

# THE USE OF *AGROBACTERIUM* FOR PLANT IMPROVEMENT

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SHARMANE MACRAE

A thesis submitted to the faculty of Science, University of Natal, Pietermaritzburg,  
for the Degree of Doctor of Philosophy.

Pietermaritzburg, 1994.



## DECLARATION

I declare that the thesis which is herewith submitted for the Doctor of Philosophy in the University of Natal, Pietermaritzburg, is my own unaided work except where otherwise stated.

No part of this thesis has been previously submitted for a degree to any other university.

A handwritten signature in black ink, appearing to read 'M. MacRae', written over a horizontal line.

Sharmane MacRae

Pietermaritzburg.

July, 1994.

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## ABSTRACT

*Agrobacterium* species have potential as tools for plant improvement, in that they can be used as biological control agents against crown gall disease, vectors for gene insertion into plants and plant root-inducing bacteria.

For a bacterium to be successful as a biocontrol agent against crown gall disease, it must be able to produce an effective agrocin specific for *A. tumefaciens* pathogens and be able to colonise host plants effectively. Successful biological control of crown gall disease has been achieved on a limited range of host plants by application of *Agrobacterium radiobacter*. Strain K84 is non-pathogenic, produces an antibiotic type substance, agrocin 84, which kills a specific spectrum of pathogenic *Agrobacterium* strains, and is a good plant coloniser. The colonisation abilities of this bacterium and a number of potential biocontrol bacteria, D286, J73 and its derivatives, H8, and H6 and its derivatives, were compared *in vitro* and *in vivo*. In addition the ability of these strains to control crown gall pathogens *in vitro* and *in vivo* was assessed. Although D286 and H8 were good long term colonisers they were subsequently eliminated from the programme because D286 was unable to control grapevine crown gall disease, and H8 was a pathogen, with a narrow host range for biotype 3 pathogens, which had not been cured.

Good long term colonisation ability was shown to be critical to the success of the biological control strain. Both K84 and J73 strains produced fibrils which attached them to tomato root surfaces and have similar colonisation efficiencies up to 14 days after inoculation. However, the ability of J73 to colonise plants for longer periods was significantly less than that of K84. Thus, the presence of fibrils is not sufficient to ensure colonisation. No correlation was found between hydrophobicity and colonisation. Poor *in vivo* biological control of tomatoes and grapevines by J73 and its derivatives has been shown to be due to poor long term colonisation. The additions of the agrocin 84 plasmid to J73 improved *in vitro* biological control by J73 but was not effective *in vivo*. Thus although J73 produced a broad host range agrocin it was ineffective as a biocontrol agent due to its poor long term colonisation ability. H6 and its derivative proved to be good long term colonisers of the grape vine rhizosphere and were able to effectively control crown gall disease on grapevines.

*Eucalyptus grandis* was propagated *in vitro* from axillary buds. The effect of the gelling agents gelrite, agarose and agar on propagation was determined. Shoot multiplication and elongation on gelrite-containing media was found to be superior to that obtained on agarose- and agar-containing media. Rooting was enhanced with gelrite as the gelling agent. *In vitro* clone development from seed of selected *E. grandis* genotypes and the integration of this into vegetative clonal propagation

programmes is proposed. Not all clones developed in this manner rooted well under *in vitro* conditions. Thus the potential use of *A. rhizogenes* to improve rooting was investigated.

*A. rhizogenes* is a bacterial pathogen which causes 'hairy root' disease on certain plants. *Eucalyptus* species do not fall into the natural host range of this organism, however, it is able to infect these plant species. In an attempt to improve rooting and root quality of *in vitro* and *in vivo* propagated *Eucalyptus* species and clones, the root-inducing genes on the Ri plasmids of a number of *A. rhizogenes* strains were inserted into *Eucalyptus* by inoculating *in vitro* and *in vivo* stem cuttings with the selected *A. rhizogenes* strains. The resultant chimeric plants have transformed roots and normal shoots. Root development was monitored *in vitro* and after the plantlets had hardened off, and *in vivo*. Only transformed roots grew as root cultures in hormone-free liquid medium. The potential use of this procedure for improving rooting of clonal material is discussed. Under *in vitro* conditions for example, one of the broad host range *A. rhizogenes* strains, LBA9402, was able to induce up to 80 % rooting on *E. grandis*, *E. nitens* and *E. dunnii* explants. While under nursery conditions for example, one of the two *E. globulus* clones tested, HM15, developed up to ten times as many roots in response to two strains of the *A. rhizogenes* bacterium (LBA9402 and TR8,3) while the other clone failed to respond. Not only the inherent rooting abilities of the numerous *Eucalyptus* genotypes and clones

tested, but also hormone induced-rooting and *Agrobacterium*-mediated rooting were found to vary from clone to clone and genotype to genotype. *A. rhizogenes* strains were however, able to overcome the genetic control on rooting of certain clones but this was found to be dependent on both the genotype of the bacterium and the plant.

The novel concept of using bacterial cocktails to improve rooting was investigated, with the aim of overcoming the limited host range of individual *A. rhizogenes* strains. Both *in vitro* and nursery trials were established to test the potential use of bacterial cocktails as a means of improving rooting of a range of *Eucalyptus* genotypes and clones. *Agrobacterium* cocktails not only improved the ability of certain clones and genotypes to root but also the quality of the roots.

The anatomy of transformed roots from chimeric plants was compared to that of non-transformed roots, revealing no difference in root anatomy of these roots. Nutrient uptake studies using a radioisotope nutrient uptake bioassay showed no difference in phosphorus, potassium and nitrogen uptake by transformed and non-transformed roots.

The potential of *A. rhizogenes* strains to improve rooting of two other species, *Anacardum occidentale* (cashews) and *Pinus* (a number of pine hybrids) was also shown in preliminary trials.

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## LIST OF ABBREVIATIONS

Agr <sup>+</sup>	agrocin producer
Agr <sup>-</sup>	agrocin non-producer
Agr <sup>s</sup>	agrocin sensitive
AS	acetosyringone
BA	benzylaminopurine
bp	base pairs
°C	degrees centigrade
cfu	colony forming units
chr	chromosomal
chv	chromosomal virulence
dpm	disintegrations per minute
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
g	grams
Gm	gentamycin
HCl	hydrochloric acid
hr(s)	hour(s)
IBA	indolebutyric acid
K	potassium
kb	kilobase
kg	kilogram
kDa	kilodalton
Km	kanamycin
kv	kilovolt
l	litre
LHR	limited host range

mg	milligrams
min	minutes
ml	millilitre
mm	millimetre
mM	millimolar
mic	minimum inhibitory concentration
MS	Murashige and Skoog medium
NAA	naphthaleneacetic acid
No.	number
<i>noc</i>	nopaline catabolism
<i>nos</i>	nopaline synthase
N	nitrogen
Nm	neomycin
Nx	naladixic acid
<i>occ</i>	octopine catabolism
<i>ocs</i>	octopine synthase
OD	optical density
<i>onc</i>	oncogenic
<i>ori</i>	origin of replication
P	phosphorous
pCry	cryptic plasmid
pg	picograms
pNOC	nopaline catabolism plasmid
PVP	polyvinylpyrrolidone
r	resistant
Rb	rubidium
Ri	root inducing
Rf	rifampicin

RNA	ribonucleic acid
RNase	ribonuclease
<i>rol</i>	root locus
rpm	revolutions per minute
<i>s</i>	sensitive
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	spectinomycin
TBE	tris-borate buffer
Tc	tetracycline
T-DNA	transfer deoxyribonucleic acid
TEA	tris-acetate buffer
Ti	tumour inducing
<i>tml</i>	tumour morphology large
<i>tmr</i>	tumour morphology rooting
Tn	transposon
<i>tra</i>	transfer
Tris	tris(hydroxymethyl)amino methane
$\mu\text{g}$	micrograms
$\mu\text{l}$	microlitre
$\mu\text{m}$	micrometre
$\mu\text{M}$	micromolar
$\mu\text{molm}^{-2}\text{s}^{-1}$	micromoles per square meter per second
V	voltage
vol	volume
vir	virulence
WHR	wide host range
wt	weight

## PREFACE

*Agrobacterium* strains are natural soil bacteria that have great potential in plant improvement :-

Non-pathogenic *A. tumefaciens* strains in the control of grown gall disease  
and

*A. rhizogenes* strains in improving rooting of a number of dicotyledonous  
plants.

This thesis explores the potential use of these strains for these purposes.

Crown gall disease is caused by pathogenic strains of *A. tumefaciens*. This disease affects many dicotyledonous plants causing severe economic losses in many countries. As a result a worldwide search for ways to control this disease has been underway for many years. The most promising approach has been the use of a biological control agent. The non-pathogenic *Agrobacterium radiobacter* strain K84 can control disease on certain hosts (NEW and KERR, 1972; KERR and HTAY, 1974; MOORE and WARREN, 1979; ELLIS *et al.*, 1979; KERR, 1980). The effectiveness of this strain as a biocontrol agent can be attributed to its ability to produced an antibiotic-type substance, called agrocin 84, and its ability to colonise host plants efficiently. Agrocin 84 is however, only active against certain pathogenic *A. tumefaciens* strains. As biological control of grown gall disease by strains K84

is limited to specific pathogenic strains and specific host plants (THOMSON, 1987; MOORE, 1988) the search for other potential biocontrol strains has been carried out in many different countries.

In South Africa crown gall disease was considered to pose a threat to the grape harvest in the summer rainfall areas of the country (STAPHORST *et al.*, 1985). It was therefore necessary to extend the biological control of the disease to grapevines. The interest in the extension of biological control of crown gall disease in South Africa was initiated by the joint collaboration of Dr B. Strydom and Dr J. Strydom, Plant Protection Research Institute, Department of Agriculture, Pretoria, South Africa and Professor J. A. Thomson, formerly the Director of the CSIR, Laboratory for Molecular and Cell Biology (LMCB), University of the Witwatersrand, Johannesburg, South Africa. The initial isolations of potential biocontrol *Agrobacterium* strains were carried out by various research institutes of the Department of Agriculture. The most promising strains were selected and their genetic analysis was carried out by LMCB researchers under the supervision of Professor J. A. Thomson.

The aim of this research was to determine the role of colonisation in the biological control process and to screen a number of potential biocontrol strains for their abilities to control crown gall disease on grapevines.

Clonal propagation of *Eucalyptus* species is playing an increasingly important part in meeting the South Africa timber demands. This involves vegetative propagation of selected trees by stem cuttings. While this technique results in the production of clones and stabilizes a genotype, it is limited by the inherent ability of the genotype to root. Although this phenomenon is still not well understood it is undoubtedly under genetic control. However, a number of physiological and environmental factors are known to influence the ability of a genotype to root. As a result tremendous variation in rooting between clones from a genotype and between genotypes has been observed. Although nursery conditions have been optimised, certain clones with desirable wood properties still cannot be rooted successfully on a commercial basis. The potential role that tissue culture can play as another means of vegetative propagation was explored. Here again rooting was one of the major limiting factors.

*A. rhizogenes* is known to infect a range of dicotyledonous plants initiating the development of numerous secondary functional roots at a wound site (TEPFER, 1983b; WHITEMAN RUNS HIM *et al.*, 1988). STROBEL and NACHMIAS (1985) and STROBEL *et al.* (1988) demonstrated the potential of *A. rhizogenes* strains to improve rooting of bare-root almond and olive stocks. This stimulated research into the potential use of these bacterial strains to improve rooting of *Eucalyptus* clones and genotypes.

The ultimate aim of this research was to illustrate the important and valuable role that natural *Agrobacterium* strains could play in plant improvement.

The research data in Chapters 2, 3 and 4 have been published (MACRAE, THOMSON and VAN STADEN 1989; MACRAE and VAN STADEN, 1990; MACRAE and VAN STADEN, 1993) and the data in Chapters 5 and 6 is presently being prepared for publication.

I would like to acknowledge the CSIR, Division of Forest Science and Technology, Pretoria, South Africa for funding and technical support needed for this project which was completed on a part time basis. My grateful thanks to my supervisor Professor Johannes van Staden for his patience, encouragement and guidance during the completion of this thesis, and to my co-supervisor Professor Jennifer A. Thomson for her guidance and encouragement in the crown gall disease research. Her comments and recommendations on a number of chapters of this manuscript were also greatly appreciated. I am indebted to my colleagues at the CSIR for their help and advice. My thanks to Dr Jocelyn Webster for her helpful discussion on the crown gall research aspect of this project. I would like to thank Mrs Siobhan Jackson, Mrs Michelle Binedell and Miss Thirumeni Naidoo, for their support and technical assistance during the latter part of my research (both *in vitro* and nursery rooting trials carried out in Durban). The assistance of Siobhan Jackson with the

preparation of some of the graphs and with the final editing is also acknowledged. The technical assistance of Gert Malan, Andre Nel, Michael Segage and a number of CSIR technical assistants during the nursery trials is acknowledged. My grateful thanks to Dr Richard Beckett, University of Natal, Pietermaritzburg (colonisation data), Mr Stephen Verryn CSIR, Forestek, Pretoria (data from one nursery trial) and Mrs Robin Seillier for their assistance with the statistical analysis of my research data. Stephen Verryn, CSIR, Forestek, Pretoria is also acknowledge for his advice on experimental nursery design and his comments on how micropropagation could be integrated into tree breeding. I would like to acknowledge the staff of the EM Unit, University of Natal, Pietermaritzburg, particularly Vejay Bandu, for their technical assistance with electron microscopy and Mr James Wesley-Smith and Miss Debbie Sweby, Department of Biological Sciences, University of Natal, Durban, for their assistance with the light microscopy. The Department of Biological Sciences, University of Natal, Durban, is also acknowledged for making their light photographic microscope available to me. My sincere thanks to Dr Mike Smith for his comments and recommendations on Chapter 6 of this thesis. My thanks to Dr Colin Dyer, CSIR, FORESTEK, Pretoria, for his encouragement and consideration during the process of writing this manuscript.

I acknowledge and thank the Plant Protection Research Institute, Pretoria South Africa (pathogenic *A. tumefaciens* strains and *A. rhizogenes* TR8,3); Professor M.

