentiated eucalypt tissue to the increased ability to produce polyphenolics after wounding and inoculation.

Sugars and phenolic compounds released from the plants and membrane-binding protein can differ according to plant genotypes, thus producing differing responses to *A. rhizogenes* (Damiano and Monticelli, 1998). In this study, leaf explants exuded phenolics into the medium and because these appeared to be oxidized (brownish exudate) by the time explants were co-cultured with bacterial cells, it is possible that oxidized phenolics were toxic (explants died ultimately) or did not attract bacteria. Further, is has been reported that once explants of eucalypts exude phenolics into the medium, most of them perish (Durand-Cresswell and Nitsch, 1977; Das and Mitra, 1990) or the wounded area becomes browned (George, 1993; Figs. 2.11 and 2.12), prohibiting the passage of the bacterial cells to 'living' tissue. In this study, oxidized phenolics probably led to the death of cells around wounded areas and could be responsible for blocking root development.

As previously mentioned, tissue transformed with *A. rhizogenes* appears to be more sensitive to auxin than untransformed tissue (Shen *et al.*, 1988, 1990). The presence of

IBA in the medium improved rooting frequency of *Juglans nigra* explants treated with *A. rhizogenes* (strain 1855) from 58.5% (no IBA) to 62.9% (with 2 $mg.l^{-1}$ IBA) although callus was produced in large amounts in the presence of that auxin (Caboni *et al.*, 1996). However, in the present study, the presence of IBA did not promote rooting but increased the size of the tumours/callus produced in its absence (Section 2.3.4.2). Even the presence of acetosyringone did not promote rooting by inducing the virulence of wild-type *A. rhizogenes* strains.

In summary, transgenic roots were not obtained on cold-tolerant clones of *Eucalyptus grandis* x *nitens* (GN121 and GN107). Based on the observed results (Fig. 2.11), it is assumed therefore that these clones were either transformed inefficiently or not at all by this system. The fact that tumours/calli were produced by the HR1 strain on hormone-free medium suggests that GN clones could be susceptible to some *A. rhizogenes* strains (Nilsson[3], pers. comm., 1999). One possibility is that the T-DNA was transferred into the plant cells (as observed by tumour/callus induction by HR1 strain) but was not successfully integrated into the host genome, or either it was transferred and integrated but not expressed (Walden, 1988). There is evidence to suggest that methylation of the DNA in transformed tissue can seriously affect its expression (Hepburn *et al.*, 1983). Van Wordragen *et al.* (1991) reported that in some cases the T-DNA might be imperfectly transferred to the host plant genome.

There are many possible reasons why the wild-type strains of *A. rhizogenes* failed to induce roots on explants of GN clones. The block in root induction may have resulted from either the inability of the bacterium to transfer its T-DNA to the plant cell or the plant cell not responding to changes in hormone level (Walden, 1988). Gheysen *et al.* (1987) suggested that host DNA synthesis is required for the integration of T-DNA, thus successful transformation may depend on the ability of the plant cell to carry out one or more cell cycles (Binns, 1990). According to Tinland (1996) certain plants (such

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as monocotyledons) may be difficult to transform with *Agrobacterium* because they are equipped with better proofreading systems that do not allow the stable entry of foreign DNA into their genomes, hence the process of T-DNA integration may be aborted. Furthermore, the wild-type strains tested could have diverged in the two laboratories (CSIR, Pretoria and University of Natal, Durban) and a mutation in the T_L region of agropine strains abolished their ability to induce root proliferation by itself (Vilaine and Casse-Delbart, 1987). Finally, the tested strains could have been weakly motile and acetosyringone did not elicite virulence of strains at the concentrations tested (Winans, 1992). Therefore, conditions that would favour successful transfer, integration and expression of the T-DNA of the pRi need to be established in order to produce chimeras of *E. grandis* x *nitens*.

CHAPTER 3: ASSESSMENT OF THE HYDRAULIC EFFICIENCY OF ROOTS PRODUCED BY *in vitro* VS. CUTTING PROPAGATION

3.1 LITERATURE REVIEW

Vegetative propagation of selected genotypes of *Eucalyptus* via cuttings and *in vitro* techniques is commonly used to establish clonal plantations because of rapid genetic gain and reduced variability among planted individuals (Yang *et al.*, 1995). The advantages and procedures for the clonal propagation of cold-tolerant clones of *Eucalyptus grandis* x *nitens* were discussed in the previous chapters. Although there have been a few field trials comparing cuttings with tissue culture-produced plants, not enough is known about properties of roots of plants propagated by those methods. To be of value, micropropagated trees should be similar or better physiologically than those derived from cuttings. In this regard, studies on hydraulic conductivity are important because differences in growth responses between macropropagated and micropropagated plants may be related to their ability to transport water from the roots to the shoot. Therefore, the objective of the present investigation was to contribute to the understanding of this issue by comparing root anatomy and hydraulic conductivity of the roots of clones GN121 and GN107 produced *in vitro* (Chapter 2) and from cuttings (provided by Mountain Home Laboratory, Mondi Forests).

3.1.1 The nature and origin of roots

Audus (1959) defined a root as a cylindrical organ, often branched, growing vertically or obliquely downwards into the substratum on which the plant lives, and serving thereby two important functions: (1) anchorage and support for the aerial portions of the plant; and (b) absorption of water and dissolved mineral salts which are essential for

continued plant growth. Hartmann and Kester (1975) divided the process of development of adventitious roots in stems of cuttings into three stages: (1) cellular differentiation followed by the initiation of groups of meristematic cells (the root initials); (2) the differentiation of these cell groups into recognizable root primordia; and (3) the growth and emergence of the new roots, including rupturing of other stem tissues, and the formation of vascular connections with the surrounding tissues of the cutting. Continuous sclerenchyma rings between the phloem and cortex exterior to the point of adventitious roots, may constitute an anatomical barrier to rooting. In a study of olive stem cuttings, such rings were associated with difficult-to-root cuttings, whereas those that were easy-to-root were characterized by discontinuities of the sclerenchyma (Clampi and Gellini, 1963, *loc.cit.* Hartmann and Kester, 1975). Biricolti *et al.* (1994) noticed pronounced changes in the stem of shoots of *Castanea sativa* x *crenata* during the initial stages of root development. Briefly, there was an interruption of the rhythmic sclerenchyma rings and their replacement by less differentiated structures, the transition of the medullary rays from uniseriate to multiseriate, and the exceptional increase of parenchyma tissues in the hyperplastic cortex. In woody perennial plants, where one or more layers of the secondary xylem are present, adventitious roots in stem cuttings usually originate in the young secondary phloem (Hartmann and Kester, 1975).

It is well known that *in vitro* microcuttings form adventitious roots in greater number and more quickly than *ex vitro* cuttings (McClelland *et al.*, 1990). However, it is unclear whether the majority of original *in vitro* roots are tenacious in the *ex vitro* environment, or are replaced by more competent *ex vitro* roots after transplanting (Torrey, 1986; McClelland *et al.*, 1990). Roots are highly variable; major differences are seen among species, habitats and even along the length of an individual root. This means that their ability to transport water may differ because of anatomical differences, therefore, results of hydraulic conductivity obtained with one experimental system cannot be automatically applied to all roots (Steudle and Peterson, 1998).

3.1.2 The hydraulic architecture of roots

The mechanism of sap ascent in the xylem was explained by the cohesion theory (Dixon and Joly, 1895; Tyree and Sperry, 1989; Tyree and Ewers, 1991; Milburn, 1996). Water ascending trees usually does so under negative pressure down a gradient of increasing negative pressure (Tyree and Ewers, 1991). The negative pressure is physically equivalent to a tension (a pulling force) transmitted to soil water via a continuous water column, and any break in the column necessarily disrupts water flow (Tyree and Sperry, 1989). Hydrogen bonding promotes cohesion between water molecules and allows water to remain liquid under tension (metastable state) (Tyree and Sperry, 1989; Milburn, 1996).

Under conditions of transpiration, water is not taken up actively by the roots, but instead it moves passively through the root in response to a water potential gradient set up by transpiration (Steudle and Peterson, 1998). The structure of the water conducting system of roots, i.e. the hydraulic architecture, influences water transport from the surface of the roots to the root xylem. Water moves through a series of tissues each with a hydraulic conductivity (i.e. the ability to conduct water) that can change with root development and with the availability of soil moisture (North and Nobel, 1996). By applying Ohm's law to water flow through the root cylinder of young maize root, Steudle and Peterson (1998) suggested that the water potential will drop along the different tissues which are arranged in series (epidermis, cortex, pericycle, parenchyma and tracheary element walls) because their resistances are additive.

Three main parallel pathways of water flow that play an important role during the passage of water across the different tissues have been distinguished (i.e. apoplastic, symplastic and transcellular paths) and these form the basis of the composite transpot model of water across roots (Peterson and Enstone, 1996; Steudle, 1997; Steudle and Heydt, 1997; Steudle and Peterson, 1998). Apoplastic transport occurs through the cell

walls, skirting the protoplasts of the cells; symplastic transport occurs when a substance already in the cytoplasm passes into the cytoplasm of a neighbouring cell through plamodesmata; and transcellular movement occurs when the substance passes through both the plasmalemma and tonoplast on its way through the cell, thus moving through the vacuole (Peterson and Enstone, 1996).

Root hydraulic conductivity for most plants is limited by radial conductivity of the tissues outside the xylem, although the xylem or axial conductivity can be limited near the root tip where conduits are immature (McCully and Canny, 1988; Frensch and Sperry, 1989). The development of suberin lamellae in the radial and transverse walls of the endodermal and exodermal cells (characterized by presence of the Casparian band) places two hydrophobic resistances in the transcellular path of water flow through cells (Peterson and Enstone, 1996). The function of a suberinized Casparian band is to channel some of the water movement from the apoplast into the symplast.

In roots undergoing secondary (thickening) growth, the endodermis loses its function as an apoplastic barrier but only after being replaced by a suberized periderm which can also influence water uptake (Moon *et al.*, 1986; McKenzie and Peterson, 1995). According to the composite transport model of Steudle and co-workers (Steudle *et al.*, 1987; Steudle *et al.*, 1993; Steudle and Frensch, 1996; Steudle and Heydt, 1997), the differences in the degree of suberization of the endo- and exodermis between species may account for the differences in hydraulic conductivity of their roots. The model further proposes that in transpiring plants with a demand for water from the shoot, root hydraulic conductance would be high (low hydraulic resistance), and there would be considerable apoplastic flow of water. At water shortages and low or zero rates of transpiration, the apoplastic path will be less used and flow would be largely osmotic which would require a high osmotic pressure in the root xylem to take up water. On the other hand, a high hydraulic resistance of the root would reduce water losses to a dry soil (Steudle and Heydt, 1997). Furthermore, the composite transport model assumes

that axial hydraulic resistances for primary roots of seedlings (e.g. maize roots) are small compared with radial resistances. However, according to the authors of the model, the approach is a good approximation for short roots but may be questioned for long roots or extended root systems, when the axial resistance is relatively high compared with the radial resistance (Steudle and Heydt, 1997).

Passage cells frequently occur in the endodermis and exodermis of roots and since they lack suberin lamellae, they would constitute a lower resistance pathway for water flow into the stele (Peterson and Enstone, 1996). Passage cells are limited to the zones of cell division, cell elongation and cell differentiation along the root, whereas cells in the zone of cell maturation cannot serve as passage cells because they have developed suberin lamellae (Peterson and Enstone, 1996). Along cell-to-cell path, water channels (aquaporins) in the plasma membrane play an important role in water transport. It is thought that aquaporins are highly selective for just water because of their narrow diameter (hydrophilic pore) that allows the passage of water molecules just one by one in a single file (Steudle and Heydt, 1997). Transport of water through the channels enhances the hydraulic conductivity of cell membranes several times, and it is thought to be purely passive flow following water potential gradients across the membranes (Steudle and Heydt, 1997). Since roots show considerable changes in cell and root hydraulic conductivity in response to environmental factors, Steudle and Heydt (1997) hypothesized that in addition to the apoplastic path, the cell-to-cell path for water could be controlled as well, namely by opening and closing of water channels. Steudle and Peterson (1998) further proposed that water channel activity accounts for 'fine adjustment' of water uptake under conditions of water shortages in young roots, or when the endo- and exodermis have suberin lamellae with the exception of passage cells.

3.1.3 Hydraulic conductivity

Vascular plants have evolved two types of highly modified cells, tracheids and vessel members, strands of which provide an axial pathway with an exceedingly low resistance to water flow. In the tracheids, this is accomplished by loss of protoplasts, and in some species degradation of the primary wall material in pits intervening between adjacent elements. In the case of vessel members, there is a similar loss of the protoplast and also a partial or complete removal of end walls between axially adjacent cells. These modifications reduce resistance to water flow (Steudle and Peterson, 1998).

The axial component within the lumena of tracheary elements is purely a bulk flow that is propelled by hydrostatic gradients set up by transpiration. In the root cylinder, the xylem acts as a duct that collects the water taken up radially and rapidly transfers it to the shoot. This however, requires that the axial hydraulic conductance is much larger than that of the radial pathway across the root cylinder (Steudle and Peterson, 1998). According to Poiseuille's law, the diameter of the conducting channel has a huge effect on its hydraulic conductance (which varies with its fourth power), and consequently the narrow diameters of the conduits offer considerable resistance to water flow (Zimmermann, 1983; Tyree and Ewers, 1991; Steudle and Peterson, 1998). According to February *et al.* (1996), vessel diameters and vessel element lengths generally decrease while vessel frequencies increase with increasing aridity.

Hydraulic conductivity is typically measured on excised xylem samples attached to a hydraulic system for measuring the pressure difference (ΔP) of a fluid (usually water) across the sample and the mass flow rate (m) through the sample (Sperry *et al.*, 1988). The pressure-flow relationship has been expressed in a variety of ways, the simplest being hydraulic conductivity (k_h), where $k_h = m/\Delta P$. In the case of roots, specific root mass hydraulic conductivity (K_h) can be compared amongst samples by expressing conductivity per unit mass or surface area ($K_h = m/(\Delta P/g)$ or $K_h = m/\Delta P/\text{area}$).

Data for hydraulic conductivity of roots of seedlings (especially primary maize roots) has been obtained by the root pressure probe technique (Rudinger *et al.*, 1994; Steudle and Meschcheryakov, 1996; Steudle and Heydt, 1997). With that method, root hydraulic conductivity was expressed on the basis of root surface area. However, Tyree *et al.* (1994b) cautioned that this approach is appropriate only if flow is approximately uniform over the whole root surface area and the main barrier to flow is at the outer surface. According to Ford and Harrison-Murray (1997), those conditions would not necessarily be satisfied by the newly formed adventitious root system of a cutting. For example, a tenuous vascular connection at the junction between roots and the stem might present a major resistance to flow. Furthermore, accurate measurements of the root surface are difficult (Ford and Harrison-Murray, 1997). Therefore, either root fresh mass or dry mass is used to express hydraulic conductivity of an adventitious root system.

For branched adventitious roots, the hydraulic method of Sperry *et al.* (1988) was modified by several authors (Kolb *et al.*, 1996; Ford and Harrison-Murray, 1997) and was deemed more reliable than other methods of measuring hydraulic conductivity by Tyree and Sperry (1989). Branched root systems have lower conductances than their unbranched counterparts, and because it is easier to measure flow from one entry point, vacuum pressure instead of above-atmospheric pressure is normally used to drive the flow (Kolb *et al.*, 1996; Ford and Harrison-Murray, 1997). The use of vacuum pressure allows for a maximum pressure difference (and increased flow) with less danger of refilling embolized conduits in the process (Kolb *et al.*, 1996). According to Ford and Harrison-Murray (1997), a potential problem with vacuum-type measurement is the formation of air bubbles in the water emerging from the cut stem, which may lead to erroneously high estimates of water flux. Under hand-lens magnification, those authors observed bubbles originating from the cortical regions of the stem, suggesting that pressurizing the root system might be causing infiltration of water into the intercellular air spaces of the root cortex, such that displaced air was being forced out through the

cortex at the cut end of the system. Ford and Harrison-Murray (1997) noticed that "decorticating" about 1 cm of the stem before attaching the silicone tubing to the root system reduced the air bubble problem significantly.