J. C. Rhodes , Norwich Laboratory, UK (*A. rhizogenes* LBA9402); Dr E. Nester, University of Washington, U.S.A. (*A. rhizogenes* R1601); and Dr David Tepfer, INRA, France (*A. rhizogenes* A4, *A. tumefaciens* pGA643 and *A. tumefaciens* pGA643-12) for making *Agrobacterium* strains available for this study.

My thanks to SAPO (South African Plant Improvement Organisation), Stellenbosch, South Africa (grapevine cuttings); ICFR (Institute of Commercial Forestry Research), Pietermaritzburg, South Africa (*E. grandis*, *E. dunni* and *E. nitens* seed); CSIR, Division of Forest Science and Technology, Pretoria, South Africa and Department of Water Affairs and Forestry (DWAF), Pretoria South Africa (*E. grandis* seed and cuttings); CELBI, Quinta do Furadouro, Portugal (*E. globulus* seed and cuttings); IDC (Industrial Development Corporation), Johannesburg, South Africa and KFC (Kwa Zulu Finance Corporation, Durban, South Africa (*Acacardium occidentale* L. plants); and CSIR, Division of Forest Science and Technology, Futululu, South Africa (pine hybrid cuttings), for supplying the plant material required for this study.

SAFCOL, Frankfort, South Africa is acknowledge for making their nursery facility available for two of the nursery trials.

I acknowledge and thank Dr Paul Cotterill, Forestry Director, CELBI, Quinta do

Furadouro, Portugal for making the CELBI development nursery facilities and *E. globulus* clones and seed available to me. My grateful thanks to Dr Philip Wilson, CELBI, Portugal, for assisting me with the *E. globulus/A. rhizogenes*-mediated rooting trial and to the CELBI nursery staff, including the students Jo Sasse and Ana Cunha, for technical assistance.

Last but by no means least I thank my husband for his patience, understanding and encouragement which made the completion of this thesis possible.

I would like to dedicate this thesis my late father, John Basil Perrow and my mother Norma Yvonne (Perrow) Leslie whose love and encouragement has been a great inspiration to me through the years.

## CHAPTER 1

### *AGROBACTERIUM* A POTENTIAL TOOL IN PLANT IMPROVEMENT

#### 1.1. INTRODUCTION

For many centuries people have been tailoring plants to meet their needs by manipulating genes through selection, breeding and testing. Until the 1970's it was only possible to transfer genetic material from one individual to another by sexual means, through fusion of the egg with the generative pollen nucleus to give a fertilized egg, from which an individual can develop with characteristics derived from both maternal and paternal lines. The discovery of *Agrobacterium* and its natural ability to transfer a segment of DNA into plants offered an alternative means of genetic manipulation. It enabled plant scientists to capitalise on molecular biology technology to manipulate the T-DNA of *Agrobacterium* for the development of gene vectors to produce transgenic plant material using tissue culture technology. Extensive research, by numerous researchers in many countries throughout the world over the past 15 years, has resulted in good progress being made towards a greater understanding of oncogenesis in general and vector development (EL-FIKI and GILES, 1981; TEMPÈ and SCHELL, 1982; SCHELL, 1987; ARMITAGE *et al.*, 1988; DRAPER *et al.*, 1988; ZAMBRYSKI, 1988; GRANT *et al.*, 1991). Research

results have proved the potential of *A. tumefaciens* in plant improvement.

Non-oncogenic vectors were first constructed as early as 1982, and much of the fundamental research on the control of plant gene expression, studying modified or chimeric genes in transgenic plants, has been performed using *Nicotiana tabacum*. The reasons for this have been the ease with which tobacco can be manipulated in culture to achieve genetic transformation and, more importantly, the efficient regeneration of transformed plants. Although most dicotyledons are susceptible to tumour formation by at least some oncogenic strains, problems still remain in the selection of tissue transformed by a non-oncogenic T-DNA and even more importantly in the regeneration of transformed shoots from such tissue (DRAPER *et al.*, 1988). As a result of these difficulties, genetic transformation systems based on the use of *A. tumefaciens* and *A. rhizogenes*, and components of the Ti and Ri plasmids, have as yet been developed for relatively few plant varieties.

This review will address the use of native *Agrobacterium* species as a tool for plant improvement. The basis of crown gall and hairy root formation will be discussed, followed by information on tumorigenic and rhizogenic genes, an in depth description of the infection process and factors affecting host range. The potential use of non-pathogenic *A. tumefaciens* strains for the control of crown gall disease caused by pathogenic strains of this bacterium will be examined, as well as the

mechanism involved in biological control. In addition, the use of *A. rhizogenes* in plant improvement will be reviewed. *A. rhizogenes* and its root inducing ability will be given particular emphasis. Finally, the role of vegetative propagation of forest tree species by cuttings and through micropropagation will be briefly reviewed with the focus on rooting ability and its importance. The potential use of *A. rhizogenes* to induce adventitious rooting of tree species will also be addressed.

## 1.2 *AGROBACTERIUM*

### 1.2.1 Species

Members of the genus *Agrobacterium*, which has been classified as part of the *Rhizobiaceae* family, are characterised primarily by their pathogenicity. This genus comprises four species: *A. radiobacter*, the non-pathogenic member of the genus; *A. tumefaciens*, the causative agent of crown gall disease; *A. rhizogenes*, which causes hairy root disease; and *A. rubi*, which is responsible for cane gall (DELLAPORTA and PESANO, 1981; KERR and BRISBANE, 1983).

*Agrobacterium* is a gram negative soil-dwelling bacterium that infects wound sites on a wide range of plant species, inducing crown gall tumours and hairy root

development. These neoplastic growth responses result from a natural genetic engineering event (TEMPÈ and SCHELL, 1982), in which a specific region of DNA (T (transfer)-DNA) from a Ti (tumour inducing) or Ri (root-inducing) plasmid is transferred from *Agrobacterium* to a plant cell, where it is integrated and expressed in the nuclear genome. The T-DNA encodes enzymes responsible for the biosynthesis of phytohormones and/or proteins affecting the sensitivity of plants to phytohormones (AKIYOSHI *et al.*, 1984; BARRY *et al.*, 1984; INZÉ *et al.*, 1984; SCHÖDER *et al.*, 1984; VAN ONCKELEN *et al.*, 1986). The expression of these genes results in the development of tumours or hairy roots. In addition it also encodes genes specifying enzymes involved in the production and secretion of opines (amino acid derivatives) which the agrobacteria can utilise as a food source (FIRMIN and FENWICK, 1978; PETIT *et al.*, 1978b; GUYON *et al.*, 1980; DESSAUX *et al.*, 1986). *Agrobacterium* itself does not appear to express genes on the T-DNA.

Saprophytic strains of *Agrobacterium* without Ti and Ri plasmids are also common. These strains do not induce crown gall or hairy root formation. Some saprophytic strains of *A. tumefaciens* for example, produce antibiotic type substances which are able to control pathogenic strains and therefore are a powerful tool for biological control of crown gall disease (NEW and KERR, 1972; HTAY and KERR, 1974; KERR and HTAY, 1974). This aspect will be discussed later (see section 1.3).

*A. tumefaciens* was first isolated in 1897 by Cavara from galls that had developed on the stem of a grapevine and he proved that this organism was responsible for the disease. However, it was not until 1907 that the organism was described in detail and named *Bacterium tumefaciens* by Smith and Townsend (EL-FIKI and GILES, 1981). Subsequently this organism was found to infect a wide range of hosts and to be the cause of an agricultural disease of major economic importance. *A. tumefaciens* has been shown to cause disease in 61 widely separated plant families including at least 142 genera of dicotyledonous plants (DELLAPORTA and PESANO, 1981). Gall formation on monocotyledonous plants are rare with only a few species being susceptible.

### 1.2.2 The Ti and Ri Plasmids

The Ti and Ri plasmids are named according to the *Agrobacterium* strain from which they were originally isolated. These plasmids are large, ranging in size from 140 - 250 kilobase pairs (kb) (MELCHERS and HOOYKAAS, 1987; ARMITAGE *et al.* 1988). The T-DNA region which is transferred to plant cells ranges in size from 14 to 42 kb and is bordered by a 25 base pair sequence (VAN LAREBEKE *et al.*, 1974; ZAMBRYSKI, 1988; PETERSEN *et al.*, 1989; SRINIVASAN *et al.*, 1989). Any sequence between these borders may be integrated into plant nuclear DNA.

There are two other important regions on the Ti and Ri plasmids, one is the virulence region which encodes the genes responsible for the excision, transfer and integration of T-DNA from *Agrobacterium* into the genome of plant cells. The other region encodes genes responsible for the catabolism of opines.

### 1.2.3 Tumours and Hairy Roots

After excision from the inoculation site, *in vitro* tumour and hairy root cultures continue to grow and proliferate, without exogenously supplied hormones. This phenomenon is due to the stable integration of the T-DNA genes. These genes are responsible for either phytohormone production (the *onc* [oncogenicity] genes of the Ti plasmids) or increased sensitivity to auxins (the *rol* [root locus] genes of the Ri plasmids). Tumour tissue continues to proliferate as a disorganised callus culture, whereas, hairy roots proliferate into a highly branched plagiotropic root system (TEPFER, 1989).

Similarities between different Ti and Ri plasmids have been reported. For example, octopine Ti plasmids and agropine Ri plasmids have non-continuous T-DNA's known as the TL-DNA and TR-DNA, each of which are bound by two border sequences. Nopaline and succinamopine Ti plasmids and mannopine Ri plasmids, on

the other hand, have one continuous T-DNA flanked by two border sequences (MELCHERS and HOOYKAAS, 1987; SRINIVASAN *et al.*, 1989).

ENGLER *et al.* (1981) found homologous DNA sequences in nopaline T-DNA and octopine TL-DNA which contain the tumour-producing phytohormone genes, encoding enzymes in the isopentenyl - AMP (a cytokinin) and indole - acetic acid (an auxin) production pathways. More recently an *onc* gene (6b) was characterised and found to have growth-inducing properties (HOOYKAAS *et al.* 1988), inducing tumours on *Nicotiana glauca*, *N. rustica*, *Kalanchoe tubiflora* and grapevines. It is thought to act by reducing the inhibitory effects of high auxin concentrations, thereby maintaining cells in an undifferentiated state (TINLAND *et al.*, 1990).

The Ri plasmid's T-DNA has a series of 4 *rol* genes known as A, B, C and D, which when transferred to, and expressed in plant cells, lead to the development of hairy roots (TEPFER, 1989). Although the exact effect of these *rol* genes is not clearly understood, they are known to enhance the sensitivity of plant cells to endogenous auxins (SHEN *et al.*, 1988). Research by GELVIN (1990) showed that in agropine Ri plasmids the TR-DNA contains auxin biosynthetic genes which play a role in the development of hairy roots. BOUCHEZ and CAMILLERI (1990) identified a putative *rolB* gene in a 1270 bp *Bam*HI fragment which was located between the auxin synthesis and agropine synthesis regions of the TR-DNA of the

*A. rhizogenes* A4 Ri plasmid. In addition, tissue specific expression of the *rolC* promoter has been found in both monocotyledons and dicotyledons (MATSUKI *et al.*, 1989). More recently, the site for initiation of the *rolC* gene (ORF12) in the TL-DNA of the Ri plasmid of *A. rhizogenes* was located 27 bp upstream from the first ATG codon of the *rolC* gene (KANYA *et al.*, 1990).

#### 1.2.4 Opines

In addition to the oncogenes, *Agrobacterium* also transfers DNA containing genes that direct the synthesis and exudation of opines into plant cells (PETERSEN *et al.*, 1989; HABEEB *et al.*, 1991). The transformed plant cells are able to secrete opines into the intracellular region of the tumour or the hairy root rhizosphere where *Agrobacterium* lives (PETIT and TEMPE, 1985). These compounds serve as a source of carbon and nitrogen for the bacteria only, as the plant cells cannot metabolise these compounds. The type of opine produced by the tumour or hairy root cells is determined by the opine synthase genes carried on each Ti and Ri plasmid. A strain carrying a specific opine synthase gene on its T-DNA also carries genes for the catabolism of these specific opines elsewhere on its Ti or Ri plasmid (PETERSEN *et al.*, 1989; GRANT *et al.*, 1991; HABEEB *et al.*, 1991). Hence, *Agrobacterium* strains and their Ti and Ri plasmids are often classified on the basis

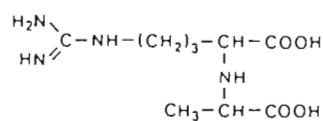
of opine, type (Table 1) (PETIT and TEMPÈ, 1985; DAVOIUD *et al.*, 1988; GRANT *et al.*, 1991).

**Table 1.** Ti and Ri plasmids classified on a biological basis.

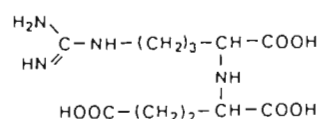
Opine type	Representative opines present in neoplastic growths	<i>Agrobacterium</i> strain examples
<b>Ti plasmids</b>		
Octopine	Octopine, octopinic acid, lysopine, histopine, agropine, agropinic acid, mannopine, mannopinic acid	B6, ACH5
Nopaline	Nopaline, nopalinic acid, agrocinopine A	C58, T37
Agropine	Agropine, agropinic acid, mannopine, mannopinic acid, agrocinopine C	AT1, AT4
Succinamopine	Succinamopine, succinanopine lactam, succinopine lactam	Eu6, 181
Grapevine	Octopine, cucumopine	K305, K308
<b>Ri plasmids</b>		
Agropine	Agropine, agropinic acid, mannopine, mannopinic acid, agrocinopine A	A4, TR105 LBA9402
Mannopine	Mannopine, mannopinic acid, agrocinopine C, agropinic acid	TR7, 8169
Cucumopine	Cucumopine	2655, 2657

Modified from GRANT *et al.*, 1991 (Derived from PETIT and TEMPÈ, 1985; DAVOIUD *et al.*, 1988).

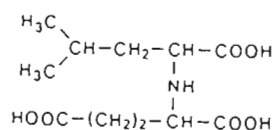
The chemical composition of opines have been characterised and the structural formulae of octopine, nopaline, leucinopine, succinamopine (asparaginopine), Agrocinospine A and Agrocinospine B are detailed in Figure 1 (HAYMAN and FARRAND, 1990; HOOYKAAS and SCHILPEROORT, 1992)



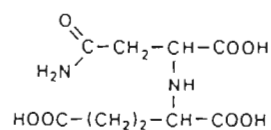
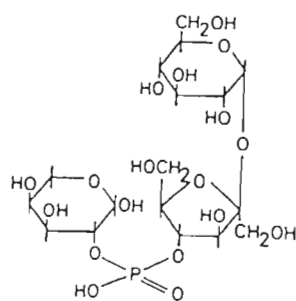
OCTOPINE



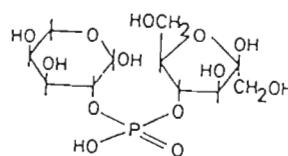
NOPALINE



LEUCINOPINE

SUCCINAMOPINE  
(ASPARAGINOPINE)

AGROCINOPINE A



AGROCINOPINE B

**Figure 1.** Structural formulae of six characteristic opines, octopine, nopaline, leucinopine and succinamopine (asparaginopine) (HOOYKAAS and SCHILPEROORT, 1992); Agrocinospine A and Agrocinospine B (HAYMAN and FARRAND, 1990).

### 1.2.5 Biotypes

*Agrobacterium* strains may be classified into three distinct biovars or biotypes, 1, 2 and 3, with pathogenic and non-pathogenic forms in each. Biotypes are based on a set of biochemical characteristics that are chromosomally encoded. Biotype 1 strains are ubiquitous soil microorganisms found on a wide variety of dicotyledonous hosts. Biotype 2 strains are most commonly found on stone fruit. Biotype 3 strains have a limited host range occurring only on grapevines (KERR and PANAGOPOULOS, 1977; PANAGOPOULOS *et al.*, 1978; OPEL and KERR, 1987) and generally contain octopine Ti plasmids.

The theory that the biotype of a strain cannot determine host range is supported by the fact that a LHR, biotype 3 strain can be converted to a WHR strain by integrating the cytokinin biosynthesis gene into its Ti plasmid (BUCHHOLZ and THOMASHOW, 1984; HOEKEMA *et al.*, 1984). However, transfer of the Ti plasmid from a biotype 3 strain to a biotype 2 strain and vice versa did not change the host range of these strains (OPEL and KERR, 1987). Their colonisation ability was essentially the same as the wild-type parent strains. These results therefore suggested that colonisation is primarily a function of the chromosomal background and that the determination of a strain's natural host range is encoded by the Ti plasmid. Although the Ti plasmid is not directly involved in a strain's biotype, there

is however a correlation between the biotype of a strain, the host range and the Ti plasmid it carries.

*A. tumefaciens* biotype 3 strains have been found to cause not only crown gall disease but also root decay of grape. MCGUIRE *et al.* (1991) analyzed biotype 1, 2 and 3 *Agrobacterium* strains for tumorigenicity, presence of Ti plasmid, ability to cause grape seedling root decay and pectolytic activity. All biotype 3 strains regardless of their tumorigenicity or presence of a Ti plasmid caused root decay and were pectolytic whereas none of the biotype 1 and 2 strains had these capabilities. Polygalacturonase was found to be produced by biotype 3 strains and the results from this study suggested that this compound played a role in grape seedling root decay (MCGURIE *et al.*, 1991).

#### 1.2.6 Host Range

A wide range of plants are susceptible to tumour and hairy root formation induced by *Agrobacterium* strains. The hosts for *Agrobacterium* are predominantly dicotyledonous plants, but also include a few gymnosperms (DE CLEENE and DE LEY, 1976; DE CLEENE and DE LEY, 1981) and a few monocotyledonous plants (SUSEELAN *et al.*, 1987; DOMMISSE and CONNER, 1989; DOMMISSE *et al.*

1990). The susceptible monocotyledons are limited to the *Liliales* and *Arales* (DE CLEENE and DE LEY, 1976). The strain specificity for plant genotypes and tissue types is well documented (BYRNE *et al.*, 1987; CHAREST *et al.*, 1989; HOBBS *et al.*, 1989; MORRIS *et al.*, 1989). Host range differences among individual *Agrobacterium* isolates have also been reported.

Specificity studies have indicated that a number of factors affect the host range of a bacterium. Although some chromosomal genes encode factors which play a role (see section 1.3.1.1), the Ti plasmid is the primary determinant of host range (LOPER and KADO, 1979; THOMASHOW *et al.*, 1980). It appears that determinants within the T-DNA region and the virulence region contribute to host specificity.

A number of researchers noted that the host range of a limited host range (LHR) strain could be extended by transferring the TL-DNA from a wide host range (WHR) plasmid or the cytokinin biosynthetic gene into it (BUCHHOLZ and THOMASHOW, 1984; HOEKEMA *et al.*, 1984 YANOFSKY *et al.*, 1985a and b). The inactivation of oncogene 4 of the plasmid pTiAch5, which codes for cytokinin biosynthesis, was found to eliminate this extension of the host range, proving the involvement of this gene in host range.

PAULUS *et al.* (1991a; 1991b) compared a number of limited and wide host range *A.* biotype 3 strains isolated from grapevine tumours and found that they carried related plasmids with two T-regions, TA and T. The WHR TA-region resembles the biotype 1 octopine region, whereas the LHR TA region was found to be a recent deletion derivative of the WHR TA region, lacking the *iaa* genes and part of the *ipt* gene. Sequences of the TA region of the ubiquitous LHR strain AB3 showed that the deleted region is replaced by an insertion sequence (IS) element, IS868, which resembles the IS51 element in *Pseudomonas syringae* subsp. *savastanal*. Biotype 3 octopine strains also carry an IS51-like sequence close to the TB *iaa* genes, and the results from this study confirm and extend earlier observations indicating that IS51-like elements in *Pseudomonas* and *Agrobacterium* are associated with *iaa* genes and played a major role in Ti plasmid evolution.

The exact mechanism of interaction between the bacteria and plant cells is still not clearly understood, and as a result of this it is necessary to screen a series of *Agrobacterium* strains on a range of plant genotypes for virulent strain/genotype combinations, if transgenic plants are to be produced. HINCHEE *et al.* (1988), for example, initially surveyed 100 cultivars for their *in vitro* response to *A. tumefaciens*-mediated transformation and then used the three most responsive strains in subsequent experiments to obtain transgenic soya bean.

A number of hypotheses have been formulated to explain the inability of *Agrobacterium* to form tumours or hairy roots on most monocotyledonous species and other susceptible plants. These include :-

- \* inability of *Agrobacterium* to bind to plant cell walls (RAO *et al.*, 1982);
- \* reduced activity of T-DNA promoters (GRAVES and GOLDMAN, 1986);  
and an
- \* 'abnormal' auxin-cytokinin balance in monocotyledonous cells (SCHAFER *et al.*, 1987).

The lack of a pronounced wound response is also thought to be an important factor, since any plant or tissue that proliferates large populations of wound-adjacent cells is considered competent for efficient transformation (POTRYKUS, 1990).

## **1.2.7 The Infection Process**

### **1.2.7.1 Colonisation and Attachment**

Colonisation is the close association that a microorganism forms with a plant. The most dense populations of *Agrobacterium* occur in the rhizosphere, the zone of soil that is influenced by root exudates. *Agrobacterium* numbers increase 100 - 1000 fold per gram of root than per gram of soil (MOORE and COOKSEY, 1981). These

bacteria have not only been isolated from plant roots but have also been detected in the vascular systems of some plants (LECHOCZKY, 1971; LECHOCZKY, 1979; BURR and KATZ, 1983; BOUZAR and MOORE, 1987).

Attachment is one of the first events in the *Agrobacterium*/plant interaction. Scanning electron microscopy has shown the presence of fibrils surrounding bacteria attached to tissue culture cells (MATTHYSSE *et al.*, 1981). These fibrils, which are synthesised by the bacteria, are composed of cellulose and form a network that anchors the bacteria to the plant surface. They can grow to such an extent that they trap other bacteria forming large clusters. Eventually large aggregates of plant cells, attached and entrapped bacteria and bacterial cellulose fibrils are formed, which can be seen with the naked eye. MATTHYSSE (1983) developed *A. tumefaciens* cellulose deficient mutants which lacked the ability to synthesise cellulose fibrils. These mutants were still able to attach to plant cells and induce tumours but did not form aggregates. This suggests that the function of cellulose fibrils is to anchor the bacteria firmly to the plant cell surface, thereby enabling the bacteria to colonise a plant efficiently.

Attempts to induce tumours without wounding have rarely been successful (KHAL, 1982), thus proving that wounding of a plant is required before tumour induction can take place. Wounding can occur during seed germination, accidentally during pruning

or cultivation, or as a result of insect and nematode damage (MOORE and COOKSEY, 1981). It was initially thought that a wound site was necessary in order to remove mechanical barriers, such as the cuticle, thus enabling bacterial attachment. However, it has now been shown that compounds produced by the wounded plant tissue are required to induce the *vir* gene region of the Ti plasmid (BOLTON *et al.*, 1986; DE CLEENE, 1988; SHIMODA *et al.*, 1990; STACHEL *et al.*, 1986). This is likely to be the situation for the Ri plasmid as well. The bacterium also possesses a highly sensitive chemotaxis system which responds to a variety of sugars, amino acids and phenolic compounds present in the wounded plant tissue (ASHBY *et al.*, 1987; DE CLEENE, 1988; LOACKE *et al.* 1988).

A range of phenolic compounds, believed to be precursors or breakdown products of lignin, are secreted by the damaged plant tissue. At least one of these, acetosyringone (AS), is a chemotactic attractant to *A. tumefaciens* strains such as C58 (ASHBY *et al.*, 1987). The optimal concentration of this compound for C58 attachment was  $10^{-7}$  M, which implies the constitutive expression of a very efficient specific recognition system in the bacterial cell. Cells lacking the Ti plasmid did not respond to AS (even though they were fully capable of other chemotactic responses) showing that the protein which responds to this compound is determined by the Ti plasmid. The specific receptor must be efficiently integrated into the bacterial chemotaxis and motility function, which is chromosomally determined, so that the

whole system operates efficiently (HEDGES and MESSENS, 1990). This provides a good example of the principle that chromosomes of bacteria belonging to the family *Rhizobiaceae* determine bacterial/plant interaction mechanisms, while the plasmids determine the specificity of both signalling and recognition of plant signals.

PARKE *et al.* (1987) disputed the ability of AS to act as an attractant but ASHBY *et al.* (1988) proved that it was an chemotactic attractant and was Ti plasmid encoded. Recent studies with narrow host range, biotype 3 *A. tumefaciens* strains (grapevine isolates) suggested that these strains were unable to respond to AS. The *virA* gene of these strains showed a marked difference from the homologies in typical wide host range strains and did not respond to AS or any other exudate from a plant which acts as host for only wide host range strains, implying that the *virA* gene product is a major specificity determinant (LEROUX *et. al.*, 1987)

Research has suggested that chemotactic responses initially attract the bacterium to specific wound sites. Following this, cells become attached to a specific plant cell wall receptor. The nature of the plant cell receptor to which *Agrobacterium* binds is unknown, but availability of receptors for attachment is considered to be one factor important in determining the host range of *Agrobacterium* (MATTHYSSE, 1986). The bacterial cell receptor for plant/*Agrobacterium tumefaciens* interactions was initially thought to be a lipopolysaccharide. *Agrobacterium* attachment to plant

cells was blocked when the plant cells were incubated with lipopolysaccharide extracted from *A. tumefaciens* (MATTHYSSE *et al.*, 1978). The cell wall receptor was also thought to be a galacturan (PUEPPKE and BENNY, 1981) but evidence now suggests that it could be a glycoprotein (BINNS and THOMASHOW, 1988).

In order to determine which genes and their products are involved in bacterial attachment, a number of mutants have been isolated which are unable to attach to plant cells (DOUGLAS *et al.*, 1982; PUVANISARAJAH *et al.*, 1985; CANGELOSI *et al.*, 1987; MATTHYSSE *et al.*, 1987a and b; THOMASHOW *et al.*, 1987). Although plasmid genes have been implicated in the attachment of *A. tumefaciens* to plant cells (MATTHYSSE, *et al.*, 1978) more recent research has focused on the chromosomal genes involved in this process. Four chromosomal genes were found to code for bacterial attachment to plant cells, *chvA*, *chvB*, *exoC* (*pscA*) and *att*.

The *chvB* and *exoC* genes are required for the synthesis of cyclic  $\beta$ -(1-2) glucan. The *chvA* gene product is thought to be an *A. tumefaciens* inner membrane protein which is involved in the secretion and modification of  $\beta$ -(1-2) glucan (IÑON DE LANNIO and UGALDE, 1989; CANGELOSI, *et al.* 1989). The requirement for  $\beta$ -(1-2) glucan in attachment has not been explained, but it is thought that glucans may provide osmotic protection, which is necessary for synthesis, stability and functioning of molecules which are directly involved in attachment (CANGELOSI,

*et al.* 1989).

Tn-5 induced mutants of *Agrobacterium* (*att*) were unable to attach to carrot cells and were avirulent. All *att* mutants analysed were found to be lacking particular polypeptides, which reappeared in revertants of the mutants. This suggests that particular polypeptides are also required for attachment to plant cells (MATTHYSSE *et al.*, 1987a) .