The junctions of tree branches in gymnosperms and some angiosperms were reported to be hydraulically constricted (Tyree *et al.*, 1983; Ewers *et al.*, 1989). The function of this hydraulic segmentation would be to favour the main pathway over any side branches (Zimmermann, 1983). Therefore, it is possible that the junction of the adventitious roots and the stem represent an area of high resistance to water flow. In times of stress when water is limiting, secondary and tertiary roots may be sacrificed first according to the hydraulic segmentation hypothesis (Tyree and Ewers, 1991). Although micropropagated eucalypts produce more adventitious roots and thus grow faster for the first few years, filed studies have shown that cutting-derived plants catch up in height after a few years (Watt *et al.*, 1995; Yang *et al.*, 1995). It is possible that the rate of growth in micropropagated plants is slowed down or reduced because of the increasing hydraulic resistance at the shoot-root junction with increasing height, since more roots are connected to the stem than in cutting-derived plants.

3.1.4 Vulnerability to cavitation

The occurrence of cavitation and subsequent embolism in the xylem decreases hydraulic conductance and may diminish the ability of a plant to maintain water transport and carbon uptake (Zimmermann, 1983; Tyree and Ewers, 1991). Cavitation occurs when a void of sufficient radius forms in water held under negative pressure in the xylem conduits. It results in a vapour–filled xylem conduit that gradually becomes air-filled as air diffuses in from surrounding tissues (Tyree and Ewers, 1991; Tyree *et al.*, 1994a). These air-filled or embolized conduits are incapable of water transport and reduce the overall conductance of the xylem (Kolb *et al.*, 1996) until water potential returns to near atmospheric pressure for the air bubbles to dissolve (Tyree and Ewers, 1991). As

opposed to the radial hydraulic resistance discussed previously, the magnitude of axial hydraulic resistance appears to depend on the number of xylem vessels affected by embolism. Surface tension usually makes the void/water interface stop at the pit membranes between adjacent conduits and prevents its from advancing to adjacent conduits (Tyree and Ewers, 1991). At water potential near zero, surface tension can raise the pressure of the air bubble above atmospheric pressure, causing the embolism to dissolve over a period of days (Tyree and Sperry, 1989).

Four mechanism were proposed for the nucleation of cavitation in xylem vessels, and the most widely accepted is the air-seeding hypothesis of Zimmermann (1983) (see review by Tyree *et al.*,1994a). According to the hypothesis, air seeding occurs when an air bubble is sucked into a water-filled lumen via a pore from adjacent air spaces. Embolisms are the natural consequence of foliar abscission, herbivory, wind damage and other mechanical fates that might befall a plant (Tyree *et al.*, 1994a). The literature does not provide a solid indication of whether roots are equally susceptible to cavitation like shoots, nor does it show whether the water potential of the roots and shoots are equal or similar.

A correlation exists between large conduits and vulnerability to cavitation within an individual, but it does not necessarily hold among species (Tyree and Sperry, 1989). Increasing the permeability of pits to air by changing the surface tension of the xylem sap, or by other means (e.g. pathogens) also increases the vulnerability of the xylem to embolism. Thus rather than conduit diameter, it is pit membrane pore diameter that determines a conduit's vulnerability; the larger the pore, the more vulnerable the conduit (Tyree and Sperry, 1989). According to Tyree and Sperry (1989), there are trade-offs between vulnerability to cavitation and vessel size: smaller pores confer resistance to cavitation and on the other hand, they may reduce the hydraulic conductivity of the xylem.

In essence, the hydraulic conductivity of the xylem of excised plant segments is measured before and after removal of air embolism by high-pressure flushes (e.g. 175 kPa) (Sperry *et al.*, 1988). Xylem embolism is then quantified by expressing the initial conductivity as a percentage of the maximum obtained after flow impeding air emboli have been removed. In this way, the cumulative effect of all cavitations that have occurred (and not been repaired) is measured (Tyree and Sperry, 1989). Reduction in the conductivity of the roots is visualized by determining a "vulnerability curve", which is a plot of the percent loss in hydraulic conductivity versus the xylem pressure potential required to induce that loss (Sperry *et al.*, 1988; Tyree and Ewers, 1991; Kolb *et al.*, 1996). Although this is a destructive method of cavitation assessment, it simply involves the measurement of the hydraulic conductivity of excised root or shoot segments dehydrated to known water potentials, before and after the removal of air embolisms by a high pressure treatment (Kolb *et al.*, 1996). Measurements of the vulnerability curves of roots and the hydraulic architecture can provide valuable insight into the possible drought resistance of trees and limitations imposed on the species by environmental stresses (Tyree and Ewers, 1991).

3.1.5 Aim of this investigation

The present investigation was focused on assessing the anatomy and hydraulic conductivity of roots produced by *in vitro* vs. cutting propagation. Root anatomy of micropropagted plants was determined before and six months after hardening-off and of cuttings after six months. Hydraulic conductivity and the vulnerability of cavitation were determined following dehydration to known water potentials.

3.2 MATERIALS AND METHODS

3.2.1 Root hydraulic conductivity

a) *Measurement apparatus*

The hydraulic conductance of adventitious roots was measured according to Kolb *et al.* (1996). Plants were prepared for hydraulic measurements by de-topping explants outside water to avoid re-imbibing dehydrated material. The root system with the original potting medium left undisturbed was turned up side down and the stem stump immediately submerged in water. The second cut was then made under water about 2 cm below the first. The stump was then fitted via tubing to a supply of filtered (0.22 μm), degassed and acidified distilled water (pH 2, 32% (v/v) HCl). This filtered, low pH solution was reported to inhibit microbial growth within the tubing system that can lead to clogging of the xylem during conductance measurements (Sperry *et al.*, 1988). Tests have shown no influence of that solution on hydraulic conductance compared with distilled water or organic acid solutions (Sperry and Saliendra, 1994). The water was degassed to minimize the formation of air bubbles and filtered to prevent particulate matter from blocking the conduits (Sperry *et al.*, 1988; vander Willigen, 1996).

The end of the stem stump was wrapped with seal tape and inserted into plastic tubing connected to a water reservoir (i.e. a volumetric flask half-filled with filtered, acidified distilled water) (Fig. 3.1). The undisturbed adventitious root system was then placed in a 9 x 50 cm polyvinyl chloride (PVC) pipe (pressure chamber) capped at both ends by removable Plexiglas lids. The lids were provided with holes; for fitting the pressure gauge at one end of the chamber and for the proximal end of the stem stump to protrude at the other end. The stump was then pushed through a rubber stopper to provide an airtight seal after it was connected to the tubing. The water reservoir was placed on a balance connected to a computer. The surface of the water reservoir was placed about 25 cm below the pressure chamber to ensure no gravity-induced flow into the adventitious root system.

Distal end of the tubing was attached to a 10 ml pipette (bent at 90°) dipped into the water reservoir (Fig. 3.1).

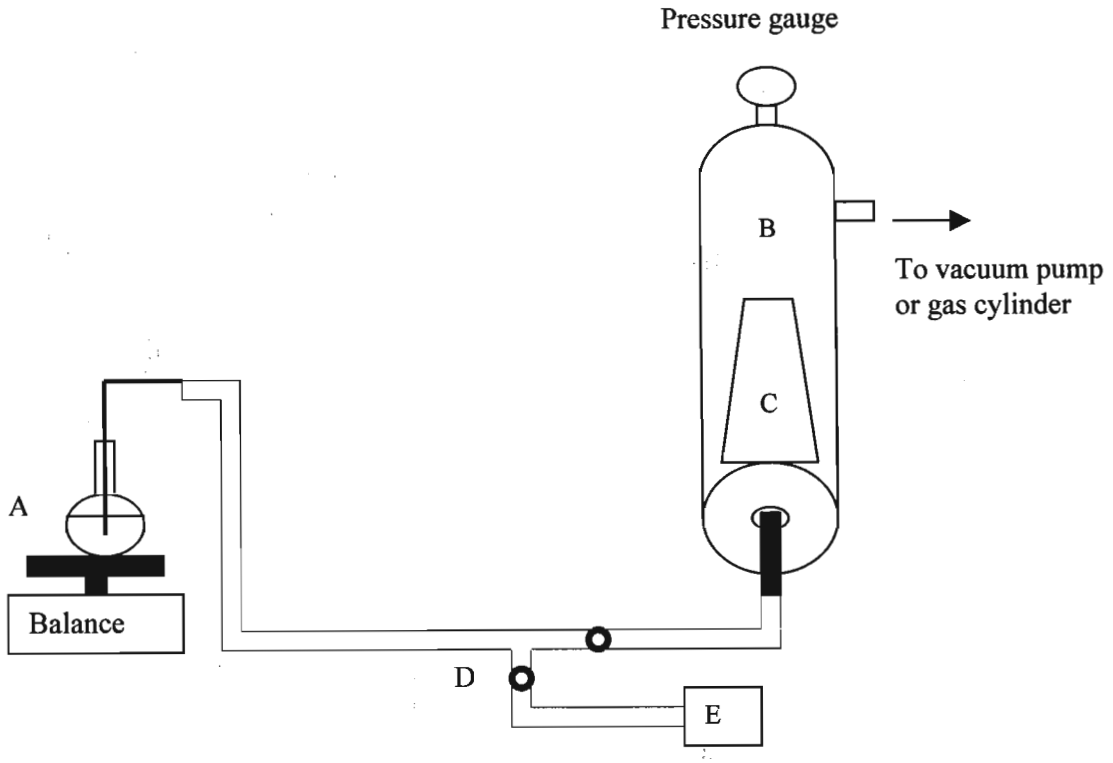


Fig. 3.1: The apparatus used for measuring the hydraulic conductance of undisturbed potted roots. Solution was supplied to the roots from a reservoir (A) located on a balance (which was interfaced with a computer). Roots were enclosed in a PVC pipe (B) with their original pot (C) and vacuum/flushing pressure applied. A two-way valve (D) was used to control the supply of solution from the reservoir or from a 20 ml syringe (E) which was used to refill the reservoir and also to release any air bubbles trapped within the system.

Flow through the xylem was induced by vacuum pressure at 40 kPa and the slope of the curve (flow rate in $g.s^{-1}$) prior to removal of emboli provided initial hydraulic conductance (k_i). After measuring k_i the pressure chamber was half-filled with distilled water, which was then forced under a 160 kPa pressure (provided by compressed air from a gas cylinder) through the xylem in the direction opposite to normal flow for 30 minutes. Following removal of emboli, a fresh cut was made across the stem stump and any bubbles introduced into the tubing were released before reconnection of the tubing to the stump under water. Flow rate was then measured and the slope of the curve after all emboli had been removed provided the maximum hydraulic conductance (k_m). After measuring k_m the supporting medium was carefully washed from the roots with minimum damage and the wet masses were determined.

Representative plots of hydraulic conductance for *in vitro* and cutting-derived adventitious roots were determined using non-stressed plants (water potential less than 100 kPa) across a vacuum pressure difference (40, 60, 80 and 100 kPa). At the end of each initial and final measurement, the tubing was allowed to relax for 10 minutes to return to its pre-pressurized state because under vacuum conditions, the walls of the tubing may be slightly compressed and when pressurized they may expand.

b) *Conductivity calculations*

Roots, with some of the potting material still attached were oven dried at 80°C for 48 hours to a constant dry mass (in grams). Thereafter, potting debris was removed and the actual dry mass was determined by subtracting the mass of potting debris. Specific root mass hydraulic conductivity (k_r) was then determined by dividing the hydraulic conductance by the root dry mass. The leaf area of the whole shoot was measured using a Cl-251 Leaf Area Meter (CID, Inc., Vancouver, Canada) and used to determine leaf specific conductivity.

The following formulae were used to calculate hydraulic conductivity:

Hydraulic conductivity (k_h) = flow rate ($g.s^{-1}$)/pressure difference (kPa)
Specific root mass hydraulic conductivity (k_r) = k_h (in $g.kPa^{-1}.s^{-1}$)/root dry mass (g)

The leaf specific conductivity was calculated by dividing hydraulic conductivity ($g.kPa^{-1}.s^{-1}$) by the leaf area (m^{-2}) supplied by those roots.

3.2.2 Plant material

Four-week old rooted cuttings of GN107 clone were obtained from Mountain Home Laboratory, Mondi Forests. These had been rooted under misthouse conditions in 55 cm^3 inserts containing a mixture of 1 vermiculite : 3 perlite (v/v). Micropropagated plants of GN121 and GN107 clones (Chapter 2) were hardened-off in 250 cm^3 pots containing a potting mixture of 1 river sand : 9 pine bark (v/v). All plants were watered daily and sprayed with fungicides and fertilizers weekly (Section 2.2.1). Plants propagated by the two methods were grown for six months in the greenhouse in original containers before hydraulic characteristics were studied.

Before measurements of hydraulic conductance, two preliminary tests were conducted to ascertain if cuttings were properly sealed at the base of the stem. Stems of four cuttings were trimmed approximately 7 cm from the root system. Two of these were joined through the stump to tubing connected to a gas (air) cylinder. The supporting medium was gently rinsed from root system (with minimal damage) which was subsequently submerged in a beaker filled with water. Air was forced at 200 kPa for 30 minutes through the cut end of the stem stump and the base of the stem was observed for release of air bubbles. After that period, no bubbling occurred. The other two cuttings were used in the second test, where a solution of safranin (0.05% w/v) was forced through the roots from the cut-end of the stump at 200 kPa for 1½ hours. Cross sections of the stem stump and roots revealed safranin only in the root and stem xylem above the rooting zone but not below that zone.

These experiments confirmed that cuttings were properly sealed and water flow would be through the roots.

3.2.3 Loss of hydraulic conductivity

Experiments were conducted in summer when temperatures in the greenhouse reached 35°C during the day. Twenty to twenty-five explants were selected at random, watering was withheld and the plants allowed to dehydrate over a period of two to three days in the greenhouse. At two-hour intervals during that period, two plants were tightly enveloped in plastic bags and transferred to the laboratory where they were incubated in the dark for 24 hours to allow water to equilibrate throughout the shoot and root systems. The shoot system was covered in a 14 x 17 cm bag and the whole plant with its insert/pot enveloped in a 22 x 30 cm bag. The stems were then excised, water potential measured using a pressure chamber and k_i and k_{max} determined as described above.

Vulnerability calculations

The percentage loss of hydraulic conductivity (PLC) was then calculated as

$$PLC = 100\ (k_{max} - k_i)/k_{max}$$

where k_{max} and k_i represent maximum and initial hydraulic conductance, respectively.

Measurements were conducted until all the leaves had dried and that occurred by the end of day two for cuttings and day three for *in vitro*-produced plants. Vulnerability curves were constructed by plotting the loss of hydraulic conductance against water potential. Each point on the curves represented a separate individual plant.

3.2.4 Root anatomy

a) *Wax embedding*

Root anatomy of micropropagated GN107 and GN121 clones was studied before and six months after hardening-off. The anatomy of roots from cuttings of GN107 clone was examined after six months of growth in the greenhouse. Samples of root midsections and root tips (± 1 cm in length) were excised and fixed in formalin : acetic acid : alcohol (FAA) for 24 hours (Appendix). They were then dehydrated in a series of butanol:ethanol:water, thereafter taken through wax/butanol solutions. Samples were incubated overnight in Paraplast paraffin wax (100%) then embedded into moulds in fresh wax.

b) *Sectioning and staining*

Embedded samples were sectioned at 10 – 15 µm with a rotary microtome (AO 820, American Optical, Buffalo, NY, USA), adhered to slides pretreated with Haupt's adhesive and double stained with 1% (w/v) safranin and 0.5% (w/v) fast-green both in 70% (v/v) ethanol (Appendix). Sections were cleared in xylene and coverslips were mounted with DPX (Unilab Saarchem, South Africa) for light microscopy.

c) *Light microscopy and Photography*

Slides of cross sections of samples were viewed under a Wild M3 stereomicroscope and recorded using a Wild Photoautomat MPS55 system.