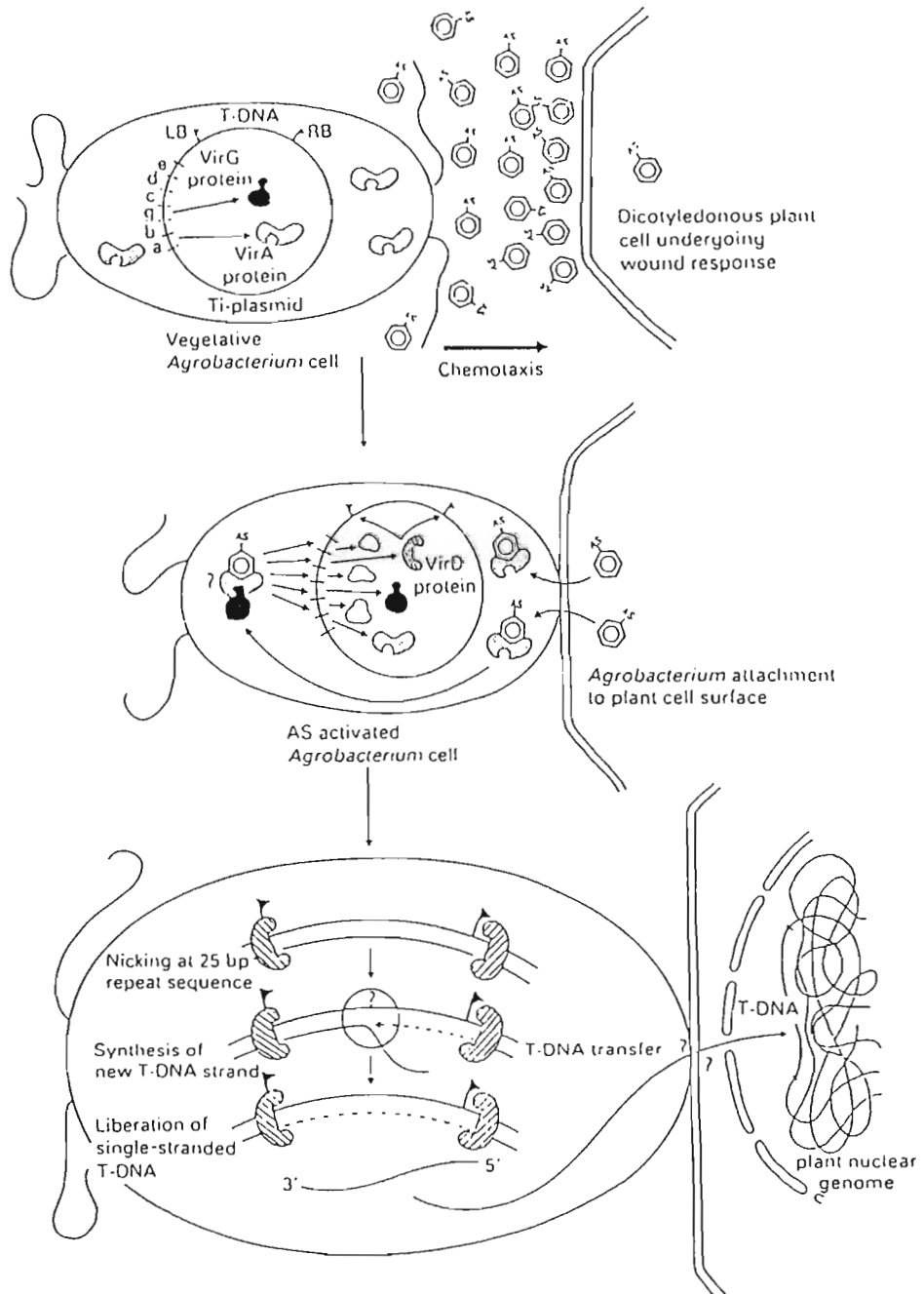
#### 1.2.7.2 Mechanism of T-DNA Transfer

Figure 2 details the *Agrobacterium*/plant interaction and the mechanism of T-DNA transfer. Products of the Ti and Ri plasmid virulence (*vir*) and chromosomal virulence (*chv*) loci direct the T-DNA transfer process. The *chv* loci specify binding of *Agrobacterium* to the plant cell (see section 1.3.1.1; DOUGLAS *et al.*, 1985).

##### 1.2.3.2.1 T-DNA Border Sequences

In the *Agrobacterium* Ti and Ri plasmids, the segment of DNA which is transferred (T-DNA) to a recipient cell is flanked by border sequences, which are 25 bp

imperfect direct repeats (ARMITAGE *et al.*, 1988; PETERSEN *et al.*, 1989; SRINIVASAN *et al.*, 1989). They are the only elements required in the *cis* orientation for mobilisation of the DNA into the plant cell (ZAMBRYSKI, 1988). T-DNA transfer is blocked by deletions of the first 6 bp and last 10 bp of these 25 bp sequences (WANG *et al.*, 1987a). Any DNA sequence placed between these borders can be transferred into plant cells. T-DNA transfer is initiated at the right hand border, consequently, the correct orientation is important; inversion results in a substantial reduction in efficiency of T-DNA transfer. Adjacent to the right hand border is a 24 bp enhancer element, known as overdrive (*ode*) which contributes to the efficiency of T-DNA transfer. Although *ode* is located 10-14 bp from the right hand border, it is active up to 7 kb away and can function in either orientation (PERALTA *et al.*, 1986).



**Figure 2.** *Agrobacterium*/plant interaction and mechanism of T-DNA transfer (AS = Acetosyringone) (From ARMITAGE, *et al.*, 1988).

#### 1.2.7.2.2 Virulence Regions of the Ti and Ri Plasmids

The virulence regions of the Ti and Ri plasmids are segments of approximately 40 kb and occur outside the T-DNA region. Although some *vir* gene products have been shown to be constitutively expressed, the *vir* loci are tightly regulated and their full activation initiates the transfer process. The *vir* region consists of six distinct operons (in order: *virA*, *virB*, *virG*, *virC*, *virD* and *virE*, reading clockwise towards the T-DNA) encoding trans-acting factors essential for T-DNA transfer (DE FRAMOND *et al.*, 1983; STACHEL *et al.*, 1985; BOLTON *et al.*, 1986; GELVIN and HABECK, 1990; SHIRASU *et al.*, 1990; GRANT *et al.*, 1991). The varying number of open reading frames which exist within each of these operons are strongly coordinately induced by phenolic compounds leached from the wound site on plants (SHIMODA *et al.*, 1990; STACHEL *et al.*, 1985; BOLTON *et al.*, 1986; SHORASU *et al.*, 1990; GRANT *et al.*, 1991). As mentioned (Section 1.3.1.1) the ability of plants to produce these signal molecules may be an important factor contributing to the host range of *Agrobacterium*. Numerous compounds activate the *vir* region and MELCHERS *et al.* (1989) surveyed 50 inducers. The results provided evidence for the basic structural features of a *vir*-inducing compound. It is a benzene molecule with an hydroxyl substituent at the R4 position and a methoxyl group at the R3 position. Six new *vir* inducers were identified of which gualacol illustrates the basic structural features required (MELCHERS *et al.*, 1989). Interestingly some

monocotyledons are unable to synthesise substances with strong *vir* inducing activity (USAMI *et al.*, 1988). This is a major barrier to gene transfer in these plant species.

*VirA* and *virG* loci are required for the induction of the remainder of the *vir* operons by external signals. They are continually expressed at low levels of expression and a substantial increase in the level of expression occurs in direct response to phenolic inducers (STACHEL and ZAMBRYSKI, 1986; WINANS *et al.*, 1988; PAZOUR and DAS, 1990). Acetosyringone and alpha-hydroxyacetosyringone were identified as the primary signal molecules present in exudates from wounded tobacco cells (STACHEL *et al.*, 1985). Other phenolic compounds were, however, also capable of partially or fully inducing the *vir* operon. In addition lignin precursors were also found to induce the *vir* genes (SHIMODA *et al.*, 1990).

VELUTHAMBI *et al.* (1989) showed that some opines enhanced the induction of the *vir* operon by phenolic compounds. In addition SHIMODA *et al.* (1990) found that a group of aldoses (L-arabinose, D-xylose, D-lyxose, D-glucose, D-mannose, D-idose, D-galactose and D-talose) could markedly enhance acetosyringone-dependent expression of the *vir* genes when the source of acetosyringone is limited, but they do not enhance expression of non-inducible genes.

The *virA* and *virG* proteins form part of a two-component positive regulatory

system, namely the sensor and activator. As the *virA* protein appears to be a trans-membrane protein, spanning the cytoplasmic membrane (WINANS *et al.*, 1989; JIN *et al.*, 1990), it is in the right position to act as the primary signal receptor (HOOYKAAS, 1989). The *virA* protein binds to the phenolic inducer, and the resulting complex is thought to act as a kinase phosphorylating the *virG* protein. The *virG* protein is converted in this way to a form capable of binding to specific DNA consensus sequences of various *vir* gene promoters. In this manner the activated *virG* protein increases transcription of its own gene and induces the transcription of *virB*, *virC*, *virD* and *virE* operons (ZAMBRYSKI, 1988). These loci are multigenic operons whose products mediate the site specific nicking of the T-DNA and its efficient transfer to the plant cell.

#### 1.2.7.2.3 T-Strand Synthesis and Transfer

Following *vir* gene activation and expression, a linear single stranded DNA molecule called the T-strand, is generated from the T-DNA region (STACHEL *et al.*, 1986a; ALBRIGHT *et al.*, 1987; JAYASWAL *et al.*, 1987; VELUTHAMBI, *et al.*, 1987). The process occurs as follow : The *virD* operon encodes two proteins, *virD1* and *virD2*, which have endonuclease activity capable of generating single-stranded site-specific nicks between the third and fourth base pairs on the bottom strand of the T-

DNA border repeats (HOOYKAAS, 1989; SRINIVASAN *et al.*, 1989; STECK *et al.*, 1990; WANG *et al.*, 1990) and are required for T-DNA processing in *A. tumefaciens* (STECK *et al.*, 1990). The *virD2* protein has been shown to function as a nuclear localizing protein in plant cells. The nuclear localization signal of *virD2* consists of two regions containing 4-5 amino acids located within the C-terminal 34 amino acids. Each region independently directs a  $\beta$ -glucuronidase reporter protein to the nucleus; however, both regions are necessary for maximum efficiency. The *virD2* protein is attached to the 5' terminus of the nicked right border T-DNA and a replicative process releases a single-stranded DNA molecule known as the T-strand (HERRERA-ESTRELLA *et al.*, 1988; ZAMBRYSKI, 1988). The process is terminated at the left border. In the case of plasmid pTiA6, nicks were found to occur between the fourth and fifth bases in both the right and left borders of the T-DNA. However, they occurred between the third and fourth bases in the right border of pTiC58 (ALBRIGHT *et al.*, 1987; WANG *et al.*, 1987b; WANG *et al.*, 1990). *VirD2* protein remains bound to the 5' end of the T-DNA (HOWARD *et al.*, 1989; SRIVINASAN *et al.*, 1989) and continues to bind tightly during the displacement of the T-strand from the T-DNA region of the Ti plasmid. The binding of the *virD2* protein is thought to act as either an unwinding protein during T-strand synthesis, or to protect the 5' end from exonucleases, or to pilot the complex to specific proteins in the bacterial membrane that facilitate its transfer to plant cells. It may however perform all these functions.

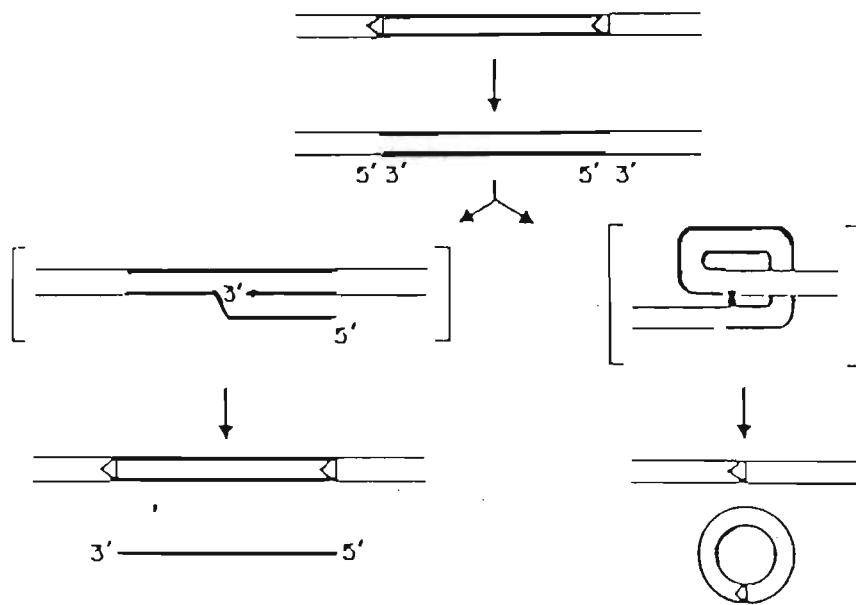
VirD2 has been shown to remain tightly bound to the 5' end of the T-strand during transfer from *A. tumefaciens* to the plant cell genome. This implies that T-strand transport is mediated by the tightly attached *virD2* protein via an import pathway common to higher eukaryotes (HOWARD *et al.*, 1992). The *virD2* protein therefore has a plant nuclear localization function. The *virE* region of the plasmid pTiA6 encodes two proteins, 7.0 and 60.5 kDa in size, both of which are required for tumour formation (WINANS *et al.*, 1987). The larger protein molecule is a non-specific single-stranded DNA-binding protein, the *virE2* protein, which is thought to bind to and protect the T-strand (DAS, 1988; SEN *et al.*, 1989). The T-strand-protein complex is believed to be an intermediary in the transfer of the T-DNA from *Agrobacterium* to plant cells. A model of its generation was developed by ARMITAGE *et al.* (1988) and is shown in Figure 3. The mechanism responsible for the release of the T-strand from the Ti plasmid is as yet not clearly understood. It is, however thought, that the T-strand formation results from replication and strand displacement. The process of T-DNA transfer has been shown to be analogous to bacterial conjugation (BINNS and THOMASHOW, 1988). This theory was substantiated by BUCHANAN-WOLLASTON *et al.* (1987) who found that the *mob* genes and *oriT* of the broad host range plasmid RSF1010 can mediate transfer of plasmid DNA from *Agrobacterium* to plant cells.

The overdrive enhancer element to the right of the right border has a highly

conserved 6 bp core and stimulates T-strand formation by interacting with the *virD2* protein and one, or both proteins encoded by the *virC* operon (PERALTA *et al.*, 1986; TORO *et al.*, 1988a and b). SRINIVASAN *et al.* (1989), however, showed that a synthetic 25 bp border sequence is sufficient for cleavage by the *virD* gene-encoded endonuclease and that overdrive sequences are not necessary for the cleavage catalysed by this endonuclease.

The DNA sequence of the *virB* operon has been analysed and was found to encode at least 11 proteins thought to form membrane-associated structures that may form a channel or channels, through which the T-strand-protein complex is exported (WARD *et al.*, 1988; HOOYKAAS, 1989).

An interesting finding is that the T-strands are not the only intermediate T-DNA forms. Circular T-DNA molecules called T-circles and double stranded T-DNA have been detected. T-circles occur at a much lower frequency than T-strands and are thought to be involved in the transfer of T-DNA to other bacteria (TIMMERMAN *et al.*, 1988). Figure 3 shows a schematic diagram of the generation of T-strands and T-circles. The double stranded T-DNA on the other hand, has been detected at high level, up to 40% of the total DNA and although its function has as yet not been determined it should be considered as a possible intermediate in T-DNA transfer (STECK *et al.*, 1989).



**Figure 3.** Release of T-DNA and possible vir-induced T-DNA rearrangements. Two potential products resulting from border nicks are the generation of single-stranded T-strands (left), or recombination-stimulated T-circle formation (right). Within the brackets are hypothetical intermediate structures. Thin lines represent Ti-plasmids sequences; thick lines T-DNA sequences; open triangles 25 bp T-region border sequences; and the interrupted thick arrow *de novo* synthesis of the bottom T-DNA strand (From TIMMERMAN *et al.*, 1988).

Other accessory virulence genes may be present in specific Ti or Ri plasmids and may help to determine host range for specific species (HOOYKAAS, 1989). An example of this is the *virF* locus found in octopine strains. This encodes a 22.4 kDa protein necessary for tumour formation on *Nicotiana glauca*. The *virF* locus is absent in nopaline strains which instead contain a *tzs* gene encoding the biosynthesis of the cytokinin trans-zeatin in *Agrobacterium*. The exact role this gene plays in the T-DNA transfer process is however, not well understood.

#### 1.2.7.3. T-DNA Integration

GHEYSEN *et al.* (1987) proposed a general model for T-DNA integration, in which:-

1. The T-strand is transferred to the plant cell as a protein DNA complex;
2. The right border and accompanying protein interact with a nick in the plant DNA;
3. This interaction causes strain on the plant genomic DNA and results in the production of a second nick on the opposite strand at varying distances from the first nick;
4. The T-strand is ligated to the plant DNA and the homologous strand

replicates; and

5. Replication and repair of the staggered nick in the plant target DNA results in both the production of a repeated sequence and additional sequence rearrangement at the ends of the inserted T-DNA element.

Molecular analysis of transformed plants has shown that T-DNA insertion can occur throughout the genome, i.e. at many different points, and that more than one insertion can occur in an individual plant cell. In addition, individual inserts can occur in repeated or single copy DNA sequences (BINNS and THOMASHOW, 1988). T-DNA integration is random with respect to the plant DNA sequences and is also often accompanied by rearrangement of both T-DNA and the target sequence (ZAMBRYSKI *et al.*, 1982). The right T-DNA/plant junction usually occurs within 10-40 bp of the right border while the left junction can be spread over 30 - 2000 bp.

#### 1.2.7.4. T-DNA Expression

After integration of the T-DNA from either a Ti or Ri plasmid into the plant nuclear genome, the T-DNA is transcribed and translated to produce proteins involved in tumour or hairy root formation and opine synthesis. Three genetic loci *tml*, *tmr* and *tms*(1 and 2) in the T-DNA have been identified by transposon and deletion

mutagenesis to be involved in tumour formation (GARFINKEL *et al.*, 1981; LEEMANS *et al.*, 1982; REAM *et al.*, 1983). Analogous genes were also found on nopaline plasmids (JOOS *et al.*, 1983). *Tml* locus (gene 6b) was found to govern size and rate of growth of the tumours as mutations in one region of the octopine Ti plasmid led to larger, faster growing tumours.

Mutations in the *tmr* locus (gene 4) resulted in root proliferation. The *tmr* gene encodes isopentenyl transferase, which catalyses the synthesis of the cytokinin isopentenyladenosine-5-monophosphate from dimethylallyl-pyrophosphate and 5'AMP (AKIYOSHI *et al.*, 1984; BARRY *et al.*, 1984).

Two other loci, *tms1* and *tms2*, were linked to shoot proliferation. The *tms1* gene codes for a product that converts tryptophan to indole-3-acetamide (VAN ONCKELEN *et al.*, 1986) and the *tms2* gene product converts indole-3-acetamide to indole-3-acetic acid which is the primary auxin in plants (INZÉ *et al.*, 1984; SCHRÖDER *et al.*, 1984).

It is the elevated levels of auxin and cytokinin, which occur as a result of the expression of the hormone biosynthetic loci, that result in cell proliferation and tumour- or hairy root formation. The transformed cells produce opines which are coded for by the T-DNA (Section 1.2.3) and these compounds are transported from

the proliferated plant tumour or hairy roots, to the surrounding agrobacteria where they are utilized as nitrogen, carbon, and in some cases phosphorus sources (MESSENS *et al.*, 1985)

Much research is still needed to understand fully the processes involved in oncogenesis and gene transfer by *Agrobacterium* before this system can be used to its full potential in plant improvement. In order to use *Agrobacterium* for plant transformation a number of steps must be optimised in both the bacteria and plant material (GRANT *et al.*, 1991). These include:-

- \* the identification of an *Agrobacterium* strain which 'infects' the appropriate plant genotype ( host susceptibility);
- \* the design and construction of modified T-DNA to allow gene expression in the plant cell;
- \* the transfer to and maintenance of the modified T-DNA in a specific *Agrobacterium* strain;
- \* the frequency of the T-DNA transfer events to plant cells being high enough to be detected; and
- \* the selection and regeneration of transformed plants cells.

These procedures, together with tissue culture developments are now widely used to create transgenic plants.

### 1.3            *AGROBACTERIUM TUMEFACIENS* AND BIOLOGICAL CONTROL OF CROWN GALL DISEASE

#### 1.3.1            General Background

Although crown gall disease is seldom fatal, gall formation causes a decrease in growth and vigour of the plant. The infected plants are less productive and more susceptible to other plant pathogens and to environmental stresses. The galls/tumours can be found on roots, crowns, leaves and stems of plants and can vary dramatically in size so that galls may weigh anything from less than one gram to over forty grams (MOORE and COOKSEY, 1981). Crown gall disease has been extensively reviewed (MOORE and WARREN, 1979; KERR, 1980; DEPICKER *et al.*, 1983; KERR and TATE, 1984; NESTER *et al.*, 1984; GHEYSEN *et al.*, 1985; GHEYSEN *et al.*, 1987; GLENISTER, 1987; THOMSON, 1987; POWELL and GORDON, 1989; HEDGES and MESSENS, 1990; RYDER and JONES, 1990; WEBSTER, 1990; NESTER and GORDON, 1991) and many aspects of the biology of the disease have been covered in the book by KAHL and SCHELL (1982). Research has shown that the majority of the genes responsible for crown gall induction are plasmid borne (Section 1.2.3).

As mentioned in section 1.2.5, *Agrobacterium* strains are classified into three

biotypes based on carbon source utilization and other biochemical tests (KERR and PANAGOPOULOS, 1977), which cause economic loss on a number of plant species in many countries throughout the world. In Australia the main pathogenic forms are biotype 2 nopaline/agrocinopine A strains. In Spain biotype 2 strains are more prevalent on stone fruit and biotype 1 are more common on roses (LÓPEZ *et al.*, 1987). The biotype 3 strains that infect grapevines have worldwide distribution and are also an economic problem in South Africa. The host range of biotype 3 is comparatively small and is usually determined by the Ti plasmid (THOMASHOW *et al.*, 1980; RYDER and JONES, 1990; Section 1.2.4).

### 1.3.2 Biological Control of Crown Gall Disease

KERR and associates (NEW and KERR, 1972; HTAY and KERR, 1974; KERR and HTAY, 1974) developed a successful biological control system for crown gall disease which has been sold commercially in Australia since 1973 and is currently used in many countries world-wide. The system involves inoculating planting stock with non-pathogenic *A. radiobacter* strain K84. This will be discussed in more detail later. Research results have shown that a successful biocontrol agent must firstly be able to produce sufficient amounts of an effective antibiotic-like substance (bacteriocin) and secondly be able to colonize plants efficiently. Strain K84 has both

these properties, hence its success.

Although a number of commercially available antibacterial chemicals have been used to control crown gall (SCHROTH *et al.*, 1971; MOORE, 1977), biological control using strain K84 is often used in preference to these compounds as it is less expensive and more efficient. MOORE (1977), for example, found that K84 performed better than commercial chemical treatments in preventing crown gall on cherry seedlings.

As with other methods of crown gall control, strain K84 can only be used as a preventative measure and cannot stop the disease after infection has taken place. In addition it does not control crown gall in all situations. Octopine strains of biotype 1 as well as biotype 3 strains that cause crown gall on grapevines are resistant to agrocin 84, a bacteriocin produced by strain K84, and are therefore not controlled by it. However, on occasions, strain K84 has been found to be able to partially control agrocin 84 - resistant strains of biotype 1 and 2, possibly due to its ability to colonize roots effectively (MOORE, 1977; LÓPEZ *et al.*, 1987) thereby out-competing the agrocin 84-resistant pathogen for attachment sites.

Another strain of *A. radiobacter*, HLB-2, has been found which reduced the incidence of crown gall disease on grapevines and sunflower seedlings by 85.6 and

83.5 % in field experiments, while the disease incidence on naturally infected grapevines was reduced by 100% (YOU *et al.*, 1991).

### 1.3.3 Biological Control Using *A. radiobacter* Strain K84

Non-pathogenic, biotype 2 *A. radiobacter* strain K84 was isolated from soil around a crown gall-affected peach tree near Adelaide, South Australia in 1972 (NEW and KERR, 1972). It was shown that when peach seedlings were potted in soil artificially inoculated with K84 and a pathogen, 100 % inhibition of gall formation was achieved. Since then, there have been numerous publications on the experimental use of strain K84 to control crown gall disease. MOORE (1979) tabulated the use of strain K84 against crown gall on different host plants, including *Rosaceae*, *Jugandaceae*, *Compositae* and *Salicaceae*, under both experimental and nursery conditions. Although the degree of control achieved varied, strain K84 was very effective in most cases, often giving complete control in naturally infested soils.

The control method involves dipping or spraying planting stock, bare-rooted root stock, cuttings, seed, seedlings, aerial grafts and plants with a suspension of non-pathogenic *A. radiobacter* strain K84 at  $10^7$  to  $10^9$  viable cells per ml immediately before planting (MOORE, 1988). This treatment allows strain K84 to colonize the

plants, cuttings etc. thereby protecting any wound sites that may occur in the cultivation process. A high population of K84 is necessary to maintain adequate titre of the strain in the rhizosphere. The rhizosphere populations of K84 were found to decline with time. Six months after inoculation and planting only 10% of strain K84 was recovered from root washings. This population level is however, thought to be adequate for protection (MOORE, 1988). The protection afforded by such a low titre of K84 can be explained by the fact that the soil pathogenic strains of *Agrobacterium* are outnumbered by non-pathogens (MOORE and COOKSEY, 1981).

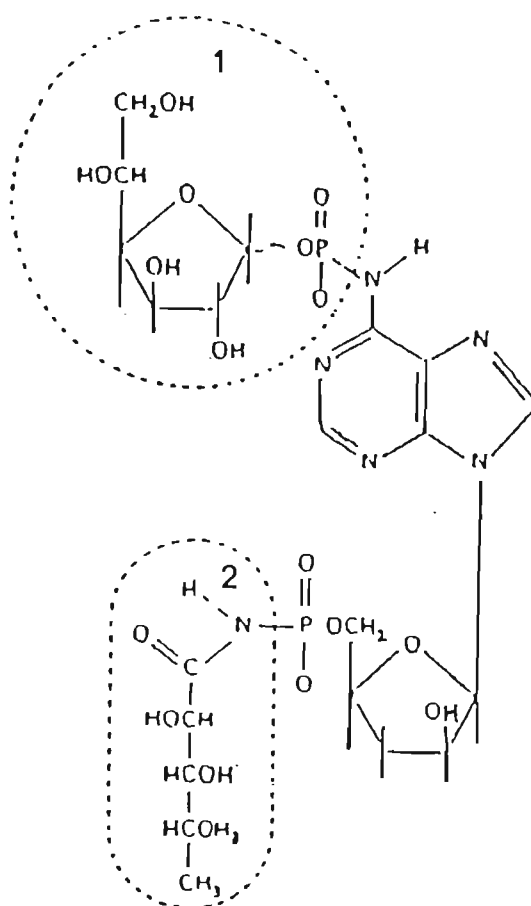
The widespread commercial use of this method is a testament to its practical nature and usefulness, and the success of strain K84 is remarkable considering the range of conditions in which it has been used (RYDER and JONES, 1990).

#### **1.3.3.1 Mechanism of Control of Crown Gall Disease in Strain K84**

Three factors contribute to the success of strain K84 as a biological control agent:- the production of agrocin 84, the ability to survive in the soil and the ability to colonize root surfaces efficiently (KERR and TATE, 1984).

#### 1.3.3.1.1 Agrocine 84 Structure, Mechanism of Action, Production and Sensitivity

The antibiotic agrocine 84, is an adenine nucleotide with a 9-(3-deoxy- $\beta$ -D-threopentofuranosyl)-adenine core and two substituent groups (Figure 4; TATE *et al.*, 1979; THOMPSON *et al.*, 1979; MURPHY *et al.*, 1981) and is thought to act as an inhibitor of DNA synthesis (DAS *et al.*, 1978; MURPHY and ROBERTS, 1979). MURPHY *et al.* (1981) demonstrated, using  $^{32}\text{P}$  labelled N<sup>6</sup>-monophosphorylated nucleotide lacking the 5'-substituent, that this compound was taken up by the cell and would inhibit the uptake of agrocine (substituent 1, Figure 4). Toxicity was found to be due to the presence of the 5'-phosphoramidate (substituent 2, Figure 4). Agrocine 84 is especially toxic to *Agrobacterium* strains carrying a nopaline / agrocinopine A type Ti plasmid and K84 is normally only effective against such strains (KERR and HTAY, 1974; ROBERTS and KERR, 1974). Agrocine 84 is taken up by these nopaline/agrocinopine A strains via agrocinopine permease (ELLIS and MURPHY, 1981). Part of the agrocine 84 molecule mimics the structure of agrocinopine A, the usual substrate recognised by agrocinopine permease. HAYMAN and FARRAND (1988) confirmed the involvement of agrocine 84 in the biological control process, by showing that bacterial mutant strains that were not able to take up agrocinopine were no longer sensitive to agrocine 84.