3.3 RESULTS AND DISCUSSION

3.3.1 Hydraulic characteristics

Figure 3.2 is a representative plot of the vacuum pressure flow relationship before and after the removal of emboli. A straight line was fitted through the data by linear regression (R^2 values ranged from 0.88 to 0.99 for *in vitro*-produced roots and from 0.86 to 0.99 for those of cuttings), and the slope of the line was used as an estimate of the root system hydraulic conductance. The initial hydraulic conductance (k_i) of a cutting-derived root system (A) was 5.0 x 10^{-5} $g.s^{-1}.kPa^{-1}$ and of an *in vitro*-derived root system (B) was 10 x 10^{-5} $g.s^{-1}.kPa^{-1}$. The maximum hydraulic conductances (k_m) after removal of all emboli were 8.0 x 10^{-5} and 25 x 10^{-5} $g.s^{-1}.kPa^{-1}$ for cutting- and *in vitro*-derived roots respectively. The root systems were 38% (A) and 60% (B) embolized.

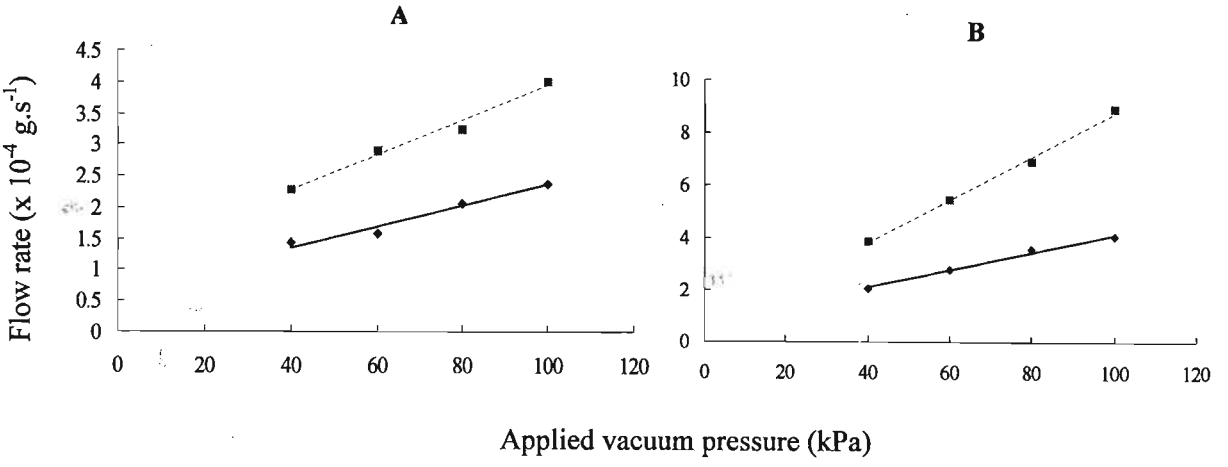


Fig. 3.2: Specimen graph showing the rate of water flow through the root systems of cuttings (A) and *in vitro*-produced roots (B) of GN107 clone before (♦) and after flushing (■) with filtered, acidified distilled water.

Figure 3.2 indicates that after a 30-minutes flushing with filtered, acidified distilled water, the slope of the maximum hydraulic conductance for *in vitro*-produced roots was

closer to a zero intercept than the slope of the initial conductance, suggesting that most of the native embolisms have been dissolved. Zimmermann (1983) suggested three causes of the initial non-zero intercept: osmotic uptake of water by the symplast, gradual dissolution of air emboli in xylem conduits, and capillary uptake of water in intercellular space. In any case, flushing the xylem with solution would eliminate or reduce the intercept (Kolb *et al.*, 1996). However, the maximum hydraulic conductance of roots of cuttings was further from zero than the initial conductance, suggesting that flushing increased hydraulic resistance or some xylem vessels were somehow blocked as a result of flushing, even though the perfusing solution was filtered. This observation may suggest an experimental error or that roots of cuttings require higher pressures during flushing to dissolve embolisms.

It was surprising that roots of fully hydrated plants showed a 38% and 60% reduction in xylem conductance. A similar observation was reported by Kolb *et al.* (1996) for seedling roots of a desert shrub (*Artemisia tridentata*), and those authors suggested that perhaps root xylem was more vulnerable to embolism than stem xylem. Therefore, the water potential of shoots of well-hydrated plants (< 100 kPa) may not necessarily reflect the water potential status of the roots even when transpiration is minimized by covering the plants in plastic. With eucalypt trees, February *et al.* (1995) reported that xylem vessel diameters tend to be greater in roots than stems and greater in the stem than branches. Hence, roots may be more efficient in transporting water than stems and branches.

At any rate, *in vitro*-produced roots were capable of transporting about twice the amount of water to the shoot (Fig. 3.3). Specific root mass hydraulic conductivity (k_r) and leaf specific conductivity (k_l) were calculated by dividing the hydraulic conductivity by the root dry mass (in grams) and leaf area (in m^2), respectively. Values for *in vitro*-produced roots were found to be significantly higher (Student *t*-test, $P \leq 0.05$), compared with those of cuttings (Fig. 3.3) and such differences were attributed to

differences in leaf areas (1: 2, cuttings : *in vitro*) and dry root masses (1: 3, cuttings : *in vitro*). In some studies, root fresh mass was found useful for expressing hydraulic conductivity of roots because of a significant increase in hydraulic conductivity with an increase in root wet mass (e.g. in *Corylus maxima and Weigela florida*, Ford and Harrison-Murray, 1997). However, fresh root mass may not be reliable because it depends on factors such as root density, amount of water absorbed by the roots and other soil debris attached to the roots. In the present study, larger (dry) root mass of *in vitro*-produced roots resulted in consistently high specific hydraulic conductivity (k_r) values (Fig. 3.3).

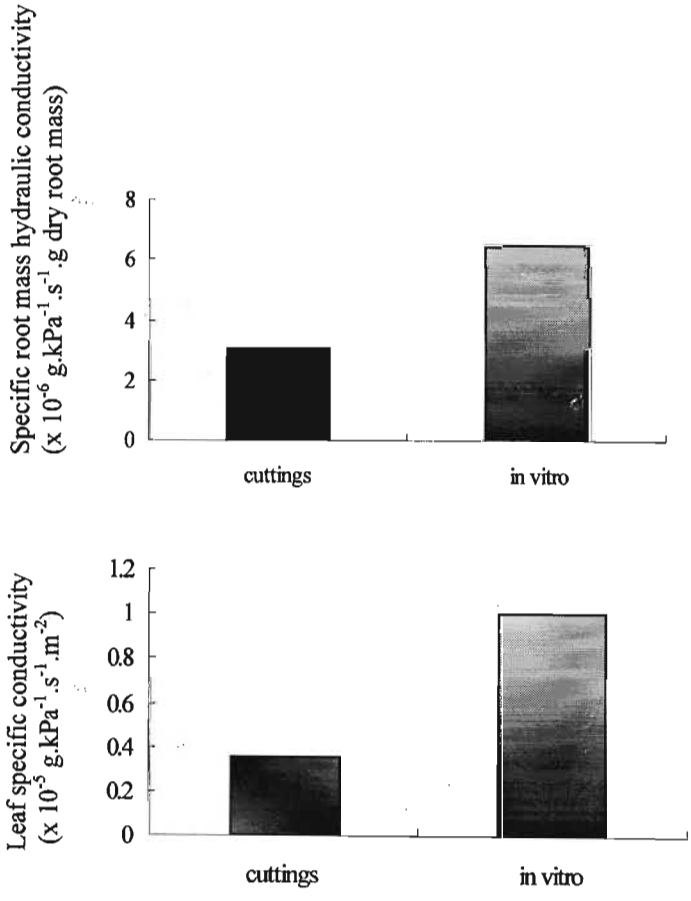


Fig. 3.3: Some hydraulic characteristics of six month-old adventitious roots produced *in vitro* and from cuttings of GN107 clone.

3.3.2 Vulnerability to cavitation

The vulnerability of roots to cavitation was measured as the percent loss of hydraulic conductivity with decreasing water potential (Fig. 3.4). Both types of roots appeared vulnerable to cavitation at low water potentials regardless of the significant differences in root mass. Further, there appeared to be no significant differences between the curves (Fig. 3.4). This suggests that conductivity is lost over a narrow range of water potentials for adventitious roots of clone GN107. The lack of any differences in the vulnerability of the xylem to cavitation between cutting-derived and *in vitro*-produced roots further suggests that even though the former had a greater xylem area than the latter (Fig. 3.6), this was not large enough to result in any change in vulnerability. Although, there are some reservations about the data presented in Figure 3.4, it came as no surprise that conductivity of the roots was reduced at low water potentials since well-hydrated roots showed high percentages of embolism at low shoot water potentials (Fig. 3.2).

As mentioned previously, one of reservations concerns the water potential of the roots dehydrated to 'known' water potentials. As shown in Figure 3.4 some root systems at low water potentials (< 50 kPa) experienced up to 70% loss of hydraulic conductivity. This is in contrast to what was previously observed for shoots obtained from field-grown and greenhouse-grown cuttings of *Eucalyptus grandis* hybrid clones (February *et al.*, 1995; vander Willigen, 1996). Therefore, it is possible that the water potential of the roots in this study was not the same as the water potential of the shoot after 24 hours of equilibration. However, since the root system has to be at lower water potential than the shoot system for water to flow into root cells, it is tempting to agree with Kolb *et al.*'s (1996) suggestion that roots may be more vulnerable to xylem dysfunctions (such as embolism) than shoots.

On the other hand, it may be possible that a 40 kPa vacuum pressure facilitated refilling of embolized vessels, thus reducing the difference between the initial and maximum hydraulic conductivities. This in turn would have resulted in high PLC values at low water potentials. The suggestion is supported by a previous report of Cochard *et al.* (1994). Those authors showed that with culms of *Rhipidocladum racemoflorum*, high vacuum pressures (> 2 kPa) refilled embolized vessels.

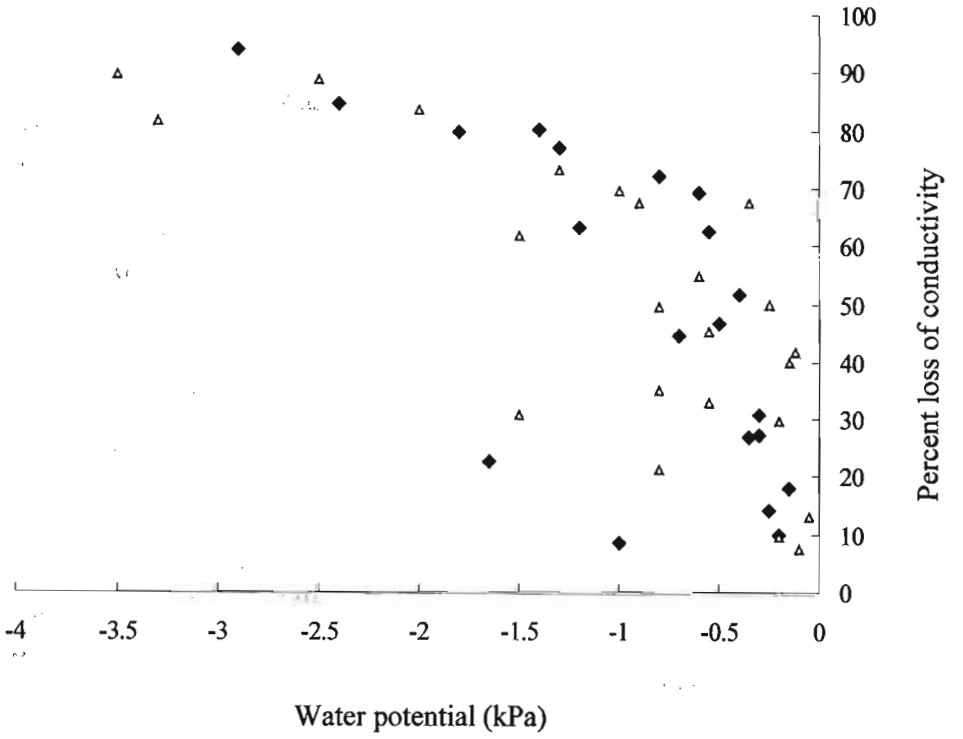


Fig. 3.4: Vulnerability curves of adventitious roots of clone GN107 using the method of Kolb *et al.* (1996). Roots produced *in vitro* (♦) and from cuttings (Δ) were allowed to dehydrate over two to three days whilst data was recorded.

The pathway for water flow through the roots involves two components, radial (root surface to xylem) and axial (longitudinal in the xylem). Working with two desert

succulents (*Agave desertii* and *Ferocactus acanthodes*), North *et al.* (1992) found that embolisms caused by dehydration reduced the axial conductance at the junction of the main root-lateral root whereas the radial conductivity increased at those areas. The stellar anatomy at main root-lateral root junctions was found to be different from that in a main root or a lateral root. For example, in nonjunction regions, xylem vessels were at least surrounded by relatively thin walled parenchyma cells whereas the proximal tissue of the lateral root at the junction consisted of thick, suberized and lignified phloem cells. (North *et al.*, 1992). Therefore, it is possible that following dehydration, the overall hydraulic conductivity of roots is reduced depending on the degree of increased water potential caused by a decrease in axial conductivity. Further, North *et al.* (1992) proposed that anatomical changes accompany an increase in radial conductivity during drying. Those authors found that for *A. desertii*, the thin walled parenchyma cells between the thickened, sclerified layers of the main root and the lateral root may have lost turgor and separated during drying, thereby opening a channel for more water movement from the root surface to the stele. For the present investigation, the above observation could imply that flushing with an acidic filtered solution significantly increased the flow rate because the passage of water was increased when certain cells collapsed during drying, considering the juvenile age of those roots.

Xylem pressure potentials of the roots may differ from those of shoot systems because of the existence of root pressure in some plant species. Root pressure is a force developed in the root cells when there is excess water in the soil and quantities have been moving into the root cells (Wilson *et al.*, 1971; Dittmer, 1972). However, fast-growing and efficient ground-water consuming crops such as eucalypts may not exhibit root pressure. In species that exhibit this phenomenon (e.g. *Rhipidocladum racemiflora*, Cochard *et al.*, 1994), root pressure may occasionally be useful in forcing air out of xylem vessels and re-establishing broken water columns, but is certainly of no value in lifting quantities of water in transpiring plants (Wilson *et al.*, 1971; Dittmer, 1972).

3.3.3 Relationship between hydraulic conductance and physical status of roots

In the present investigation, root hydraulic conductance was measured on intact, undisturbed roots of clone GN107 propagated *in vitro* and through cuttings, by placing the root system with its original container into the pressure chamber. As shown earlier (Fig. 3.2), a line drawn through the data points was not as straight as in those studies whereby roots were excavated and washed free of soil before determination of hydraulic conductance (Kolb *et al.*, 1996; Ford and Harrison-Murray, 1997). Ford and Harrison-Murray (1997) noted that when hydraulic conductance was measured on an undisturbed root system of *Corylus maxima*, a plot of hydraulic conductance against root fresh mass was more scattered than when soil was washed-off the roots. However, there was no significant difference between the slopes when both sets of data were modelled by linear regression constrained through the origin.

Kolb *et al.* (1996) removed the root tips of seedlings of *Artemisia tridentata* prior to assessment of hydraulic conductance in order to expose the xylem at both ends of the flow path so that flow could be measured in parallel to all severed root tips. In that study, it was not reported whether removal of root tips had a significant increase in hydraulic conductance but authors agreed that it may at least be difficult to remove all root tips of a branched root system or at most impossible to make comparisons between root systems. Ford and Harrison-Murray (1997) reported that excision of root tips of *Cotylus maxima* led to an initial increase in hydraulic conductance but the rate of flow decreased with time presumably because of rapid blocking of the conduits by micro-organisms and particles entering the cut. Hence any broken roots were sealed with molten lanolin. According to those authors, the effect of excising the root tips indicated that radial resistance in intact roots provided the major barrier to water flow. Roots of trees have been suggested to be less permeable to sap than those of herbaceous crops probably due a higher degree of suberization of the endo- and exodermis as well as the periderm (Steudle and Heydt, 1997). Steudle and co-workers (Frensch and Steudle,

1989; Steudle and Peterson, 1998) demonstrated experimentally that in young maize roots it is the radial rather than the axial resistances that limit water uptake. Therefore, removal of the root tips may provide incorrect hydraulic conductivity of roots because conductivity depends on both radial and axial water flow.