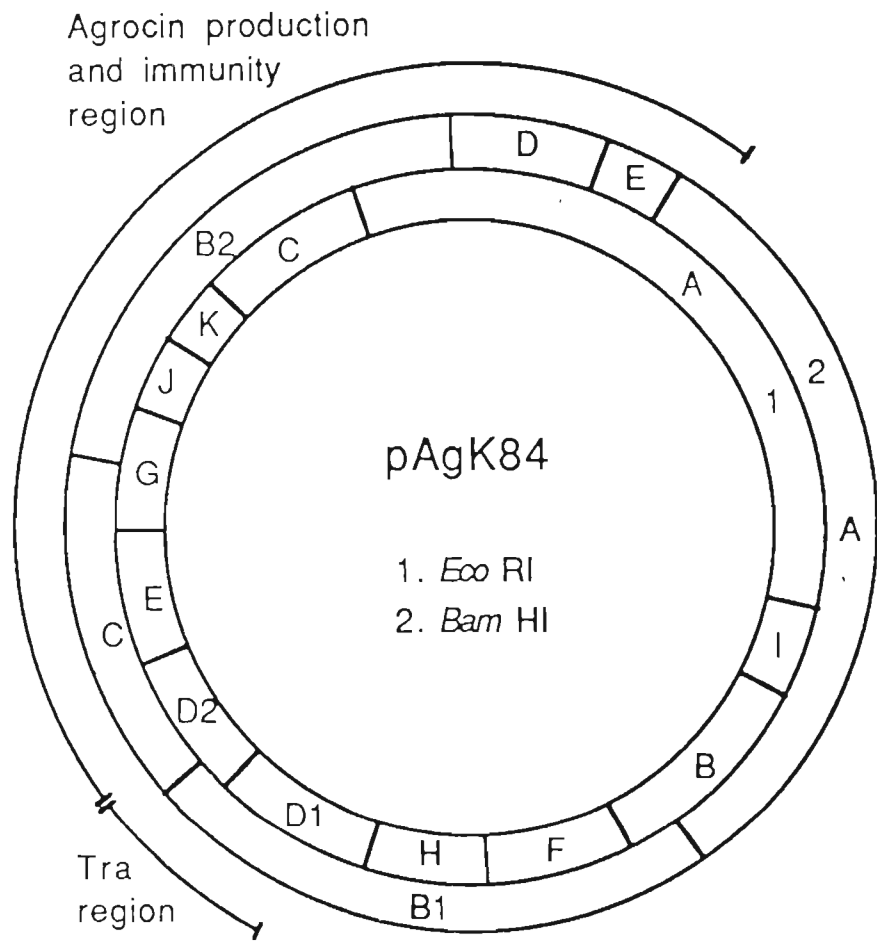
**Figure 4.** The structure of agrocin 84. Substituent 1 determines uptake specificity, while substituent 2 confers antibiotic activity (MURPHY *et al.*, 1981).

Two key lines of evidence have materialised for the involvement of agrocin 84 in the control of crown gall and they can be summarised as follows (KERR, 1984) :-

- \* Only agrocin-sensitive strains such as nopaline / agrocinopine strains (which are responsible for most crown gall disease in orchards and nurseries) are controlled by strain K84 (KERR and HTAY, 1974; KERR and PANAGOPOULOS, 1977; KERR and TATE, 1984).
- \* The transfer of pAg84 to other agrobacteria confers on them the ability to synthesise agrocin and also to control crown gall (ELLIS and KERR, 1979).

#### 1.3.3.1.2 Agrocin 84 Plasmid

*A. radiobacter* strain K84 carries three plasmids (MERLO and NESTER, 1977), a cryptic plasmid, a 200 kb plasmid called pAgK84b (or pNOC) which encodes nopaline catabolism, and a small 47.7 kb plasmid called pAgK84 which carries the genes for agrocin biosynthesis (ELLIS *et al.*, 1979; SLOTA and FARRAND, 1982). This latter plasmid has been physically and genetically characterised and regions coding for agrocin production, immunity to agrocin and plasmid transfer have been located (Figure 5; ELLIS *et al.*, 1979, SLOTA and FARRAND, 1982; FARRAND *et al.*, 1985; RYDER *et al.*, 1987).



**Figure 5.** BamHI and EcoRI restriction map of pAgK84 showing the regions coding for plasmid transfer, agrocin 84 synthesis and immunity to agrocin. (From RYDER and JONES, 1990).

The agrocin plasmid not only has its own transfer function (FARRAND *et al.*, 1985), but can also be mobilized by other agrobacteria at the same time as pNOC, the nopaline catabolic plasmid of strain K84 (ELLIS *et al.*, 1979). The transfer frequency of pAgK84 is highest in the presence of nopaline, which induces the conjugal transfer of pNOC, and in the presence of pNOC. FARRAND *et al.* (1985) mapped the transfer (*tra*) region of pAgK84, which codes for conjugal transfer, by Tn5 mutagenesis. It was found to be 3.5 kb long (Figure 5). As pAg84 is self-conjugal the construction of a *tra*<sup>-</sup> mutant of pAg84 was proposed to overcome this problem. A *tra*<sup>-</sup> mutant, strain K1026, was constructed which is just as effective as strain K84 at controlling gall formation, and therefore has potential as a new biocontrol strain (CLARE *et al.*, 1990; JONES *et al.*, 1988; JONES and KERR, 1989; SHIM *et al.*, 1987; WEBSTER, 1990).

#### **1.3.3.1.3 Other Factors Involved in Biological Control of Crown Gall by Strain K84**

Although there is a high correlation between *in vitro* sensitivity to agrocin and biological control by strain K84 evidence is mounting to show that other factors are involved in the success of this system. These include survival and growth of *A. radiobacter* K84 in the soil and rhizosphere and its ability to colonise plants

efficiently (ELLIS *et al.*, 1979; SHIM *et al.*, 1987; MACRAE *et al.*, 1989).

Not all strains carrying pAgK84 are effective biocontrol agents. The chromosomal background (biotype) in which pAgK84 resides was found to influence the ability of the strain to control crown gall. When agrocin production was, for example, transferred from K84, a biotype 2 strain, to biotype 1 strains, the new strains produced agrocin but did not control the disease as effectively as K84 (ELLIS *et al.*, 1979; SHIM *et al.*, 1987). The biotype 2 strains containing pAgK84 were found to be more efficient long term colonisers of almond seedlings roots than the biotype 1 pAgK84 containing strains (SHIM *et al.*, 1987). Colonisation by these strains was initially similar, but by about three months after inoculation biotype 2 populations in the rhizoplane (root surface) were consistently three to four times higher than those of the biotype 1 strains.

The host plant also appears to affect the mechanism of control by K84. DU PLESSIS *et al.* (1985) showed that gall formation by an agrocin resistant strain, K285, was controlled by strain K84 on tomato seedlings but not on chrysanthemums. This could possibly be due to the fact that K84 may not be able to attach to the wound site as efficiently as some pathogens on certain hosts .

Evidence from colonisation studies has clearly demonstrated that the ability of the biocontrol bacterium to colonise and maintain a sufficient population density for a

sufficient length of time is critical to the success of this method.

#### **1.3.3.1.4 Breakdown of Biological Control of Crown Gall by Strain K84.**

Strain K84 usually controls crown gall disease caused by the nopaline/ agrocinopine A type pathogenic *Agrobacterium* strains. However, biological control can break down and there are several reason why this could happen. Three problem areas will be discussed, all of which appear to be associated with agrocin 84 production. Other factors important in biological control, such as root colonisation, appear to be stable for K84.

**a. Pathogen immunity due to transfer of the agrocin plasmid to pathogenic strains:** PANAGOPOULOS *et al.* (1979) identified a serious problem that can occur when using strain K84 to control crown gall, viz., genetic transfer between strain K84 and the pathogen. Results from field trials showed that the agrocin plasmid, pAgK84, had been conjugally transferred to the pathogen, which was then able to produce agrocin, becoming immune to the toxin and therefore no longer subjected to biological control. It is believed that the occurrence of these events was undoubtedly due in part to the high population densities of both the pathogen and strain K84 (1:1), which were each introduced artificially during experiments.

Nevertheless, the possibility exists that this could also occur under normal biological control practice using K84. ELLIS *et al.* (1979) also demonstrated this type of break down in biocontrol by K84 under *in vitro* conditions. Although the transfer frequency *in vitro* was markedly higher in the presence of nopaline, it would also presumably be higher in the vicinity of a gall containing nopaline, than in the soil or on the root at the same distance from a gall.

**b. The biocontrol strain becomes pathogenic by the transfer of a Ti plasmid to K84:** Biological control can also be broken down by the transfer of the Ti plasmid from a pathogenic strain to K84. This results in the biocontrol agent becoming pathogenic while retaining agrocin 84 production and immunity (RYDER and JONES, 1990). No data is available on the transfer frequency of the Ti plasmid into strain K84 and occurrence of such an event is probably less common than the transfer of pAgK84 from strain K84 to a pathogen. This is due to the fact that K84 also carries a Ti-like plasmid (pNOC) which codes for the catabolism of nopaline, but does not confer virulence on strain K84. Ti plasmids of pathogenic strains which are successfully controlled by K84 are incompatible with the plasmid pNOC of strain K84. These plasmids therefore cannot coexist in the same cell. Thus for this event to occur, strain K84 would first (or simultaneously) have to lose the nopaline catabolism plasmid (pNOC) in order to accept the incoming Ti plasmid. pNOC is however, stably maintained in strain K84 so this event appears very unlikely and has

never been observed experimentally (RYDER and JONES, 1990).

**c. Resistance to agrocin as a result of spontaneous mutation:** In laboratory studies *Agrobacterium* strains sensitive to agrocin 84 were found to mutate at relatively high frequencies to become resistant to the antibiotic (SÜLE and KADO, 1980; COOKSEY and MOORE, 1982b; COOKSEY, 1986). Some of these resistant mutants retained their pathogenicity while other appeared to have lost their Ti plasmid (COOKSEY and MOORE, 1982b). The genetic locus for sensitivity to agrocin 84 is located on the Ti plasmid (ENGLER *et al.*, 1975; HAYMAN and FARRAND, 1988) and this is presumably where the mutation to resistance occurs, either by a deletion or a point mutation.

It is not known whether resistant mutant strains occur in the soil, and if they do, with what frequency they arise. Agrocin-resistant mutants would, however, be difficult to combat (RYDER and JONES, 1990). Some protection against such spontaneous agrocin 84-resistant pathogenic mutants could be achieved by using strain K84 in combination with another agrocin producing strain, in the form of a mixed inoculum.

Although the three mechanisms described above could cause the breakdown of biological control they are not the main deficiencies attributed to strain K84. This

strain is an extremely successful biocontrol agent for crown gall disease, but only on certain hosts. Furthermore, it is most effective only when the pathogens are strains that harbour nopaline/agrocinopine A Ti plasmids, and in some cases it was unable to control pathogenic strains that were susceptible to agrocin 84 (KERR and PANAGOPOULOS, 1977; MOORE, 1979; PANAGOPOULOS *et al.*, 1979). In other instances strain K84 was able to afford a certain measure of control against pathogens resistant to agrocin 84 (MOORE, 1977; LÓPEZ *et al.*, 1989). Reports have also shown that K84 is unable to control crown gall disease on grapevines which is caused by biotype 3 pathogens (PANAGOPOULOS *et al.*, 1978; PERRY and KADO, 1982).

The limited biological control of crown gall afforded by strain K84, has resulted in a search by numerous researchers throughout the world, for potential biocontrol strains that exhibit broader host ranges than K84 (HENDERSON *et al.*, 1983; RYDER and JONES, 1990; WEBSTER, 1990; HASS *et al.*, 1991). Research to extend the effectiveness of K84 has also been undertaken, with the focus being on the development of a derivative of K84, such as K1026, in which the agrocin plasmid would be stable and no longer transferrable to pathogenic strains (CLARE *et al.*, 1990; RYDER and JONES, 1990).

#### 1.3.3.1.5 Search for Alternative Biocontrol Agents

As already mentioned, biological control by strain K84 is limited to a narrow host range of pathogenic *A. tumefaciens* strains which carry nopaline / agrocinopine A Ti plasmids, which are sensitive to agrocin 84. The incidence of crown gall on commercially produced roses in Australia has been reduced to 1 % using Strain K84. The high success rate of controlling crown gall disease in Australia can be attributed to the fact that the population of *A. tumefaciens* in that country appears to be homogeneous (MGOORE and WARREN, 1979). Most Australian pathogens are apparently biotype 2 strains carrying nopaline type Ti plasmids. However, the diverse populations of pathogenic *A. tumefaciens* strains found in other countries has necessitated a search for alternative biocontrol strains.

Seventeen of the 620 bacteriocin-producing *A. tumefaciens* strains isolated from Greek soils by KERR and PANAGOPOULOS (1977) were found to produce a bacteriocin with a different host range to that of strain K84. Unfortunately none of the strains were effective as biocontrol agents. Only two bacteriocin-producing biotype 2 *A. tumefaciens* strains were isolated in New Zealand (SPIERS, 1980). Their antibiotics were effective *in vitro*, however, no reports have yet been published on their *in vivo* effectiveness.

In Israel, although all three biotypes can potentially induce crown gall disease on grapes, *A. tumefaciens* biotype 3 is the greatest potential threat to nurseries and vineyards (HASS *et al.*, 1991). Crown gall disease of deciduous fruit trees, grapevines and some ornamental plants, eg., roses, is a common and costly disease in South Africa (DU PLESSIS *et al.*, 1984). All three biotypes have been isolated locally and many of these *A. tumefaciens* strains, particularly the grapevine isolates, are resistant to agrocin 84. Fungal (*Penicillium* and *Aspergillus*) and bacterial (*Bacillus*, *Pseudomonas* and *A. radiobacter* strains) antagonists were also isolated and tested for biological control of crown gall (COOKSEY and MOORE, 1980). Although these organisms reduced the incidence of crown gall on cherry seedlings, they were not as effective as K84.