3.3.4 Root external morphology and anatomy

In vitro-propagated plants of clone GN107 (and GN121) hardened-off in 250 cm^3 pots containing 1 river sand : 9 pine bark, produced more roots than cuttings in 55 cm^3 inserts containing 1 vermiculite : 3 perlite after six months (Fig. 3.5). On average, cuttings produced one thick main root and a few fine roots (smaller root volume) a short distance above the stem base, whereas *in vitro*-propagated plants produced over three thick main roots from the stem base and more fine roots (larger root volume) (Fig. 3.5). Most roots produced from *in vitro*-propagated plants appear to have been produced *ex vitro* because an average number of two roots were produced *in vitro* after 28 days of rooting (Table 2.8). For the sake of clarity and distinction between roots originally produced *ex vitro* from cuttings, those roots of *in vitro*-propagated plants will hereafter be referred to as '*in vitro*-produced' roots.

The different types of supporting medium must have affected the number and size of roots produced. Initial attempts to harden-off *in vitro*-propagated plantlets in 55 cm^3 inserts containing 1 vermiculite : 3 perlite resulted in 100% mortality rate, probably because of rapid loss of moisture from the supporting medium. Differences between the two types of roots were also observed in the root tips. The root tips of cuttings appeared 'swollen' and sausage shaped probably because they were exposed outside the support medium, whereas those of *in vitro*-propagated plants were normally tapered and did not grow out of drainage holes of containers (Fig. 3.5).

The rooting habit of *Eucalyptus* has an important bearing on the ability of many members of the genus to grow aggressively and survive in drought conditions where less productive species may fall (Adlard, 1987; Mueller-Dombois, 1992). In the present investigation, plants propagated by cuttings and *in vitro* methods were far smaller than their field-grown counterparts at six months, and it was assumed that their growth had been slowed down once the roots became pot-bound. Schuch and Pittenger (1996) reported that that plants of *Eucalyptus citriodora* growing in tall (10 x 40 cm in height) vs. regular (16 x 17.5 cm in height) containers had 58% more root dry weight and 39% more shoot dry weight three months after transplanting. Therefore, the small containers used in this study to grow cutting-derived and *in vitro*-produced plants could have impeded growth and extension of the adventitious roots.

Fig. 3.5: Root morphology of cuttings (left) and *in vitro*-propagated plants (right) of clone GN107 after six months of growth in 55 cm^3 and 250 cm^3 containers. Bar = 1.7 cm.

A direct, comprehensive morphological or anatomical comparison of the two types of roots (i.e. *in vitro*- and cutting-produced roots) after transplanting and hardening-off was first reported by McClelland *et al.* (1990) with *Acer rubrum*, *Betula nigra* and

Malus x *domestica*. In the present investigation, 10 – 15 μm sections of roots of clones GN121 and GN107 were double stained with safranin and fastgreen (Fig. 3.6). After 28 days of *in-vitro* rooting, roots of both clones appeared polyarch, i.e. primary xylem differentiated with arms (> 4) radiating from a common center like points of a star; GN121 had 12 and GN107 had six protoxylem elements. In addition, the epidermis of GN107 comprised of two layers of cells, whereas GN121 had a single layer of epidermal cells (Fig. 3.6). The cortical cells of clone GN121 were much more uniform and compactly organized than those of GN107 clone. However, those cells of GN107 clone could have been damaged during sectioning, as the specimen could have been improperly infiltrated with wax or sectioned at a different angle.

Material for examining the anatomy of GN107 cuttings 28 days after rooting and also of GN121 cuttings (28 day-old and six-month-old) was not available. Therefore, for clone GN107, lateral roots were sectioned because they represent the youngest part of the root (Fig. 3.6). The anatomy of lateral roots resembled that of *in vitro* roots of GN107 clone in terms of being polyarch, and the cortical cells were also not as compact as those of clone GN121. McClelland *et al.* (1990) observed anatomical differences between *in vitro*-produced and *ex vitro*-produced roots of *Acer rubrum* during the first 4 – 5 weeks after root initiation, however, after 16 - 20 weeks *in vitro* and *ex vitro* roots had acquired similar characters such as a greater proportion of vascular tissue relative to the root cross-section.

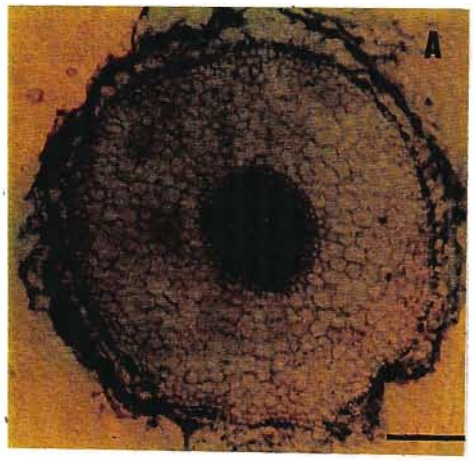
After six months of growth in the greenhouse, there were no traces of cortical cells and roots appeared matured with late metaxylem vessels (Fig. 3.6). The bark of the root (i.e. the periderm plus phloem) was thicker in *in vitro*-produced roots than those derived from cuttings, and was made up of square and rectangular cells. Cells of the phellogen (one of three layers of periderm) were identified by dark coloured substances frequently identified as tannins (Cuttler *et al.*, 1987). The uniseriate medullary rays were well developed especially in the *in vitro*-produced roots, and appeared heterocellular, i.e.

cells were rectangular in the periderm and square to oval in the xylem. In both types of roots, the xylem area was made up of wide solitary vessels arranged rows, but the total xylem area was smaller in the *in vitro*-produced roots than in those produced from cuttings (Fig. 3.6). Similar observations were recorded by McClelland *et al.* (1990).

The larger xylem area of roots of cuttings suggests that they may be more physiologically mature than their *in vitro*-produced counterparts since a larger proportion of mature eucalypt dry mass is made up of wood. Xylem vessel frequency and size were not measured in this study but from Figure 3.6 it appears that there are no significant differences in the size of xylem vessels between the two types of roots (i.e. cutting and *in vitro*-produced) although the xylem vessel frequencies may be different.

Differences were also observed in the staining reactions of the two types of roots. Roots produced from cuttings absorbed more fast-green in the periderm than their *in vitro*-produced counterparts. This observation suggests that there may be differences in the chemical composition of roots at that juvenile age. For example, phloroglucinol staining confirmed a higher lignin composition for *ex vitro* root sections of *Acer rubrum* than *in vitro*-produced sections after five weeks of hardening-off, probably due to their more advanced vascular development (McClelland *et al.*, 1990).

Fig. 3.6: Root anatomy of GN121 and GN107 roots produced *in vitro* and from cuttings. (A) cross-section of a 28 day-old *in vitro*-produced root of GN121 clone showing 12 protoxylem elements surrounded by the pericycle and compact cortical cells; (B) cross-section of (A) after six months, note the thick root bark; (C) cross-section of a 28 day-old *in vitro*-produced root of GN107 clone showing six protoxylem elements and a double layer of epidermal cells; (D) cross-section of (C) after six months, note the developing lateral root; (E) cross-section of a lateral root of a six-month-old GN107 cutting-derived root; and (F) cross-section of a GN107 cutting-derived root after six months, note large xylem area compared to (B) and (D). Bar = 200 μm.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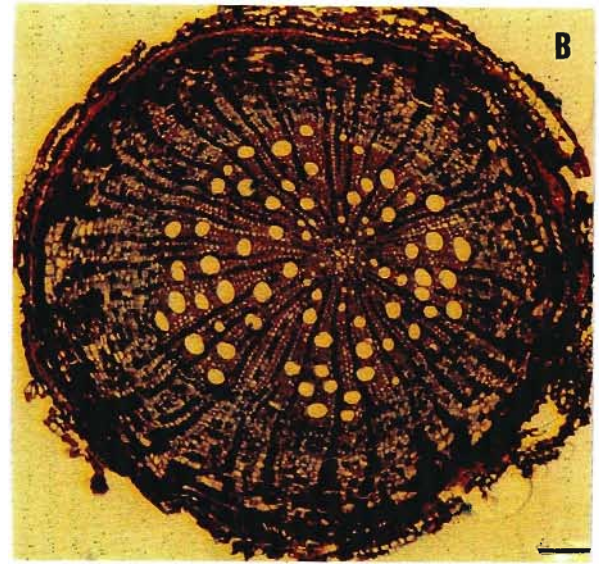
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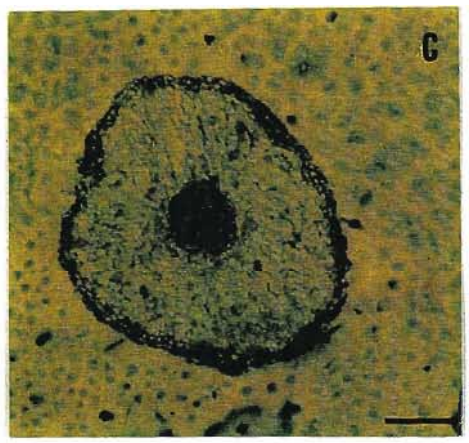
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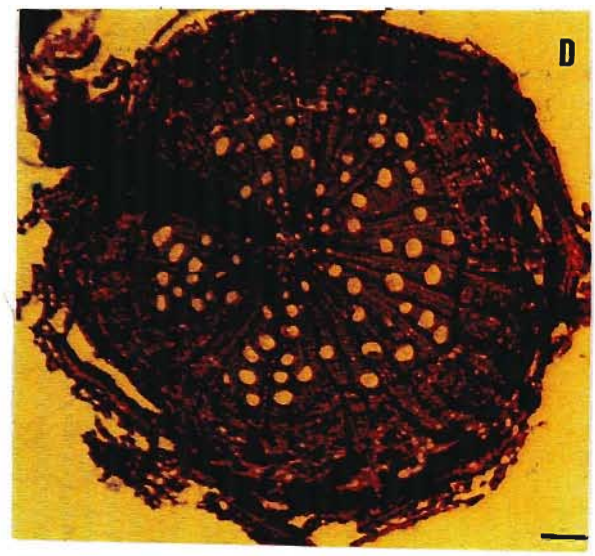
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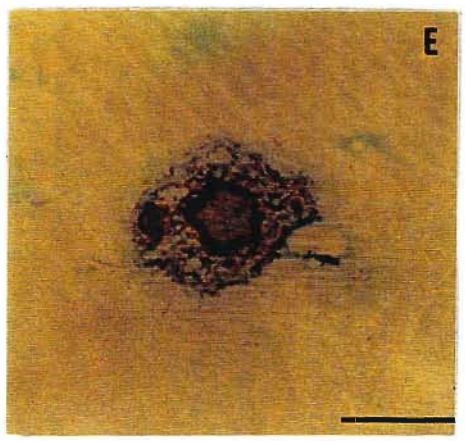
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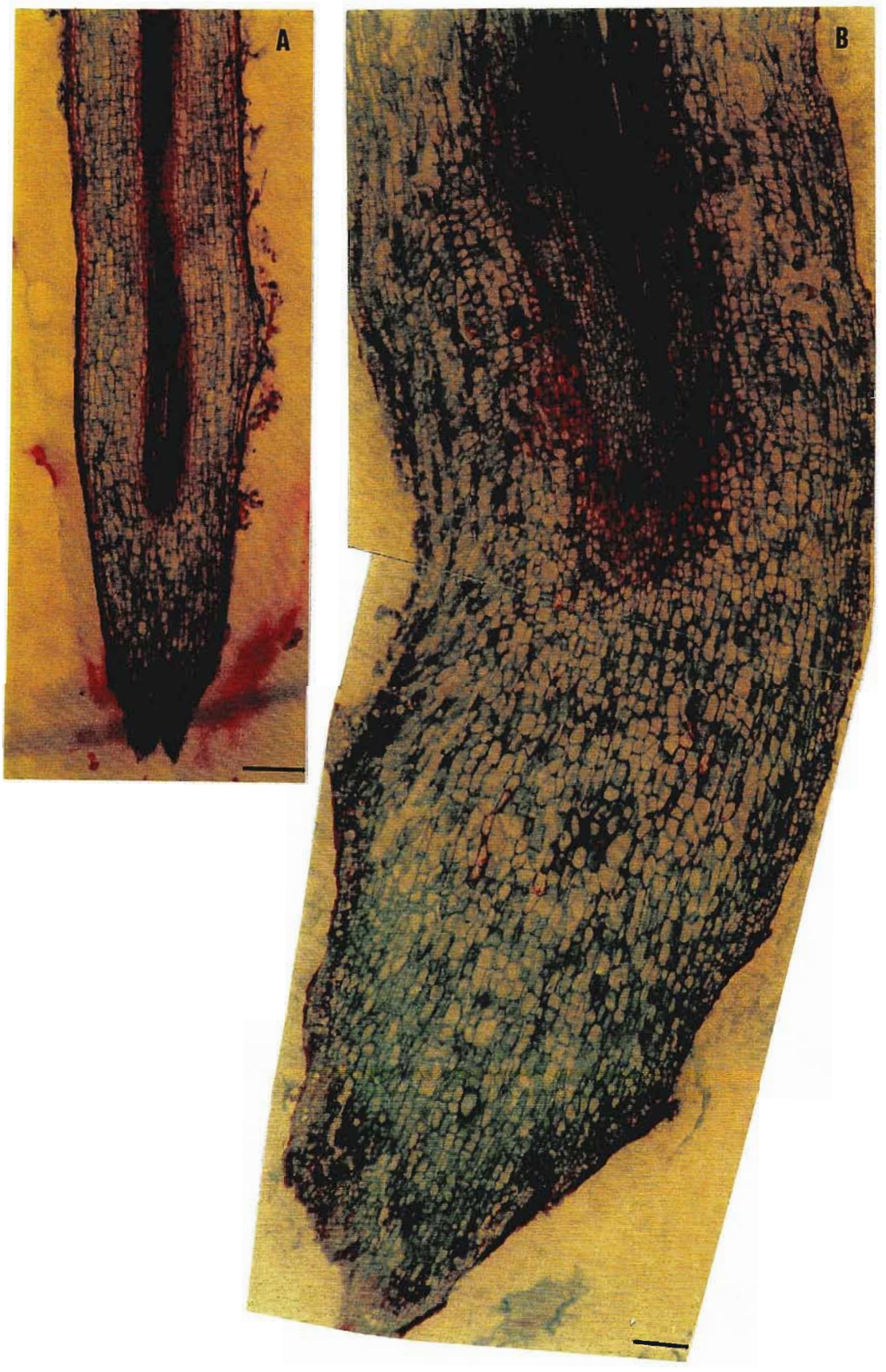
F

Although the tips of roots of cuttings were morphologically different from those of *in vitro*-produced roots after six months (Fig. 3.5), there were no anatomical differences with regards to tissue organization (data not shown). Further, there were also no significant differences between 28 day-old *in vitro*-produced root tips and six month-old root tips from cuttings (Fig. 3.7). In roots of cuttings, isodiametric cells in the meristematic zone of the root tip were the only cells stained with safranin, probably because they were more actively dividing than cells at any part of the root. However, the whole vascular area of *in vitro*-produced roots 28 days after rooting was stained with safranin (Fig. 3.7) and that difference was probably related to the physiological age of the roots.

Fig. 3.7: A comparison of root tips of clone GN107 after 28 days of *in vitro* rooting (A) and of cuttings at six months (B). In (A), the whole vascular area was stained with safranin whereas in (B), only cells in meristematic tip were stained with safranin. Bar = 208 μm.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B

In summary, the hydraulic characteristics of adventitious roots propagated *in vitro* and through cuttings of a clone of *Eucalyptus grandis* x *nitens* (GN107) were investigated in this preliminary study. The loss of hydraulic conductivity at low water potentials may suggest that roots are more vulnerable to cavitation than shoots, or may suggest that the experimental method caused the hydraulic conductivity to increase. Therefore, future research is aimed at modifying the technique of measuring the hydraulic conductivity of roots. Further, those factors that were not taken into account, such as the size of potting containers and potting mixture will also be considered.

This preliminary study has shown that i*n vitro*-propagated plants may be able to grow faster than those derived form cuttings initially, because they are capable of conducting about twice the amount of water to the shoot than cuttings. Observations were related to differences in root architecture, which was in turn probably determined by the size of the potting container and the supporting medium.

Differences were also observed in the staining reactions between cutting-derived and *in vitro*-produced roots. As mentioned previously, this may suggest differences in presence and/or level of secondary metabolites at that juvenile age.

Finally, roots are difficult to study; perhaps this is why this field of hydraulic architecture and drought tolerance is often overlooked (vander Willigen, 1996). As previously suggested by vander Willigen (1996), detailed hydraulic maps would provide a more extensive information on the hydraulic and vulnerability segmentation of *Eucalyptus grandis* hybrids.

CHAPTER 4: CONCLUDING REMARKS AND FUTURE RESEARCH STRATEGIES

The achievements of this work and proposed future research are summarized in Table 4.1.

4.1 Production of GN clones for commercial plantations

An efficient *in vitro* rooting protocol was established in this study for two cold-tolerant clones of *Eucalyptus grandis* x *nitens* GN121 and GN107). Of all the parameters tested, rooting was affected most by a step-wise increase in light intensity and temperature. The level of macronutrients (Ca^{2+} and Mg^{2+}) in the rooting medium also played a significant role especially in preventing callus formation at basal ends of shoots. With the established *in vitro* rooting protocol and subsequent hardening-off success (Tables 2.6, 2.8 and 2.9), over 500, 000 (GN121) and 300, 000 (GN107) plants can be produced from one axillary bud within one year. Such results are good, considering the low success obtained by others working with cuttings.

Clonal variations were observed in percentages of rooting and hardening-off rates (Tables 2.6, 2.8 and 2.9). It is suggested therefore, that the established *in vitro* rooting protocol should be tested on other GN clones for its applicability on a wide range of genotypes, particularly commercially important ones. In this manner, superior selected genotypes can be multiplied and integrated into forestry clonal and production programmes. Tissue culture may be used in clonal programmes to increase plant output for planting in dry, cold marginal areas or improve productivity of existing plantations.

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

Protocol	Results
Establishment of an *in vitro* rooting protocol	
- Modification of medium and culture conditions.	Achieved (Section 2.3.2.2)
- *Agrobacterium rhizogenes*-mediated root induction on GN clones.	Not yet achieved (Section 2.3.4.2), ongoing investigations
Outstanding work	
- Construction of a root-inducing vector with a disarmed supervirulent strain of *A. tumefaciens*.	Proposed future research.
- Establishment of a root-induction protocol with a modified *A. tumefaciens* (carrying the pRi) for micropropagated shoots and cuttings.	
Comparison of roots from macro- and microcuttings	
- Hydraulic conductivity.	Preliminary study completed. Protocol requires modification. (Section 3.3.1)
- Root anatomy.	Preliminary study completed. (Section 3.3.4). More detailed investigations required.
Outstanding work (with all root types)	
- Determination of hydraulic conductivity of roots grown under identical conditions.	Proposed future research.
- Anatomy and hydraulic characteristics of transformed roots once protocol is established.	
- Measurement of xylem area, frequency and size.	
- Investigations into different staining reactions.	

4.2 Progress towards the production of *E. grandis* x *nitens* chimeras

Transformation work on GN clones with wild-type strains of *Agrobacterium rhizogenes* was not successful. Wild-type strains that produced tumours and transgenic roots on carrot discs (Table 2.11, Fig. 2.10) and shoot explants of pure *E. grandis* and *E. nitens* shoots (from seedlings) (Table 2.12) did not produce roots on GN clones. However, the results with HR1 strain indicated that some transformation events occurred on GN leaf and nodal explants. Tumours/calli were produced in the absence of growth regulators. Although it could be argued that such neoplastic growths could have been caused by plant cytokinins (Section 2.4), the fact that they were not produced on the controls (Fig. 2.11) suggests the involvement of the root inducing plasmid (pRi). Towards improving the *in vitro* rooting ability of *Prunus padus*, Grant[4] (pers.comm. 1999) transformed that species with the AUX1 gene (a putative cellular auxin influx carrier) from *Arapidopsis thaliana*. Unfortunately, in that study, transformants rooted poorly compared with their control counterparts and that observation was attributed to the 35S CaMV (cauliflower mosaic virus) promoter which may have interfered with gene expression. Hence, it may be necessary to use plant tissue-specific promoters (such as those occurring on the plasmid DNA of *Agrobacterium* species) to regulate expression of genes that could improve rooting frequency.

In the present study, a more detailed investigation is required to establish why the T-DNA, if it was transferred into the plant cell, was either not integrated into the host genome or not expressed. It may be possible that cold-tolerant clones of *E. grandis* could be more susceptible to wild-type strains of *A. tumefaciens* than to *A. rhizogenes* since the former has the widest host range. Puonti-Kaerlas *et al.* (1989) showed that supervirulent strains of *A. tumefaciens* can be disarmed and genes of interest can be placed between the 25 bp borders. Therefore, by replacing the tumour-inducing T-DNA of *A. tumefaciens* (e.g. C58 strain) with root-inducing T-DNA of *A. rhizogenes* (e.g.

[4] Grant, Plant Genetics and Biotechnology, Horticulture Research International, Wellesbourne, Warwirk, Warwickshire UK

HR1 strain), successful non genotype-specific root induction may be achieved. Such an approach is planned for future transformation work with *Agrobacteria*, and should it be successful, the anatomy and hydraulic characteristics of those transgenic roots will be studied and compared with those of other *in vitro*-produced (Chapter 2) and cutting-derived roots (Chapter 3).

Of interest, studies have shown that plants with only a transformed root system (i.e. chimeras) produce a much higher biomass than their control counterparts (e.g. Strobel and Nachmias, 1985; Strobel *et al.*, 1988), a character much appreciated in the forestry industry (Hammatt, 1992).

4.3 Methods of vegetative propagation and hydraulic characteristics of roots

The hydraulic properties of roots from cuttings and micropropagated shoots were compared after six months of growth in the greenhouse. Both types of roots showed vulnerability to cavitation at high water potentials. The observations made in the present investigation suggest that roots produced *in vitro* and *ex vitro* from cuttings are equally vulnerable to drought induced cavitation, regardless of the significant differences in root mass. However, studies need to be undertaken on roots grown under the same conditions (e.g. size of pots and potting medium) and for this reason, hardening-off conditions need to be investigated.

Since root anatomical changes have been suggested to accompany drying (North *et al.*, 1992), it may be important to investigate if such changes occur in GN clones. Roots of micropropagated GN clones as well as those derived from cuttings appeared to have already undergone secondary growth after six months (Fig. 3.6). This may contribute towards an explanation of the fast growth of GN clones.

Field trials will also be conducted to determine the maximum hydraulic conductivity as well as vulnerability to cavitation of roots in situ, in both 'wet' and 'dry' treatments. Towards a much broader understanding of the physiological function of roots, one of the objectives planned for future research is to study the application of the hydraulic segmentation hypothesis (Tyree and Ewers, 1991) to adventitious roots of GN clones propagated *in vitro* (through tissue culture and by *Agrobacterium rhizogenes*) and *ex vitro* via cuttings. The other objective is to investigate solute transport in those roots. For example, roots would be grown in a solution of radioactively labeled potassium solution and the pathway of that nutrient traced and quantified throughout the roots.

These studies will aim to contribute to the understanding of root physiology in *Eucalyptus* trees and whether or not roots produced by different propagation methods have significant physiological differences that may have economical implications for the industry.

CHAPTER 5: APPENDIX

5.1 Wax embedding method

Fixation

Fix root samples (± 1cm in length) in approximately 2 – 3 ml of formalin : acetic acid : alcohol (FAA) for 24 hours at room temperature.

Ethanol (95%)	50 ml
Acetic acid (glacial)	5 ml
Formalin (37 – 40% formaldehyde)	10 ml
Distilled water	35 ml

Dehydration

Dehydrate samples in a butanol : ethanol : water series. Use 2 - 3 ml of mixture at each step.

Butanol	Ethanol (95%)	Water	Time (min)
10	20	70	30
15	25	60	30
25	30	45	30
40	30	30	30
55	25	20	45
70	20	10	60
85	15	0	90
100	0	0	overnight

Embedding

Embed samples in paraffin wax-melting point 52°C.

Take samples through a series of wax/butanol (w/w) mixtures. Use enough mixture to cover samples.

Wax	Butanol	Time (hour)
25	75	2
50	50	3
75	25	3
100	0	overnight

Replace 100% wax after overnight incubation with fresh molten wax (100%) before proceeding with mould preparation.

Moulds

Melt wax to about 65 – 70°C (waterbath usually most convenient), pour into moulds and position sample at the base of the mould with a pair of forceps while is still molten (speed is essential as wax hardens rapidly). Place a paper label with the sample identification on the surface of the wax and allow to solidify at room temperature. Store at room temperature in an airtight container.

Sectioning

Pre-treatment of microscope slides with an adhesive preparation is required. Place one drop of Haupt's Adhesive on the surface of a clean glass slide and smear thinly across the slide. Allow to dry. When sections are obtained, float on the surface of a drop of water on the slide. Flatten the sections by placing the slide on a hot tray. Do not allow wax to melt. Remove slide from hot tray and allow to dry overnight before staining.

Haupt's Adhesive:	gelatin	1g
	water	100 ml
	glycerol	15 ml
	phenol crystals	2g

Mix the gelatin with the water and melt in a waterbath at 30°C. Add the glycerol and phenol and mix together.

5.2 Staining procedure

Safranin/fast green for wax- embedded samples

1.	Xylene	2 min.
2.	Xylene	30 sec.
3.	Xylene/ethanol (1:1, v/v)	1 min.
4.	95% ethanol	30 sec.
5.	70% ethanol	30 min.
6.	1% safranin in 70% ethanol	15 min.
7.	95% ethanol	30 min.
8.	Absolute ethanol	1min.
9.	Absolute ethanol	1 min.
10.	Xylene/ethanol (1:1, v/v)	1 min.
11.	0.5% fast green in xylene/ethanol	10 sec.
12.	Xylene/ethanol (1:1, v/v)	30 sec.
13.	Xylene	1 min.

Allow to dry and mount with DPX.

CHAPTER 6: LITERATURE CITED

ADENDORFF, M.W. AND P.P SCHÖN. 1991. Root strike and root quality: the key to commercial success. In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 30 - 38.

ADLARD, P.G. 1987. Review of the ecological effects of *Eucalyptus*. Oxford Forestry Institute. pp. 1 – 59. (prepared for Wiggins Teape, Butler's Court, Beaconsfield).

ALBRIGHT, L.M., M.F. YANOFSKY, R. LE ROUX, D. MA AND E.W. NESTER. 1987. Processing of the T-DNA of *Agrobacterium tumefaciens* generates border nicks and linear, single stranded T-DNA. *Journal of Bacteriology* 169: 1046 – 1055.

ALTMAN, A. AND P.F. WAREING. 1975. The effect of IAA on sugar accumulation and basipetal transport of ^{14}C-labelled assimilation in relation to root formation in *Phaseolus vulgaris* cuttings. *Physiologia Plantarum* 33: 32 – 38.

AMMIRATO, P.V. 1986. Control of morphogenesis in culture. In: Withers, L.A. and P.G. Anderson (eds.). Plant Tissue Culture and its Agricultural Applications. Butterworths, Durban. pp. 23 – 45.

ANON. 1998. Envirotech: Forestry and Environment, Vol. 6. No.1. March 1998. McBean, B.S. (ed.). pp. 3 – 6.

ARMSTRONG, A.J., G. BENN, A.E. BOWLAND, P.S. GOODMAN, D.N. JOHNSON, A.H. MADDOCK AND C.R. SCOTT-SHAW. 1998. Plantation forestry in South Africa and its impact on biodiversity. *Southern African Forestry Journal* 182: 59 – 65.

AUDUS, L.J. 1959. Plant growth substances. Plant Science Monographs. Leonard Hill [Books] Limited, London. pp. 126- 157.

BAADSMAND, S. AND A.S. ANDERSEN. 1984. Transport and accumulation of indole-3-acetic acid in pea under two levels of irradiance. *Physiologia Plantarum* 61: 107 – 113.

BAJAJ, Y.P.S. 1986. Biotechnology of tree improvement and biomass energy production. In: Bajaj, Y.P.S. (ed.). Biotechnology in Agriculture and Forestry, Vol. 1: Trees 1. Springler-Verlag Berlin Heidelberg. pp. 1 – 13.

BANDYOPADHYAY, S., K CANE, G. RASMUSSEN AND J.D. HAMILL. 1999. Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species – *Eucalyptus nitens* and *E. globulus*. *Plant Science* 140: 189 – 198.

BARALDI, B., G. BERTAZZA, S. PREDIERI AND J.D. COHEN. 1993. Uptake and metabolism of indole-3-butyric acid during the *in vitro* rooting phase in pear cultivars (*Pyrus communis*). *Acta Horticulturae* 329: 289 – 291.

BARWALE, U.B. AND J.M. WIDHOLM. 1987. Somaclonal variation in plants regenerated from cultures of soybean. *Plant Cell Reports* 6: 365 – 368.

BEADLE, N.C.W. 1981. The vegetation of Australia. In: Walter, H. and S.W. Breckle (eds.). Vol. IV of Vegetation Monographs of the Earth. Fisher-Verlag, Stugart. p. 60.

BENNETT, J.I. AND J.A. MCCOMB. 1982. Propagation of Jarrah (*Eucalyptus marginata*) by organ and tissue culture. *Australian Forest Research* 12: 121 – 127.

BENNETT, I.J., J.A. MCCOMB, C.M. TONKIN AND D.A.J. MCDAVID. 1992. Effect of cytokinins on multiplication and rooting of *Eucalyptus globulus* and other *Eucalyptus* species. In: AFOCEL/IUFRO Symposium 'Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species'. September, 1992. Bordeaux, France. pp. 195 - 202.

BENNETT, I.J., J.A. MCCOMB, C.M. TONKIN AND D.A.J. MCDAVID. 1994. Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globulus* Labill. *Annals of Botany* 74: 53 – 58.

BERCETCHE, J., D. CHRIQUI, S. ADAM AND C. DAVID. 1987. Morphogenic and cellular reorientations induced by *Agrobacterium rhizogenes* (strains 1855, 2659 and 8196) on carrot, pea and tobacco. *Plant Science* 52: 195 - 210.

BERTAZZA, G., R. BARALDI AND S. PREDIERI. 1995. Light effects on *in vitro* rooting of pear cultivars of different rhizogenic ability. *Plant Cell, Tissue and Organ Culture* 41: 139 – 143.

BINNS, A.N. 1990. *Agrobacterium*-mediated gene delivery and the biology of host range limitations. *Physiologia Plantarum* 79: 135 – 139.

BIONDI, S. AND T.A. THORPE. 1981. Clonal propagation of forest tree species. In: Rao, A.N.(ed.). Proceedings of the COSTED Symposium on Tissue Culture, Singapore. pp. 197 – 204.

BIRICOLTI, S., A. FABBRI, F. FERRINI AND P.L. PISANI. 1994. Adventitious rooting in chestnut: an anatomical investigation. *Scientia Horticulturae* 59: 197 – 205.

BLAŹKOVÁ, A., B. SOTTA, H. TRANVAN, R. MALDINEY, M. BONNET, J. EINHORN, L. KERHOAS AND E. MIGINIAC. 1997. Auxin metabolism and rooting in young and mature clones of *Sequoia sempervirens*. *Physiologia Plantarum* 99: 73 – 80.

BLOMSTEDT, C., J. CAMERON, P. WHITEMAN AND S.F. CHANDER. 1991. Micropropagation of juvenile *Eucalyptus regnans* (Mountain Ash). *Australian Journal of Botany* 39: 179 – 186.

BOLLMARK, M., B. KUBAT AND L. ELIASSON. 1988. Variation in endogenous cytokinin content during adventitious root formation in pea cuttings. *Journal of Plant Physiology* 132: 262 – 265.