The South African search for alternative biocontrol strains has centred around the isolation of antibiotic-producing strains of *A. tumefaciens* that exhibit a broader activity spectrum than K84. *A. tumefaciens* strain D286 was the first potential biocontrol strain isolated (HENDSON *et al.*, 1983). Its activity spectrum *in vitro* was broader than that of K84, inhibiting pathogenic strains carrying nopaline, octopine and agropine Ti plasmids. However, when tested *in vivo* against sixty five *A. tumefaciens* strains, representing all three biotypes, strain K84 was able to control crown gall formation more efficiently than D286. Both strains alone or in combination failed to control biotype 3 strains (VAN ZYL, *et al.*, 1986).

The second potential strain, J73, was a biotype 2 strain (WEBSTER *et al.*, 1986). The results of *in vitro* assessments clearly showed that this strain produced a potent agrocin, which had a broad activity spectrum effective against all three biotypes and strains carrying octopine, nopaline and agropine Ti plasmids (WEBSTER, 1990). Although this strain was originally pathogenic it was cured of its Ti plasmid. The agrocin 84 plasmid, pAGK84, was also introduced into this strain with the aim of improving its biocontrol potential. However, *in vivo* biological control by J73 was disappointing and the data reported in Chapter 2 of this dissertation details reasons for the poor biological control by *A. tumefaciens* strain J73.

A number of agrocin-producing pathogenic and non-pathogenic biotype 3 strains were also isolated in South Africa (STAPHORST *et al.*, 1985). Some of these isolates reduced the incidence of crown gall disease in glasshouse experiments. Two of the non-pathogenic strains, H6 and F2/5, showed such potential for the control of crown gall on grapevines that they were selected for further investigation. Unfortunately, the non-pathogenic biotype 3 agrocin-producing strains had a narrow host range and were ineffective against biotype 1 and 2 strains. In addition, pathogenic biotype 3 strains that were effective against biotype 1 and 3 strains were not effective against the biotype 2 strains tested (WEBSTER, 1990). The role of colonisation in effective biological control by H6 is also reported in this dissertation.

## 1.4 *AGROBACTERIUM RHIZOGENES* AND ITS ROOT-INDUCING ABILITY

### 1.4.1 General Background

*A. rhizogenes* is a soil bacterium with a host range confined to dicotyledonous plants (STROBEL and NACHMIAS, 1985). Unlike *A. tumefaciens* which causes unorganised neoplastic growth (KAHL and SCHELL, 1982; NESTER *et al.*, 1984) *A. rhizogenes* infection results in the proliferation of secondary functional roots, a condition known as "hairy root" disease (RIKER, 1930; HILDEBRAND, 1934; ELLIOT, 1951). This disease occurs as a result of the genetic modification of the host genome by the insertion of a fragment of T-DNA from the Ri plasmid of this bacterium (CHILTON *et al.*, 1982). Hairy root disease, unlike crown gall disease is however, not a disease of major economic importance and therefore *A. rhizogenes* has not been given the same attention as *A. tumefaciens*.

The first transgenic plants using *A. rhizogenes* were produced in 1973 by inducing roots on tobacco plants, producing callus from these and then regenerating into plants (ACKERMAN, 1977). The presence of the Ri T-DNA in descendants of the original regenerates was confirmed by molecular hybridization (C. ACKERMAN, M. BOPP and D. TEPFER unpublished data - see TEPFER, 1990). In 1981

TEPFER and TEMPÈ regenerated plants directly from *A. rhizogenes* transformed root cultures. As a result of these findings several different research groups began studying the Ri plasmid of *A. rhizogenes* and the organisation and expression of the T-DNA in transformed tissue (CHILTON *et al.*, 1982; SPANO *et al.*, 1982; WHITE *et al.*, 1982; BIROT *et al.*, 1987; PETERSEN *et al.*, 1989; FALLIA *et al.*, 1990; SHEN *et al.*, 1990; OONO *et al.*, 1990; CHIRIQUI *et al.*, 1991; MACRAE, 1991).

#### 1.4.2 The Selectable Markers of the Ri Plasmid

The Ri T-DNA provides two selectable markers that function in root organ cultures; the first is accelerated growth and the second is increased branching and plagiotropism (TEPFER, 1990). The T-DNA of the Ri plasmid has been shown to code for at least three genes that can individually induce root formation. Together they cause hairy root formation in transformed tissue and impose a high auxin sensitivity on the infected tissue (PETERSEN *et al.*, 1989; SHEN *et al.*, 1990; Section 1.2.2). As a result of the insertion of these genes into the plant genome the phenotype of roots transformed by *A. rhizogenes* differs from untransformed roots and this allows them to grow readily in culture (TEPFER and TEMPÈ, 1981; TEPFER, 1982; TEPFER, 1983a; TEPFER, 1983b; TEPFER, 1984; CAPONE *et*

*al.*, 1989). Transformed roots do however, closely resemble roots *in situ*, they are genetically stable and easy to manipulate.

The T-DNA region of the Ri plasmid also carries a set of morphological markers (for example wrinkled leaves, short internodes, altered flower morphology) which are expressed in regenerated plants (ACKERMAN, 1977; TEPFER, 1982; TEPFER 1984; DURAND-TARDIF *et al.*, 1985; SPENA *et al.*, 1987; SCHMULLING *et al.*, 1988; SINKAR *et al.*, 1988) providing a simple and accurate way of identifying transformed material. OONO *et al.* (1990), for example, found that progeny of selfed transgenic tobacco plants carrying the hairy root-inducing *rolC* gene (ORF12 of the T-DNA of the Ri plasmid) from *A. rhizogenes* showed stable inheritance of dwarfness and reduced floral organs. Those transgenic plants that express the dwarf character also flowered earlier than normal. Interestingly, expression of these markers in regenerants was found to vary from species to species, and from clone to clone within a species and from individual to individual within a clone (TEPFER, 1984; DURAND-TARDIF *et al.*, 1985). In addition the markers segregated with the Ri T-DNA during meiosis (TEPFER, 1984).

When *A. rhizogenes* is used as a vector to insert useful foreign genes into plant tissue, eg. herbicide resistance genes, the resultant altered morphology of regenerated transgenic plants may not be desirable. This phenomenon could detract

from the potential use of *A. rhizogenes* as a vector.

#### 1.4.3 Hairy Roots and Their Uses

The ability to mass culture transformed roots in the absence of the aerial parts of the plant has been studied since the early 1980's. *A. rhizogenes* transformed root cultures have been produced from at least 133 plant species and transformed plants have been regenerated from 48 of these (Table 2; TEPFER, 1990). One of the main reasons for working with the *A. rhizogenes* system is that root organ cultures can be produced from many plant species (Table 2) which produce secondary metabolites of commercial importance. Transformed root cultures were initiated in many species as a method of producing these metabolites *in vitro* (FLORES and FILNER, 1985; KAMADA *et al.*, 1986; MANO *et al.*, 1986; RHODES *et al.*, 1986; FLORES *et al.*, 1987; JUNG and TEPFER, 1987; HAY *et al.*, 1988; PARR, 1988). More recently hairy root cultures of *Armoracca lapathitolis* (horseradish) were found to produce high levels of isoperoxidase and shown to have potential as a means of peroxidase production (SAITOU *et al.*, 1991).

Table 2. Species transformed by *A. rhizogenes*. (Modified from TEPFER, 1990).

Species	Roots <sup>*</sup>	Plants	Reference
<i>Abrus precatoris</i>	yes <sup>*</sup>	no	K. Soo Ko (Unpubl.)
<i>Ambrosia artemisiifolia</i> (ragweed)	yes	no	Mugnier, 1988
<i>Anacardium occidentale</i> L (cashew)	yes <sup>*</sup>	no	S. MacRae (Unpubl.)
<i>Anagallis arvensis</i> (pimpernel)	yes	yes	Mugnier, 1988
<i>Anchusa officinalis</i>	yes	no	Mugnier, 1988
<i>Antirrhinum majus</i> (snapdragon)	yes <sup>*</sup>	no	Hamill <i>et al.</i> , 1987
	yes	yes	Mugnier, 1988
<i>Antirrhinum majus</i> L	yes <sup>*</sup>	yes <sup>*</sup>	Handa, 1992
<i>Arabidopsis thaliana</i>	yes	yes	Pavingerova & Ondrei, 1986
<i>Arachis hypogaea</i> (peanut)	yes	no	Mugnier, 1988
<i>Aristolelia australisica</i>	yes <sup>*</sup>	no	E. Davioud (unpubl.)
<i>Artemisia annua</i>	yes <sup>*</sup>	no	E. Davioud (unpubl.)
<i>Artemisia lapathifolia</i>	yes <sup>*</sup>	yes <sup>*</sup>	Saitou <i>et al.</i> , 1991
<i>Armoracia rusticana</i> (horse radish)	yes <sup>*</sup>	yes <sup>*</sup>	Noda <i>et al.</i> , 1987
<i>Atropa belladonna</i> (belladonna)	yes	yes	Kamada <i>et al.</i> , 1986
	yes <sup>*</sup>	yes <sup>*</sup>	Jung and Tepfer, 1987
	yes	yes <sup>*</sup>	Saito <i>et al.</i> , 1991
<i>Atropa caucasica</i>	yes	no	E.Knopp & A.Strauss (unpubl.)
<i>Beta vulgaris</i> (sugar beet & red beet)	yes <sup>*</sup>	yes <sup>*</sup>	A. Yacoub & A.Tepfer (unpubl.)
	yes	no	Hamill <i>et al.</i> , 1986
	yes	no	Mugnier, 1988
<i>Bidens sulphureus</i>	yes	no	Flores & Filner, 1987
<i>Brassica chinensis</i>	yes <sup>*</sup>	no	G.-L. Chi (unpubl.)
<i>Brassica hirta</i> Moench (mustard)	yes	no	Mugnier, 1988
<i>Brassica napus</i> var. <i>Oleifera</i> (oilseed rape)	yes <sup>*</sup>	yes <sup>*</sup>	Ooms <i>et al.</i> , 1985
	yes <sup>*</sup>	yes <sup>*</sup>	Guerche <i>et al.</i> , 1987
	yes <sup>*</sup>	yes	Dusabkova <i>et al.</i> , 1989
<i>Brassica oleracea</i> (cauliflower)	yes <sup>*</sup>	no	Petit <i>et al.</i> , 1983
	yes <sup>*</sup>	yes <sup>*</sup>	David and Tempé, 1988
<i>Brassica oleracea</i> (cabbage)	yes <sup>*</sup>	yes <sup>*</sup>	J. Tourneur (unpubl.)
<i>Brassica pekinensis</i>	yes <sup>*</sup>	no	G.-L. Chi (unpubl.)
<i>Brassica rapa</i> (turnip)	yes <sup>*</sup>	no	Tanaka <i>et al.</i> , 1985
<i>Calystegia spium</i> (morning glory)	yes	no	Tepfer, 1982
<i>Cassia torosa</i>	yes	no	K. Soo Ko (Unpubl.)
<i>Cassia obtusifolia</i>	yes	no	K. Soo Ko (Unpubl.)
<i>Cassia occidentalis</i>	yes <sup>*</sup>	no	K. Soo Ko (Unpubl.)
<i>Catharanthus roseus</i>	yes <sup>*</sup>	no	Brillianceau, 1986
	yes	no	E. Aird <i>et al.</i> (unpubl.)
	yes <sup>*</sup>	no	Hamill <i>et al.</i> , 1987
<i>Catharanthus trichophyllus</i>	yes	no	E. Davioud (unpubl.)
<i>Centaurea cyanus</i> (cornflower)	yes	no	Mugnier, 1988
<i>Cichorium endivia</i>	yes	no	Mugnier, 1988
<i>Cichorium intybus</i> (endive)	yes	no	Mugnier, 1988
	yes <sup>*</sup>	yes <sup>*</sup>	G. Touraud (unpubl.)
<i>Cinchona ledgeriana</i> (peruvian bark)	yes	no	Hamill <i>et al.</i> , 1987
<i>Convolvus arvensis</i> (morning glory)	yes <sup>*</sup>	yes <sup>*</sup>	Tepfer, 1982, 1984
<i>Coriandrum sativum</i>	yes	no	Mugnier, 1988
<i>Crepis capillaris</i>	yes <sup>*</sup>	no	Ambros <i>et al.</i> , 1986
<i>Cucumis sativus</i> (Cucumber)	yes <sup>*</sup>	no	McInnes <i>et al.</i> , 1991
	yes <sup>*</sup>	yes <sup>*</sup>	Trulson <i>et al.</i> , 1986
	yes	no	Mugnier, 1988

<sup>\*</sup> Stable, axenic root cultures,      \* Biochemical confirmation of transformation obtained.