BRASILEIRO, A.C.M., J-C. LEPLÉ, J. MUZZIN, D. OUNNOUGHI, M-F. MICHEL AND L. JOUANIN. 1991. An alternative approach for gene transfer in trees using wild-type *Agrobacterium* strains. *Plant Molecular Biology* 17: 441- 452

BRESSAN, P.H., Y.J. KIM, S.E. HYNDMAN, P.M. HASEWAGA AND R.A. BRESSAN.1982. Factors affecting *in vitro* propagation of rose. *Journal of the American Society of Horticultural Science* 107: 979 – 990.

BURDON, R.G. 1988. When is cloning on an operational scale appropriate. Proceedings "Breeding Tropical Trees", IUFRO, Pataya, Thailand. In: Denison, N.P. and J.E. Kietzka. 1993. The use and importance of hybrid intensive forestry in South Africa. *South African Forestry Journal* 165: 55 – 61.

BURGER, D.W. 1987. *In vitro* micropropagation of *Eucalyptus sideroxylon*. *Horticultural Science* 22: 496 – 497.

CABONI, E., P. LAURI, M. TONELLI, G. FALASCA AND C. DAMIANO. 1996. Root induction by *Agrobacterium rhizogenes* in walnut. *Plant Science* 118: 203 - 208.

CAPONE, I., L. SPANÒ AND M. CARDARELLI. 1989. Induction and growth properties of carrot roots with different complements of *Agrobacterium rhizogenes* T-DNA. *Plant Molecular Biology* 13: 43 – 52.

CARDARELLI, M., L. SPANÒ, D. MARIOTTI, M.L. MAURO, M.A. VAN SLUYS AND P. COSTANTINO. 1987a. The role of auxin in hairy root induction. *Molecular and General Genetics.* 208: 457 – 463.

CARDARELLI, M., D. MARIOTTI, M. POMPONI, L. SPANÒ, I. CAPONE AND P. COSTANTINO. 1987b. *Agrobacterium rhizogenes* T-DNA genes capable of inducing hairy root phenotype. *Molecular and General Genetics.* 209: 475 – 480.

CARTER, A. AND M. SLEE. 1991. Propagation media and rooting cuttings of *Eucalyptus grandis.* In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 58 - 63.

CHALUPA, V. 1987. Temperature. In: Bonga, J.M. and D.J. Durzan (eds.). Cell and Tissue Culture in Forestry, Vol. 1. Martinus Nijhoff Publishers. pp. 142 – 151.

CHAREST, P.J., D. STEWART AND P.L. BUDICKY. 1992. Root induction in hybrid poplar by *Agrobacterium* genetic transformation. *Canadian Journal of Forest Research* 22: 1832 – 1837.

CHENG, B., C.M. PETERSON AND R.J. MITCHELL. 1992. The role of sucrose, auxin and explant source on *in vitro* rooting of seedling explants of *Eucalyptus sideroxylon. Plant Science* 87: 207 – 214.

CHRIQUI, D., S. ADAM, J-C. CAISSARD, M. NOIN AND A. AZMI. 1991. Shoot regeneration and *Agrobacterium* - mediated transformation of *Eucalyptus globulus* and *E. gunnii.* In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 70 - 80.

COCHARD, H., F.W. EWERS AND M.T.TYREE. 1994. Water relations of a tropical vine-like bamboo (*Rhipidocladum racemiflorum*): root pressures, vulnerability to cavitation and seasonal changes in embolism. *Journal of Experimental Botany* 45: 1085 – 1089.

COHEN, D. 1986. The influence of explant source on the establishment of plant tissue cultures. *New Zealand Journal of Technology* 2: 95 – 97.

CONSTANTINE, D.R. 1986. Micropropagation in the commercial environment. In: Withers, L.A. and P.G. Alders. Plant Tissue Culture and its Agricultural Applications. Butterworths, Durban. pp.175 – 186.

CREASY, L.L. 1968. The increase in phenylalanine ammonialyase activity in strawberry leaf discs and its correlation with flavonoid synthesis. *Phytochemistry* 7: 441 – 446.

CRESSWELL, J.R. AND R.A. DE FOSSARD. 1974. Organ culture of *Eucalyptus grandis*. *Australian Forestry* 37: 55 – 69.

CRESSWELL, J.R. AND C. NITSCH. 1975. Organ culture of *Eucalyptus grandis* L. *Planta* 12: 87 – 90.

CURIR, P., C.F. VANSUMERE, A. TERMINI, P. BARTHE, A. MARCHESINI AND M. DOLCI. 1990. Flavonoid accumulation is correlated with adventitious roots formulation in *Eucalyptus gunnii* Hook micropropagated through axillary bud stimulation. *Plant Physiology* 92: 1148 – 1153.

CUTTER, D.F., P.J. RUDALL, P.E. GASSON AND R.M.O. GALE. 1987. Root identification manual of trees and shrubs. Chapman and Hall Ltd., London.

DAMIANO, C. AND S. MONTICELLI. 1998. *In vitro* fruit trees rooting by *Agrobacterium rhizogenes* wild type infection. *Electronic Journal of Biotechnology* Vol. 1. ISSN 0717 – 3458 (http://ejb.org/content/vol/issue3/full/4/Index.asp)

DAS, T. AND G.C. MITRA. 1990. Micropropagation of *Eucalyptus tereticornis* Smith. *Plant Cell Tissue and Organ Culture* 22: 95 – 103.

DEBERGH, P.C. AND P.E. READ. 1991. Micropropagation. In: Debergh, P.C. and R.H. Zimmerman (eds.). Micropropagation. Kluwer Academic Publishers, Netherlands. pp. 1 – 13.

DE CLEENE, M. AND J. DE LEY. 1981. The host range of infectious hairy-root. *The Botanical Review* 47: 147 -- 194.

DE FOSSARD, R.A., M.T. BENNETT, J.R. GORST AND R.A. BOURNE. 1978. Tissue culture propagation of *Eucalyptus ficifolia* F. Muell. *International Plant Propagators Society* 28: 429 – 435.

DENISON, N.P. 1999. Mondi Forests Tree Improvement Research: (1968 - 1998) 30 years in perspective. Mondi Forests. pp. 28 - 39.

DENISON, N.P. AND J.E. KIETZKA. 1993. The use and importance of hybrid intensive forestry in South Africa. *South African Forestry Journal* 165: 55 – 61.

DEPICKER, A., L. HERMAN, A. JACOBS, J. SCHELL AND M. VAN MONTAGU. 1985. Frequencies of simultaneous transformation with different T-DNAs and their relevance to the *Agrobacterium*/plant cell interaction. *Molecular and General Genetics* 201: 477 - 484.

DI COLA, A., P. COSTANTINO AND L. SPANO. 1996. Cell commitment and *rol*B gene expression in the induction of root differentiation. *Plant Cell Tissue and Organ Culture* 46: 203 - 209.

DITTEMER, H.J. 1972. Modern Plant Biology. Van Nostrand Reinhold Co., New York. pp. 166 – 175.

DIXON, H.H. AND J. JOLY. 1895. On the ascent of sap. *Philosophical Transactions of the Royal Society of London B* 186: 536 - 576.

DRUART, P., C. KEVERS, P. BOXUS AND T. GASPER. 1982. *In vitro* promotion of root formation by apple shoots through darkness effect on endogenous phenols and peroxidases. *Zeitschrift fur Pflazenphysiologie* 108: 429 – 439.

DURAND-CRESSWELL, R. AND C. NITSCH. 1977. Factors influencing the regeneration of *Eucalyptus grandis* by organ culture. *Acta Horticulturae* 78: 149 – 155.

DURAND-CRESSWELL, R., M. BOULAY AND A. FRANCLET. 1982. Vegetative propagation of *Eucalyptus* species. In: Bonga, J.M., and Durzan, D.J. (eds.). Tissue culture in forestry.

ELIASSON, L. 1978. Effects of nutrients and light on growth and root formation in *Pisum sativum* cuttings. *Physiologia Plantarum* 43: 13 – 18.

ELIEZER, J. AND D.A. MORRIS. 1980. Cell length, light and ^{14}C-labelled indole-3-yl-acetic acid transport in *Pisum sativum* L. and *Phaseoulus vulgaris* L. *Planta* 149: 327 – 331.

EPSTEIN, E. AND J. LUDWIG-MÜLLER. 1993. Indole-3-butyric acid in plants: occurrence, synthesis, metabolism and transport. *Physiologia Plantarum* 88: 382 – 389.

EPSTEIN, E., S. ZILKAH, G. FAINGERSH AND A. ROTENBAUM. 1993. Transport and metabolism of indole-3-butyric acid in easy and difficult-to-root cuttings of sweet cherry *(Prunus avium* L.). *Acta Horticulturae* 329: 292 – 295.

EPSTEIN, E., S.J. NISSEN AND E.G. SUTTER. 1991. Indole-3-acetic acid and indole-3-butyric acid in tissues of carrot inoculated with *Agrobacterium rhizogenes*. *Journal of Plant Growth Regulation* 10: 97 – 100.

EWERS, F.W., J.B. FISHER AND S-T. CHIU. 1989. Water transport in the liana *Bauhinia fassoglensis* (Fabaceae). *Plant Physiology* 91: 1625 – 1631.

FEBRUARY, E. C., W.D. STOCK, W.J. BOND AND D.J. LE ROUX. 1995. Relationships between water availability and selected vessel characteristics in *Eucalyptus grandis* and two hybrids. *International Association of Wood Anatomists Journal* 16: 269 – 276.

FERGUSON, I.B. AND B.K. DRØBAK. 1988. Calcium, mineral nutririon and salinity. *HortScience* 23: 262 - 269

FENNING, T.M., S.S. TYMENS, J.S. GARTLAND, C.M. BRASIER AND K.M.A. GARTLAND. 1996. Transformation and regeneration of English elm using wild-type *Agrobacterium tumefaciens*. *Plant Science* 116: 37 - 46.

FLORENCE, R.G. 1983. A perspective of the eucalypt forests: their characteristics and role in wood production. *New Zealand Journal of Forestry* 20: 372 – 393.

FRANCLET, A. AND M. BOULAY. 1982. Micropropagation of frost resistant *Eucalyptus* clones. *Australian Forest Research* 13: 83 – 89.

FRENSCH, J. AND E. STEUDLE. 1989. Axial and radial hydraulic resistance in roots of maize (*Zea maize* L.). *Plant Physiology* 91: 719 – 726.

FORD, Y-Y. AND R.S. HARRISON-MURRAY. 1997. Water transport through the roots of ornamental shrub cuttings. *Journal of Horticultural Science* 75: 687 – 698.

GARDNER, R. 1998. Grafting of cold-tolerant eucalypt selections: 1997. ICFR Newsletter, February 1998. pp. 10 – 12.

GASPAR, T. AND M. COUMANS. 1987. Root formation. In: Bonga, J.M. and D.J. Durzan (eds.). Cell and tissue culture in forestry. Vol. 2. Martinus Nijhoff Publishers, Dordrecht. pp. 202 – 217.

GEORGE, E.F. 1993. Plant propagation by tissue culture. Part 1. 2nd edition. Exegetics Limited, Edington, Wilts. England. pp. 193 – 315.

GEORGE, E.F. 1996. Plant propagation by tissue culture. Part 2. 2nd edition. Exegetics Limited. Edington, Wilts. England. pp. 639 – 695.

GEORGE, E.F. AND P.D. SHERRINGTON. 1984. Plant propagation by tissue culture. Exegetics Limited. Edington, Wilts. England.

GHEYSEN, G., M. VAN MONTAGU AND P. ZAMBRYSKI. 1987. Integration of *Agrobacterium tumefaciens* transfer DNA (T-DNA) involves rearrangements of target plant sequences. *Proceedings of the National Academy of Sciences USA* 84: 6169 – 6173.

GODWIN, I., G. TODD, B. FORD-LLOYD AND H.J. NEWBURY. 1991. The effects of acetosyringone and pH on *Agrobacterium*-mediated transformation vary according to plant species. *Plant Cell Reports* 9: 671 – 675.

GORDON, V. 1991. Tissue culture decontamination procedures for *Eucalyptus nitens*. In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 81 - 85.

GRIMSLEY, N.H. 1990. Agroinfection. *Physiologia Plantarum* 79: 147 – 153.

GUPTA, P.K., A.F. MASCARENHAS AND V. JAGANNATHAN. 1981. Tissue culture of forest trees – clonal propagationn of mature trees of *Eucalyptus citriodora* Hook, by tissue culture. *Plant Science Letters* 20: 195 – 201.

HAINES, R. 1994. Biotechnology in forest tree improvement with special reference to developing countries. *FAO Forestry Paper* 118: 151 – 167.

HAMMATT, N. 1992. Progress in the biotechnology of trees. *World Journal of Microbiology and Biotechnology* 8: 369 – 377.

HARMS, C.T., I. BAKTIR AND J.J. OERTI. 1983. Clonal propagation *in vitro* of red beet (*Beta vulgaris* spp.) by multiple adventitious shoot formation. *Plant Cell Tissue and Organ Culture* 2: 93 – 102.

HARTMANN, H.T. AND D.E. KESTER. 1975. Plant propagation; principles and practices, 3rd ed. Prentice-Hall, Inc., Englewood Cliffs, New Jersey. pp. 211 – 270, 509.

HARTNEY, V. 1980. Vegetative propagation of the Eucalypts. *Australian Forest Research* 10: 199 – 211.

HATTA, M., C.A. BEYL, S. GARTON AND A.M. DINER. 1996. Induction of roots on jujube softwood cuttings using *Agrobacterium rhizogenes. Journal of Horticultural Science* 71: 881- 886.

HEPBURN, A.G., R.E. CLARKE, L. PEARSON AND J. WHITE. 1983. The role of cytosine methylation in the control of nopaline of nopaline synthase gene expression in the plant tumour. *Journal of Molecular and Applied Genetics* 2: 315 – 329.

HILDEBRAND, E.M. 1934. Life history of the hairy – root organism in relation to its pathogenesis on nursery apple trees. *Journal of Agricultural Research* 48: 857 – 885.

HILLS, W.E. AND A.G. BROWN. 1978. Eucalypts for wood production. CSIRO, Australia. p. 434. In: Turnbull, J.W. 1991. Future use of *Eucalyptus*: opportunities and problems. In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 2 –27.

HOOYKAAS, P.J.J. 1983. Plasmid genes essential for the interactions of *Agrobacteria* and *Rhizobia* with plant cells. In: Pühler, A. (ed.). Molecular Genetics of the Bacteria-Plant Interaction. Springler-Verlag, Berlin Heidelberg. pp. 229 - 238.

HOPE, B.A. 1994. The development of regeneration and transformation systems for *Eucalyptus* spp. MSc Thesis. Department of Biology, Faculty of Science, University of Natal Durban, South Africa.

HUANG, Y., A. DINER AND D.F. KARNOSKY. 1991. *Agrobacterium rhizogenes*-mediated genetic transformation and regeneration of a conifer: *Larix decidua. In Vitro Cellular and Developmental Biology – Plant* 27: 201 – 207.

HUGHES, K.W. 1981. Ornamental species. In: Conger, B.V. (ed.). Cloning agricultural plants via *in vitro* techniques. CRC Press, Inc., Boca Raton, Florida. pp. 6 – 50.

HUTCHINSON, J.F. 1982. Tissue culture propagation of fruit trees. In: Rao, A.N. (ed.). Proceedings of the COSTED symposium on Tissue Culture of Economically Important Plants. pp. 113 – 120.

HYNDMAN, S.E., P.M. HASEWAGA AND R.A. BRESSAN. 1982. The role of sucrose and nitrogen in adventitious root formation on cultured rose shoots. *Plant Cell Tissue and Organ Culture* 1: 229 – 238.

JAY-ALLEMAND, C., S. PENG, P. CAPELLI AND D. CORNU. 1993. Micropropagation of hybrid walnut trees: some factors involved in rooting. *Acta Horticulturae* 311: 117 – 124.

JONES, N.B. AND J. VAN STADEN. 1994. Micropropagation and establishment of *Eucalyptus grandis* hybrids. *South African Journal of Botany* 60: 122 – 126.

KADO, C.I. AND S-T. LIU. 1981. Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology* 145: 1365 - 1373.

KELLY, M.K. 1993. Benlate: old reliable fungicide gone and possibly deadly. GrowerTalks, November: 20 – 25.

KIRBY, E.G., T. LEUSTER AND M.S. LEE. 1987. Nitrogen nutrition. In: Bonga, J.M. and D.J. Durzan (eds.). Cell and tissue culture in forestry. Vol. 1: General principles and Biotechnology. pp. 67 – 88.

KIRDMANEE, C. 1999. Environmental control in micropropagation: effects of CO_2 enrichment and photosynthesis of *Eucalyptus* shoots/plantlets cultured photoautotropically *in vitro*. In: Altman, A., M. Ziv and S. Izhar (eds.). Plant

Biotechnology and In Vitro Biology in the 21st Century. Kluwer Academic Publishers. pp. 651 – 654.

KIRDMANEE, C., Y. KITAYA AND T. KOZAI. 1995. Effects of CO_2 enrichment and supporting material of *Eucalyptus* shoots/plantlets cultured photoautrophically *in vitro*. *Environmental Control in Biology* 32: 133 – 141.

KOLB, K.J., J.S. SPERRY AND B.B. LAMONT. 1996. A method for measuring xylem hydraulic conductance and embolism in entire root and shoot systems. *Journal of Experimental Botany* 47: 1805 – 1810.

KOZAI, T. 1991. Micropropagation under photoautotrophic conditions. In: Debergh, P.C. and R.H. Zimmerman. Micropropagation. Kluwer Academic Publishers. pp. 447 – 469.

KOZAI, T., Q.T. NGUYEN AND C. CHUN. 1999. Environmental control in photoautotropic micropropagation. In: Altman, A., M. Ziv and S. Izhar (eds.). Plant Biotechnology and In Vitro Biology in the 21st Century. Kluwer Academic Publishers. pp. 725 – 728.

KOZAI, T., Y. KOYAMA AND I. WATANABE. 1988. Multiplication of potato plantlets *in vitro* with sugar- free medium under high photosynthetic photon flux. *Acta Horticulturae* 230: 121 – 127.

LE ROUX, J.J. 1990. Propagation of cold-tolerant Eucalyptus L'Her. species in South Africa. MSc Thesis, Faculty of Science, University of Natal, Pietermaritzburg, South Africa.

LE ROUX, J.J. AND J. VAN STADEN. 1991a. Micropropagation of *Eucalyptus* species. *HortScience* 26: 199 – 200.

LE ROUX, J.J. AND J. VAN STADEN. 1991b. Micropropagation and tissue culture of *Eucalyptus* – a review. *Tree Physiology* 9: 435 – 477.

LIPPINCOTT, B.B. AND J.A. LIPPINCOTT, J.A. 1969. Bacterial attachment to a specific wound site as an essential stage in tumour initiation by *Agrobacterium tumefaciens*. *Journal of Bacteriology* 97: 620 – 628.

LIPPINCOTT, B.B., M.H. WHATLEY AND J.A. LIPPINCOTT. 1977. Tumour induction by *Agrobacterium* involves attachment of the bacterium to a site on the host plant cell wall. *Plant Physiology* 59: 388 – 390.

MACHADO, L.O.R., G.M. DE ANDRADE, L.P.B. CID, R.M. PENCHEL AND A.C.M. BRASILEIRO. 1997. *Agrobacterium* strain specificity and shooty tumour formation in eucalypt (*Eucalyptus grandis* x *E. urophylla*). *Plant Cell Reports* 16: 299 – 303.

MACRAE, S. 1991. *Agrobacterium* - mediated transformation of Eucalypts to improve rooting ability. In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 117 - 125.

MAKWARELA, M. 1996. The development of protocols for the diagnosis and micropropagation of cold-tolerant *Eucalyptus* cultivars. MSc Thesis. Department of Biology, University of Natal, Durban 4041, South Africa.

MARTIN, B. 1988. The benefits of hybridization. How do you breed for them? Proceedings "Breeding Tropical Trees", IUFRO, Pataya, Thailand.

MARTIN, C. 1985. Plant breeding *in vitro*. *Endeavour, New Series* 9: 81 – 86.

MCAFEE, B.J., E.E. WHITE, L.E. PELCHER AND M.S. LAPP. 1993. Root induction in pine (*Pinus*) and larch (*Larix*) spp. using *Agrobacterium rhizogenes*. *Plant Cell, Tissue and Organ Culture* 34: 53 - 62.

MCCLELLAND, M.T. AND M.A.L. SMITH. 1990. Vessel type, closure, and explant orientation influence *in vitro* performance of five woody species. *HortScience* 25: 797 – 800.

MCCLELLAND, M.T., M.A.L. SMITH AND Z.B. CAROTHERS. 1990. The effects of *in vitro* and *ex vitro* root initiation on subsequent root quality in three woody plants. *Plant Cell, Tissue and Organ Culture* 23: 115 – 123.

MCCOMB, J.A. AND I.J. BENNETT. 1986. Eucalypts (*Eucalyptus* spp.). In: Bajaj, Y.P.S. (ed.). Biotechnology in Agriculture and Forestry, Vol. 1: Trees 1. Springler-Verlag Berlin Heidelberg. pp. 340 – 362.

McCOMB, J.A. AND I.J. BENNETT. 1982. Vegetative propagation of *Eucalyptus* using tissue culture and its applications to forest improvement in Western Australia. Proceedings of the 5th International Plant Tissue and Cell Culture. pp. 721 – 722.

McCOWN, B.H. AND J.C. SELLMER. 1987. General media and vessels suitable for woody plant culture. In: Bonga, J.M., and Durzan, D.J. (eds.). Cell and Tissue Culture in Forestry, Vol. 1. Martinus Nijhoff Publishers. pp. 4 – 16.

McCULLY, M.E. AND M.J. CANNY. 1988. Pathways and processes of water and nutrient movement in roots. *Plant and Soil* 111: 159 - 170.

McKENZIE, B.E. AND C.A. PETERSON. 1995. Root browning in *Pinus banksiana* Lamb. and *Eucalyptus pilularis* Sm.1. Anatomy and permeability of the cork zone. *Acta Botanica* 108: 138 - 143.

MEADOWS, D.G. 1999. Mondi in South Africa. *TAPPI Journal* 82: 71 – 76.

MEHRA-PALTA, A. 1982. Clonal propagation of *Eucalyptus* by tissue culture. *Plant Science Letters* 26: 1 – 11.

MILBURN, J.A. 1996. Sap ascent in vascular plants: challengers to the cohesion theory ignore the significance of immature xylem and the recycling of Munch water. *Annals of Botany* 78: 399 - 407.

MILHAJEVIĆ, S., S. STIPKOVIĆ AND S. JELASKA. 1996. Increase of root induction in *Pinus nigra* explants using agrobacteria. *Plant Cell Reports* 15: 610 - 614.

MOHAMMED, G.H. AND W.E. VIVADER. 1988. Root production and plantlet development in tissue-cultured conifers. *Plant Cell, Tissue and Organ Culture* 14: 137 – 160.

MOKOTEDI, M.E.O. 1997. Development of protocols for high yielding and good quality roots of cold-tolerant hybrid clones from micropropagated shoots. BSc (Hons) Thesis. Department of Biology, Faculty of Science, University of Natal, Durban 4041, South Africa.

MOON, G.J., B.F. CLOUGH, C.A. PETERSON AND W.G. ALLAWAY. 1986. Apoplastic and symplastic pathways in *Avicennia marina* (Forsk.) Vierh. roots revealed by fluorescent tracer dyes. *Australian Journal of Plant Physiology* 13: 637 - 648.

MOORE, L., G. WARREN AND G. STROBEL. 1979. Involvement of a plasmid in the hairy root disease of plants caused by *Agrobacterium rhizogenes*. *Plasmid* 2: 617- 626.

MOZO, T. AND P.J.J. HOOYKAAS. 1992. Factors affecting the rate of T-DNA transfer from *Agrobacterium tumefaciens* to *Nicotiana glauca* plant cells. *Plant Molecular Biology* 19: 1019 – 1030.

MUELLER-DOMBOIS, D. 1992. Sustainable Forestry: The role of Eucalypts and lessons from natural and artificial monoculture systems. *South African Forestry Journal* 162: 57 – 59.

MULLER, P.H. 1988. The utilization of sawn timber from young plantation grown *Eucalyptus grandis/saligna* in South Africa. In: Proceedings of the International Forestry Conference for the Australian Biocentenary, Albury 2. pp.22.

MURASHIGE, T. 1974. Plant propagation through tissue culture. *Annual Review of Plant Physiology* 25: 135 – 166.

MURASHIGE, T. AND F. SKOOG. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473 – 497.

NASHAR, L. 1989. Micropropagation. *Australian Journal of Biotechnology* 3: 288 – 289.

NAUERBY, B., K. BILLING AND R. WYNDAELE. 1997. Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. *Plant Science* 123: 169 - 177.

NÉMETH, G. 1986. Induction of rooting. In: Bajaj, Y.P.S. (ed.). Biotechnology in Agriculture and Forestry, Vol. 1: Trees 1. Springler-Verlag Berlin Heidelberg. pp. 49 – 64.

NESTER, E.W., M.P. GORDON, R.M. AMASINO AND M.F. YANOFSKI. 1984. Crown gall: A molecular and physiology analysis. *Annual Review of Plant Physiology* 35: 387 - 413.

NILSSON, O. AND O. OLSSON. 1997. Getting to the root: The role of the *Agrobacterium rhizogenes rol* genes in the formation of hairy roots. *Physiologia Plantarum* 100: 463 – 473.

NILSSON, O., H. TUOMINEN, B. SUNBERG AND O. OLSSON. 1997. The *Agrobacterium rhizogenes rol* B and *rol* C promoters are expressed in pericycle cells competent to serve as root initials in transgenic hybrid aspen. *Physiologia Plantarum* 100: 456 – 462.

NORTH, G.B. AND P.S. NOBEL. 1996. Radial hydraulic conductivity of individual root tissues of *Opuntia ficus-indica* (L.) Miller as soil moisture varies. *Annals of Botany* 77: 133 – 142.

NORTH, G.B., F.W. EWERS AND P.S. NOBEL. 1992. Main root-lateral root junctions of two desert succulents: changes in axial and radial components of hydraulic conductivity during drying. *American Journal of Botany* 79: 1039 – 1050.

NORTON, C.R. AND M.E. NORTON. 1989. Rhododendrons. In: Bajaj, Y.P.S. (ed.). Biotechnology in Agriculture and Forestry, Vol.5: Trees II. Springler-Verlag Berlin Heidelberg. pp. 428 – 451.

OOISHI, A., H. MACHIDA, T. HOSOI AND H. KOMATSU, H. 1978. Root formation and respiration of the cuttings under different temperatures. *Journal of Japanese Society of Horticultural Science* 47: 243 – 247.

PATON, D.M., R.R. WILLING, W. NICHOLLS AND L.D. PRYOR. 1970. Rooting of stem cuttings of *Eucalyptus*: a rooting inhibitor in adult tissue. *Australian Journal of Botany* 18: 175 – 183.

PAWLICKI, N., R.S. SANGWAN AND B. SANGWAN-NORREEL. 1992. Factors influencing the *Agrobacterium tumefaciens*-mediated transformation of carrot (*Daucus carota* L.). *Plant Cell, Tissue and Organ Culture* 31: 129 – 139.

PELLEGRINESCHI, A. AND O. DAVOLIO-MARIANI. 1996. *Agrobacterium rhizogenes*-mediated transformation of scented geranium. *Plant Cell, Tissue and Organ Culture* 47: 79 – 86.

PETERSON, C.A. AND D.E. ENSTONE. 1996. Functions of passage cells in the endodermis and exodermis of roots. *Physiologia Plantarum* 97: 592 – 598.

PETIT, A., A. BERKALOFF AND J. TEMPÈ. 1986. Multiple transformation of plant cells by *Agrobacterium* may be responsible for the complex organization of T-DNA in crown gall and hairy root. *Molecular and General Genetics* 202: 388 – 393.

PETIT, A. AND J. TEMPÉ. 1985. The function of T-DNA in nature. In: Van Vloten-Doting, L., G.S.P. Groot and T.C. Hall (eds.). Molecular form and function of the plant genome. Plenum Press, New York and London. pp. 625 – 636.

PETIT, A., C. DAVID, G.A. DAHL, J.G. ELLIS, P. GUYON, F. CASSE-DELBART AND J. TEMPÈ. 1983. Further extension of the opine concept: plasmids in *Agrobacterium rhizogenes* cooperate for opine degradation. *Molecular and General Genetics* 190: 204 – 214.

PFAFF, W. AND P. SCHOPFER. 1980. Hormones are no casual links in phytochrome-mediated adventitious root formation in mustard seedlings (*Sinapis alba* L.). *Planta* 150: 321 – 329.

POOVAIAH, B.W. 1988. Molecular and cellular aspects of calcium action in plants. *HortScience* 23: 267 – 271.

PUONTI-KAERLAS, J., P. STABEL AND T. ERIKSSON. 1989. Transformation of pea (*Pisum sativum* L.) by *Agrobacterium tumefaciens*. *Plant Cell Reports* 8: 321 – 324.

PRATT, L.M. 1910. Pratt *Eucalyptus* Bulletin. Vol. 1, No.3. p. 39. Pratt *Eucalyptus* Investment Company, Los Angeles. In: Turnbull, J.W. 1991. Future use of *Eucalyptus*: opportunities and problems. In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 2 –27.

RAHMAN, S.M., M. HOSSAIN, A.K.M. RAFIUL ISLAM AND O.J. JOARDER. 1992. Effects of media composition and culture conditions on *in vitro* rooting of rose. *Scientia Horticulturae* 52: 163 – 169.

RASMUSSEN, G.F. 1991. Micropropagation of *E. nitens* and hybrid clones. In: Dean, C. (ed.). Proceedings of the 11th Research Working Group 1 (Forest Genetics) meeting, Coonawarra, South Australia. pp. 106 – 109.

RIKER, A.J., W.M. BANFIELD, W.H. WRIGHT, G.W. KEITT AND H.E. SAGEN. 1930. Studies on infectious hairy root of nursery apple trees. *Journal of Agricultural Research* 41: 507 – 540.

RIOV, J. 1993. Endogenous and exogenous auxin conjugates in rooting of cuttings. *Acta Horticulturae* 329: 284 – 287.

ROSSI, F., R. BARALDI, O. FACINI AND B. LERCARI. 1993. Photomorphogenic effects on *in vitro* rooting of *Prunus* rootstock GF 655-2. *Plant Cell, Tissue and Organ Culture* 32: 145 – 151.

RÜDINGER, M., S.W. HALLGREN, E. STEUDLE AND E-D. SCHULZE. 1994. Hydraulic and osmotic properties of spruce roots. *Journal of Experimental Botany* 45: 1413 – 1425.

RUGINI, E. AND D. MARRIOTI. 1991. *Agrobacterium rhizogenes* T-DNA genes and rooting in woody species. *Acta Horticulturae* 300: 301 – 308.

RUGINI, E., A. BAZZOFFIA AND A. JACOBONI. 1988. A simple *in vitro* method to avoid the initial dark period and to increase rooting in fruit trees. *Acta Horticulturae* 227: 438 – 440.

RUSSELL, J.A. AND B.H. MCCOWN. 1988. Highly purified subpopulations of protoplasts show superior competency for use in genetic manipulations. *HortScience* 23: 780.

SANCHEZ, M.C., M.C. SAN-JOSE, A. BALLESTER AND A.M. VIEITEZ. 1996. Requirements for *in vitro* rooting of *Quercus robur* and *Q. rubra* shoots derived from mature trees. *Tree Physiology* 16: 673 – 680.

SCHUCH, U.K. 1991. Advances in plant biotechnology and their implications for forestry research. *In Vitro Cellular and Developmental Biology – Plant* 27: 99 – 103.

SCHUCH, U.K. AND D.R. PITTENGER. 1996. Root and shoot growth of *Eucalyptus* in response to container configuration and copper carbonate. *HortScience* 31: 165.

SHEN, W.H., A. PETIT, J. GUERN AND J. TEMPÈ. 1988. Hairy roots are more sensitive to auxin than normal roots. *Proceedings of the National Academy of Sciences USA* 85: 3417 – 3421.

SHEN, W.H., E. DAVIOUD, C. DAVID, H. BARBIER-BRYGLOO, J. TEMPÉ AND J. GUERN. 1990. High sensitivity to auxin is a common feature of hairy root. *Physiologia Plantarum* 94: 554 - 560.

SLIGHTOM, J.L., M. DURAND-TARDIFF, L. JOANIN AND D. TEPFER. 1986. Nucleotide sequence analysis of the T_L-DNA of *Agrobacterium rhizogenes* agropine type plasmid. *Journal of Biological Chemistry* 261: 108 – 121.

SMITH, M.R. (ed.). 1996. *Eucalyptus* takes on "super tree" status. *Southern Hemisphere Forest Industry Journal*. Trade and Media Services Ltd. Rotorua, New Zealand. ISSN 1173-5899. pp. 1- 16.

SPANÒ, L., M. CARDARELLI, M.L. MAURO, M. POMPONI AND P. COSTANTINO. 1985. Hairy root: Molecular and physiological aspects. In: Vloten-Doting, L., G.S.P. Groot and T.C. Hall (eds.). Molecular form and function of the plant genome. Van Plenum Press, New York and London. pp. 637 - 653.

SPENCER, P.A., A. TANAKA AND G.H.N. TOWERS. 1990. An *Agrobacterium* signal compound from grapevine cultivars. *Phytochemistry* 29: 3785 – 3788.

SPENA, A., T. SCHULLING, C. KONCZ AND J. SCHELL. 1987. Independent and synegistic activity of *rol* A, B, C and D loci in stimulating abnormal growth in plants. *EMBO Journal* 6: 3891 – 3899.

SPERRY, J.S., J.R. DONNELLY AND M.T. TYREE. 1988. A method for measuring hydraulic conductivity and embolism in xylem. *Plant, Cell and Environment* 11: 35 – 40.

SPERRY, J.S. AND N.Z. SALIENDRA. 1994. Intra- and inter-plant variation in xylem cavitation in *Betula occidentalis*. *Plant, Cell and Environment* 17: 1233 – 1241.

SRIKANDARAJAH, S., R.M. SKIRVIN AND H. ABU-QUAD. 1990. The effect of some macronutrients on adventitious root formation of scion apple cultivars *in vitro*. *Plant Cell Tissue and Organ Culture* 21: 185 -189.

STACHEL, S.E., E. MESSENS, M. VAN MONTAGU AND P. ZAMBRYSKI. 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium tumefaciens*. *Nature* 318: 624 – 629.

STACHEL, S.E. AND P.C. ZAMBRYSKI. 1986a. *Vir*A and *vir*G control plant induced activation of the T-DNA transfer process of *A. tumefaciens*. *Cell* 46: 325 – 333.

STACHEL, S.E. AND P.C. ZAMBRYSKI. 1986b. *Agrobacterium tumefaciens* and the susceptible plant cell: A novel adaptation of extracellular recognition and DNA conjugation. *Cell* 47: 155 – 157.

STACHEL, S.E., E.W. NESTER AND P.C. ZAMBRYSKI. 1986a. A plant cell factor induces *Agrobacterium tumefaciens vir* gene expression. *Proceedings of the National Academy of Sciences, U.S.A.* 83: 379 - 383.

STACHEL, S.E., B. TIMMERMAN AND P. ZAMBRYSKI. 1986b. Generation of single-stranded T-DNA molecules during the initial stages of T-DNA transfer from *Agrobacterium tumefaciens* to plant cells. *Nature* 322: 706 – 711.

STEUDLE, E. 1997. Water transport across plant tissue: Role of water channels. *Biology of the Cell* 89: 259 – 273.

STEUDLE, E. AND C.A. PETERSON. 1998. How does water get through roots? *Journal of Experimental Botany* 49: 775 – 788.

STEUDLE, E. AND H. HEYDT. 1997. Water transport across tree roots. In: Rennenberg, H., W. Eschrich and H. Ziegler (eds.). Trees - Contributions to modern tree physiology. Backhuys Publishers, Leiden, The Netherlands. pp. 239 – 255.

STEUDLE, E. AND J. FRENSCH. 1996. Water transport in plants: role of the apoplast. *Plant and Soil* 187: 67 – 79.

STEUDLE, E. AND A.B. MESHCHERYAKOV. 1996. Hydraulic and osmotic properties of oak roots. *Journal of Experimental Botany* 47: 387 – 401.

STEUDLE, E., M. MURRMANN AND C.A. PETERSON. 1993. Transport of water and solutes across maize roots modified by puncturing the epidermis. Further evidence for the composite transport model of the root. *Plant Physiology* 103: 335 – 349.

STEUDLE, E., R. OWEN AND E.D. SCHULZE. 1987. Water transport in maize roots. *Plant Physiology* 84: 1220 – 1232.

STROBEL, G.A. AND A. NACHMIAS. 1985. *Agrobacterium rhizogenes* promotes the initial growth of bare root stock Almond. *Journal of General Microbiology* 131: 1245 - 1249.

STROBEL, G.A., A. NACHMIAS AND W.M. HESS. 1988. Improvements in the growth and yield of olive trees by transformation with the Ri plasmid of *Agrobacterium rhizogenes*. *Canadian Journal of Botany* 66: 2581 - 2585.

SUTTER, E. G. AND J. LUZA. 1993. Developmental anatomy of roots induced by *Agrobacterium rhizogenes* in *Malus pumila* 'M.26' shoots grown *in vitro*. *International Journal of Plant Sciences* 154: 59 – 67.

SVENSON, S.E. AND F.T. DAVIES, JR. 1995. Change in tissue mineral elemental concentration during root initiation and development of Poinsettia cuttings. *HortScience* 30: 617 – 619.

TEPFER, D. 1983. The biology of genetic transformation of higher plants by *Agrobacterium rhizogenes*. In: Pühler, A (ed.). Molecular Genetics of the Bacteria-Plant Interaction. Springler-Verlag, Berlin Heidelberg. pp. 248 - 258.

TEPFER, D. 1984. Tranformation of several spp. of higher plants by *Agrobacterium rhizogenes*: Sexual transmission of the transformed genotype and phenotype. *Cell* 37: 959 – 967.

TEPFER, D. 1990. Genetic transformation using *Agrobacterium rhizogenes*. *Physiologia Plantarum* 79: 140 - 146.

THOKOANE, N.L. 1998. The development of short-to-medium and long-term germplasm storage protocols for *Eucalyptus* spp. MSc Thesis. Department of Biology, Faculty of Science, University of Natal, Durban, South Africa.

THORPE, T.A., I.S. HARRY AND P.P. KUNAR. 1991. Application of micropropagation to forestry. In: Debergh, P.C and R.H. Zimmerman. Micropropagation. Kluwer Academic Publishers, London. pp. 311 – 336.

TIBBITS, W.N., T.L. WHITE, G.R. HODGE AND K.R. JOYCE. 1997. Genetic control of rooting ability of stem cuttings in *Eucalyptus nitens*. *Australian Journal of Botany* 45: 203 – 210.

TINLAND, B. 1996. The integration of T-DNA into plant genomes. *Trends in Plant Science* 1: 178 – 184.

TORREY, J.G. 1986. Endogenous and exogenous influences on the regulation of lateral root formation. In: Jackson, M.B. (ed.). New root formation in plants and cuttings. Marinus Nijhoff/Dr. W. Junk Publishers, Dordrecht.

TRINDADE, H. AND M.S. PAIS. 1997. *In vitro* studies on *Eucalyptus globulus* rooting ability. *In Vitro Cellular and Developmental Biology – Plant* 33: 1 – 5.

TURNBULL, J.W. 1991. Future use of *Eucalyptus*: opportunities and problems. In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 2 –27.

TYREE, M.T. AND F.W. EWERS. 1991. The hydraulic architecture of trees and other woody plants. *New Phytologist* 119: 345 – 360.

TYREE, M.T. AND J.S. SPERRY. 1989. Vulnerability of xylem to cavitation and embolism. *Annual Review of Plant Physiology and Molecular Biology* 40: 19 – 38.

TYREE, M.T., S.D. DAVIS AND H. COCHARD. 1994a. Biophysical perspectives of xylem evolution: is there a tradeoff of hydraulic efficiency for vulnerability to dysfunction? *International Association of Wood Anatomists Journal* 15: 335 – 360.

TYREE, M.T., S. YANG, P. CRUIZIAT AND B. SINCLAIR. 1994b. Novel methods for measuring hydraulic conductivity of tree root system and interpretation using AMAIZED. *Plant Physiology* 104: 189 – 199.

TYREE, M.T., M.E.D. GRAHAM, K.E. COOPER AND L.J. BAZOZ. 1983. The hydraulic architecture of *Thuja occidentalis*. Canadian Journal of Botany 61: 2105 – 2111.

VANDER WILLIGEN, C. 1996. The relationship between xylem hydraulic characteristics, growth rates and water availability in *Eucalyptus* clones. MSc Thesis, University of Natal, Durban, South Africa.

VANKOVA, R., K-C. HSIAO, C.H. BORNMAN, A. GAUDINOVA. 1991. Effects of synthetic cytokinins and respiration patterns of *Beta vulgaris* cells in suspension. *Journal of Plant Growth Regulation* 10: 197 – 199.

VAN WORDRAGEN, M. F., J. DE JONG, H.B.M. HUITEMA AND H.J.M. DONS. 1991. Genetic transformation of chrysanthemum using wild-type *Agrobacterium* strains: strain and cultivar specificity. *Plant Cell Reports* 9: 505 - 508.

VAN WORDRAGEN, M.F., P.B.F. OUWERKERK AND H.J.M. DONS. 1992. *Agrobacterium rhizogenes* mediated induction of apparently untransformed roots and callus in chrysanthemum. *Plant Cell, Tissue and Organ Culture* 30: 149 - 157.

VEIERSKOV, B. AND A.S. ANDERSEN. 1982. Dynamics of extractable carbohydrates in *Pisum sativum*. III. The effect of IAA and temperature on content and translocation of carbohyydrates in pea cuttings during rooting. *Physiologia Plantarum* 55: 179 – 182.

VELUTHAMBI, K., M. KRISHNAN, J.H. GOULD, R.H. SMITH AND S.B. GELVIN. 1989. Opines stimulate induction of the *vir* genes of the *Agrobacterium tumefaciens* Ti plasmid. *Journal of Bacteriology* 171: 3696 – 3703.

VILAINE, F. AND F. CASSE-DELBART. 1987. Independent induction of transformed roots by the TL and TR regions of the Ri plasmid of agropine type *Agrobacterium rhizogenes*. *Molecular and General Genetics* 206: 17 - 23.

WALDEN, R. 1988. The biology of tumour formation by *Agrobacterium*. In: Walden, R. (ed.). Genetic transformation in plants. Open University Press Biotechnology Series. pp. 7 - 24.

WALKER, N., R. JACQUES AND E. MIGINIAC. 1987. Action of light on rooting *in vitro* and acclimatization of *Sequoia sempervirens* to soil. *Acta Horticulturae* 212: 289 – 301.

WANG, Q. 1992. The effects of light, darkness and temperature on micropropagation of the pear rootstock BP10030. *Journal of Horticultural Science* 67: 869 – 876.

WANG, K., S.C. STACHEL, B. TIMMERMAN, M. VAN MONTAGU AND P. ZAMBRYSKI. 1987. Site-specific nick in the T-DNA border sequences following *vir* gene expression in *Agrobacterium*. *Science* 235: 587 – 591.

WARRAG, E.I., M.S. LESNEY AND D.L. ROCKWOOD. 1990. Micropropagation of field-tested superior *Eucalyptus grandis* species. *New Forests* 4: 67 – 79.

WATT, M.P., B.A. GAUNTLETT AND F.C. BLAKEWAY. 1996. Effect of anti-fungal agents on *in vitro* cultures of *Eucalyptus grandis. South African Forestry Journal* 175: 23 – 27.

WATT, M.P., F.C. BLAKEWAY, B. HERMAN AND N. DENISON. 1997. Developments in the use of biotechnology in commercial forestry tree improvement programmes in South Africa. Forests, biological diversity and the maintenance of the natural heritage. Proceedings of the XI World Forestry Congress, Antalya, Turkey. Vol. 2. pp. 189 – 194.

WATT, M.P., S. RAMGAREEB, B. HOPE, F.C. BLAKEWAY AND N.P. DENISON. 1998. Micropropagation via axillary bud proliferation from seedlings and juvenile shoots of *Pinus patula* Schiede *et* Deppe. *Southern African Forestry Journal* 181: 1 – 6.

WATT, M.P., E.A. DUNCAN, M. ING, F.C. BLAKEWAY AND B. HERMAN. 1995. Field performance of micropropagated and macropropagated *Eucalyptus* hybrids. *South African Forestry Journal* 173: 17 – 21.

WHITE, F.F., G. GHIDOSSI, M.P. GORDON AND E. NESTER. 1982. Tumour induction by *Agrobacterium rhizogenes* involves the transfer of plasmid DNA to the plant genome. *Proceedings of the National Academy of Sciences, USA* 79: 3193 – 3197.

WHITE, F.F., B.H. TAYLOR, G.A. HUFFMAN, M.P. GORDON AND E.W. NESTER. 1985. Molecular and genetic analysis of the transferred DNA regions of the root-inducing plasmid of *Agrobacterium rhizogenes. Journal of Bacteriology* 164: 33 – 44.

WHITEMAN RUNS HIM, K., R.J. SCHEFFER AND G.A. STROBEL. 1988. Factors influencing root formation in dicots by *Agrobacterium rhizogenes. Canadian Journal of Botany* 66: 642 - 644.

WILLYAMS, D., P. WHITEMAN, J. CAMERON AND S.F. CHANDLER. 1992. Inter- and intra-family variability for rooting capacity in micropropagated *Eucalyptus globulus* and *Eucalyptus nitens*. In: AFOCEL/IUFRO Symposium 'Mass Production Technology for Genetically Improved Fast Growing Forest Tree Species'. September 1992. Bordeaux, France pp. 177 - 181.

WILSON, P.J. 1998. *In vitro in vacuo. Southern African Forestry Journal* 183: 53 – 58.

WILSON, C.L., W.E. LOOMIS AND T.A. STEEVES. 1971. Botany, 5th edition. Hott, Rinehart and Winston, Inc., New York. pp. 202 - 205.

WINANS, S.C. 1992. Two-way chemical signalling in *Agrobacterium*-Plant interactions. *Microbiological Reviews* 56: 12 – 31.

YANG, J-C., J-D. CHUNG AND Z-Z. CHEN. 1995. Vegetative propagation of adult *Eucalyptus grandis* x *urophylla* and comparison of growth between micropropagated plantlets and rooted cuttings. *Plant Cell Reports* 15: 170 – 173.

ZELDIN, E.L. AND B.H. MCCOWN. 1986. The dynamics of poplar root culture and the differentiation of shoots from cultured roots. *HortScience* 21: 815 (Abstract).

ZIMMERMAN, R.H. 1984. Rooting apple cultivars *in vitro*: Interactions among light, temperature, phloroglucinol and auxin. *Plant Cell, Tissue and Organ Culture* 3: 301 – 311.

ZIMMERMANN, M.H. 1983. Xylem structure and the ascent of sap. Springer–Verlag, Berlin/Heidelberg/New York/Tokyo. pp. 1- 143.

ZOBAYED, S.M.A., F. AFREE-ZOBAYED AND T. KOZAI. 1999. Use of scale-up vessel for the large scale photoautotrophic micropropagation of woody plants. *In Vitro Cellular and Developmental Biology- Plant* 35: 156 – 157.

ZOBEL, B.J. 1993. Clonal forestry in the Eucalypts. In: Ahuja, M.R. and W.J. Libby (eds.). Clonal Forestry II: Conservation and Application. Springler-Verlag Berlin Heidelberg. pp. 139 – 148.

ZOBEL, B. 1988. *Eucalyptus* in the forest industry. *Tappi* 71: 42 – 46. In: Turnbull, J.W. 1991. Future use of *Eucalyptus*: opportunities and problems. In: Schönau, A.P.G. (ed.). Symposium on intensive forestry: the role of *Eucalyptus*. IUFRO Proceedings, Vol. 1. 2-6 September, Durban, South Africa. pp. 2 –27.

ZOBEL, B. AND J. TALBERT. 1984. Applied Forest Tree Improvement. John Wiley & Sons, New York.