continued

Table 2. continued

Species	Roots <sup>a</sup>	Plants	Reference
<i>Datura chlorantha</i>	yes	no	E.Knopp & A.Strauss (unpubl.)
<i>Datura ferox</i>	yes	no	E.Knopp & A.Strauss (unpubl.)
<i>Datura innoxia</i>	yes	no	E.Knopp & A.Strauss (unpubl.)
	yes <sup>*</sup>	no	Brillianceau, 1986
<i>Datura metel</i>	yes	no	E.Knopp & A.Strauss (unpubl.)
<i>Datura meteloides</i>	yes	no	E.Knopp & A.Strauss (unpubl.)
<i>Datura rosei</i>	yes	no	E.Knopp & A.Strauss (unpubl.)
<i>Datura sanguinea</i>	yes	no	E.Knopp & A.Strauss (unpubl.)
<i>Datura stramonium</i> (Jimson-weed)	yes <sup>*</sup>	no	Payne <i>et al.</i> , 1987
	yes	no	Mugnier, 1988
<i>Daucus carota</i>	yes <sup>*</sup>	no	Tepfer & Tempé, 1981
	yes <sup>*</sup>	no	Spano <i>et al.</i> , 1982
	yes <sup>*</sup>	yes <sup>*</sup>	Tepfer, 1982, 1983a, 1984
	yes <sup>*</sup>	yes <sup>*</sup>	David <i>et al.</i> , 1984
	yes <sup>*</sup>	no	Capone <i>et al.</i> , 1989
	yes <sup>*</sup>	no	Spano <i>et al.</i> , 1989
<i>Dendrathera grandiflora</i> (Chrysanthemum)	yes <sup>*</sup>	no	Failla <i>et al.</i> , 1990
<i>Dianthus caryophyllus</i> (carnation)	yes <sup>*</sup>	no	Wordragen <i>et al.</i> , 1992
<i>Digitalis lanata</i>	yes	no	Mugnier, 1988
<i>Duboisia myoporoides</i>	yes <sup>*</sup>	no	Brillianceau, 1986
<i>Ervatamia obtusifolia</i>	yes <sup>*</sup>	no	Deno <i>et al.</i> , 1987
<i>Eucalyptus dunnii</i>	yes <sup>*</sup>	no	Brillianceau, 1986
<i>Eucalyptus globulus</i>	yes <sup>*</sup>	no	MacRae & Van Staden, 1993
	yes <sup>*</sup>	no	Chirqui <i>et al.</i> , 1991
<i>Eucalyptus grandis</i>	yes <sup>*</sup>	no	S. MacRae (unpubl.)
<i>Eucalyptus gunnii</i>	yes <sup>*</sup>	no	MacRae & Van Staden, 1993
<i>Eucalyptus nitens</i>	yes <sup>*</sup>	no	Adam, 1987
<i>Foeniculum vulgare</i> (fennel)	yes <sup>*</sup>	no	MacRae & Van Staden, 1993
	yes <sup>*</sup>	no	Brillianceau, 1986
	yes	yes	Mugnier, 1988
<i>Fragaria annanassa</i>	yes <sup>*</sup>	yes <sup>*</sup>	C.Attal & D.Tepfer (unpubl.)
<i>Galinsoga parviflora</i> (quickweed)	yes <sup>*</sup>	no	Kyo & Shirai, 1990
<i>Gentiana lutea</i> (yellow gentian)	yes	no	Mugnier, 1988
<i>Glycine max</i> (soyabean)	yes <sup>*</sup>	no	E. Davioud (unpubl.)
<i>Gypsophila muralis</i> (babybreath)	yes <sup>*</sup>	no	A.Yacoub & D.Tepfer (unpubl.)
<i>Helianthus annuus</i> (sunflower)	yes	no	Mugnier, 1988
	yes	no	Mugnier, 1988
<i>Helianthus tuberosus</i> (Jerusalem artichoke)	yes <sup>*</sup>	no	C.Attal & D.Tepfer (unpubl.)
<i>Hyoscyamus albus</i>	yes <sup>*</sup>	no	C.Attal & D.Tepfer (unpubl.)
<i>Hyoscyamus aureus</i>	yes	no	K.Knopp & A. Strauss (unpubl.)
<i>Hyoscyamus bohemicus</i>	yes	no	K.Knopp & A. Strauss (unpubl.)
<i>Hyoscyamus muticus</i>	yes	no	K.Knopp & A. Strauss (unpubl.)
	yes	no	Flores & Filner, 1985
<i>Hyoscyamus niger</i>	yes <sup>*</sup>	yes <sup>*</sup>	C.Attal & D.Tepfer (unpubl.)
<i>Ipomoea batatas</i> (sweet potato)	yes	no	Flores & Filner, 1985
	yes	no	Eilers <i>et al.</i> , 1986
<i>Ipomoea aristolochiaefolia</i>	yes	no	Mugnier, 1988
<i>Ipomoea purpurea</i>	yes	no	Mugnier, 1988
<i>Kalanchoë daigremontiana</i>	yes	no	Mugnier, 1988
	yes	no	Mugnier, 1988
<i>Linum grandiflorum</i> (flax)	yes	no	Mugnier, 1988

continued

Table 2. continued

Species	Roots*	Plants	Reference
<i>Lithospermum erythrorhizon</i>	yes	no	Shimomura <i>et al.</i> , 1986
<i>Lotus corniculatus</i> (bird's foot trefoil)	yes*	yes*	Petit <i>et al.</i> , 1986
<i>Lupinus albus</i> (white lupin)	yes	no	Mugnier, 1988
<i>Lupinus polyphyllus</i> (lupin)	yes	no	Mugnier, 1988
<i>Lycopersicon esculentum</i> (tomato)	yes	no	Banerjee <i>et al.</i> , 1985
	yes*	yes*	Shahin <i>et al.</i> , 1986
	yes*	yes*	Morgan <i>et al.</i> , 1987
	yes*	no	Shen <i>et al.</i> , 1990
<i>Lycopersicon peruvianum</i>	yes	no	Banerjee <i>et al.</i> , 1985
<i>Macroptilium atropurpureum</i> (siratro)	yes	no	Beach <i>et al.</i> , 1986
<i>Malus</i>	yes*	no	Lambert & Tepfer, 1991
	yes	no	S. MacRae (unpubl.)
<i>Medicago arborea</i> L	yes*	yes	Damiani & Arciono, 1991
<i>Medicago sativa</i> (alfalfa)	yes	no	Beach <i>et al.</i> , 1986
	yes*	yes*	Skhapinda <i>et al.</i> , 1987
	yes*	yes*	Spano <i>et al.</i> , 1987
	yes*	yes*	Gold <i>et al.</i> , 1991
<i>Medicago tomata</i>	yes*	no	C. Attal & D. Tepfer (unpubl.)
<i>Nicotiana africana</i>	yes*	no	Parr & Hamill, 1987
<i>Nicotiana cavicola</i>	yes*	no	Parr & Hamill, 1987
<i>Nicotiana glauca</i>	no	no	McInnes <i>et al.</i> , 1989
	yes*	yes*	Taylor <i>et al.</i> , 1985
<i>Nicotiana hesperis</i>	yes*	no	Parr <i>et al.</i> , 1987
	yes	yes	Walton & Belshaw, 1988
<i>Nicotiana plumbaginifolia</i>	yes*	yes*	Jouanin <i>et al.</i> , 1987
<i>Nicotiana rustica</i>	yes	no	Hamill <i>et al.</i> , 1986
	yes	no	Rhodes <i>et al.</i> , 1986
<i>Nicotiana tabacum</i> (tobacco)	no	yes	Ackerman, 1977
	yes*	yes*	Tepfer, 1982; 1983a,b; 1984
	yes*	yes*	Constantino <i>et al.</i> , 1984
	yes*	yes*	Comai <i>et al.</i> , 1985
	yes*	no	McInnes <i>et al.</i> , 1989
	yes*	yes*	Sugaya <i>et al.</i> , 1989
	yes*	no	Kanaga <i>et al.</i> , 1990
	yes*	yes*	Oono <i>et al.</i> , 1990
<i>Nicotiana umbratica</i>	yes*	no	Parr & Hamill, 1987
<i>Nicotiana velutina</i>	yes*	no	Parr & Hamill, 1987
<i>Onobrychis viciifolia</i>	yes*	yes*	Gold <i>et al.</i> , 1991
<i>Panax ginseng</i>	yes	no	K. Soo Ko <i>et al.</i> (unpubl.)
	yes*	no	Yoshikawa & Furuya, 1987
<i>Petunia hybrida</i> (petunia)	yes	yes	Ondrej & Biskova, 1986
<i>Phaseolus vulgaris</i> (bean)	yes*	no	C. Attal & D. Tepfer (unpubl.)
	yes*	no	Hamill <i>et al.</i> , 1987
<i>Pimpinella anisum</i> (anis)	yes	no	Mugnier, 1988
<i>Pinus</i> (Pine hybrids)	yes	no	S. MacRae (unpubl.)
<i>Pisum sativum</i> (pea)	yes*	no	Bercetche <i>et al.</i> , 1987
<i>Polygonum aviculare</i> (knotweed)	yes	no	Mugnier, 1988
<i>Polygonum convolvulus</i> (corn bindweed)	yes	no	Mugnier, 1988
<i>Polygonum hydropiper</i>	yes	no	Hamill <i>et al.</i> , 1987
<i>Populus tremula</i> x <i>Populus alba</i> (poplar)	yes	no	J. Carr & F. Le Tacon (unpubl.)
<i>Populus trichocarpa</i> x <i>Populus deltoides</i>	no	yes*	Pythoud <i>et al.</i> , 1987

Continued

Table 2. continued

Species	Roots'	Plants	Reference
<i>Psophocarpus tetragonolobus</i> (winged bean)			C. Attal & D. Tepfer (unpubl.)
<i>Raphanus sativus</i> (radish)	yes	no	Tanaka <i>et al.</i> , 1985
<i>Rheum palmatum</i> (rhubarb)	yes	no	Mugnier, 1988
<i>Rumex crispus</i> (yellow dock)	yes	no	Mugnier, 1988
<i>Scopolia carniolica</i>	yes	no	E. Knopp & A. Strauss (unpubl.)
<i>Scopolia japonica</i>	yes	no	Flores <i>et al.</i> , 1986
	yes	no	Mano <i>et al.</i> , 1986
	yes	no	Nabeshima <i>et al.</i> , 1986
<i>Scopolia simaminiifolia</i>	yes	no	E. Knopp & A. Strauss (unpubl.)
<i>Sesbania rostrata</i>	yes	no	Mugnier, 1988
<i>Silene amneria</i> (catchfly)	yes	no	Mugnier, 1988
<i>Sinapis alba</i> (white mustard)	yes	no	Mugnier, 1988
<i>Solanum dulcamara</i>	yes*	no	Garland <i>et al.</i> , 1991
<i>Solanum lactiniatum</i>	yes	no	Hamill <i>et al.</i> , 1987
<i>Solanum launatum</i>	yes*	no	Brillianceau, 1986
<i>Solanum nigrum</i> (nightshade)	yes*	yes*	Wei <i>et al.</i> , 1985
	yes	no	Mugnier, 1988
	yes*	no	McInnes <i>et al.</i> , 1989
<i>Solanum systembriifolium</i>	yes*	no	Brillianceau, 1986
<i>Solanum tuberosum</i> (potato)	yes*	no	Petit <i>et al.</i> , 1983
	yes*	yes*	Ooms <i>et al.</i> , 1985; 1986
	yes	no	Oliver, 1986
	yes*	no	Quattrocchio <i>et al.</i> , 1986
	yes*	yes*	Hanisch Ten Cate <i>et al.</i> , 1988
	yes*	yes	Ondrej <i>et al.</i> , 1989
	yes*	yes*	Montanelli & Nascari, 1989
	yes*	yes*	Visser <i>et al.</i> , 1989
<i>Solanum tuberosum</i> cv Binje	yes*	no	Ottaviani <i>et al.</i> , 1990
<i>Spergula arvensis</i> (spurry)	yes	no	Mugnier, 1988
<i>Tagetes erecta</i> (marigold)	yes	no	Mugnier, 1988
<i>Tagetes patula</i> (marigold)	yes	no	Flores <i>et al.</i> , 1986
<i>Trifolium pratense</i> (red clover)	yes	no	Beach <i>et al.</i> , 1986
<i>Valerianella locusta</i>	yes	no	Mugnier, 1988
<i>Vicia sativa</i> (common vetch)	yes	no	Mugnier, 1988
<i>Vicia faba</i>	yes*	no	Ramsay & Kumor, 1990
<i>Vigna aconitifolia</i> (moth bean)	yes	yes	K. Sukhapinda & E. Shahin (unpubl.)
<i>Vigna unguiculata</i> (cowpea)	yes	no	Mugnier, 1988
<i>Vitis vinifera</i> L (grape)	yes*	no	Guellec <i>et al.</i> , 1990

*A. rhizogenes* has also been used to increase the production of secondary metabolites such as tropane alkaloids from *Nicotiana tabacum*, *Hyoscyamus muticus* and *H. niger* (FLORES and FILNER, 1985); betalain pigment from *Beta vulgaris*

(HAMILL *et al.*, 1986); saponin from *Panax ginseng* (YOSHI-KAWA and FURUYA, 1987) and scopolamine from *Datura stramonium*. In addition transformed root cultures have been used as a means of studying previously unidentified secondary metabolites (TEPFER *et al.*, 1988).

Transformed roots also have potential as a tool (a potential living substrate for growing soil microorganisms) in rhizosphere interaction studies (TEPFER and YACOUB, 1986; MUGNIER and MOSSE, 1987; BÉCARD and FORTIN, 1988; TEPFER, 1989; TEPFER, 1990).

#### 1.4.4 *A. rhizogenes* an Alternative Vector for Foreign DNA Insertion

As with *A. tumefaciens*, *A. rhizogenes* can be used to insert foreign DNA into plant species either by insertion of foreign DNA into the Ri T-DNA itself or by making use of a binary vector system, where foreign DNA is carried on a second plasmid (BIROT *et al.*, 1987; KYO and SHIRAI, 1990). This together with the ability to regenerate plants directly from transformed roots suggests that *A. rhizogenes* has potential as an alternative vector system to *A. tumefaciens*.

The observation that transformed roots are capable of regenerating into whole plants

without going through a callus formation stage is valuable in that this would circumvent problems with somaclonal variation and possibly enable transgenic plants to be produced more easily. Many plant species such as *Convolvus arvensis* (morning glory) are able to regenerate shoots from roots naturally. However, growth substances are required for the regeneration of other plant species. For example, transformed potato plants could only be regenerated from potato root cultures in the presence of hormones (ONDREJ *et al.*, 1989). To date only a limited number of woody species has been manipulated in this way, mainly due to lack of regeneration success.

#### 1.4.5 Potential of *A. rhizogenes* as a Tool in Plant Improvement

Very little research has been undertaken to determine the potential use of *A. rhizogenes* in improving rooting of cuttings and transplants. The success of cutting-based plant vegetative propagation programmes is dependent on the rooting ability of cuttings. Good root mass and vigour are essential for the establishment and survival of both cuttings and transplanted plants. Difficulties are experienced in rooting of a number of plant genotypes. The potential use of the adventitious root-inducing ability of the Ri plasmid of *A. rhizogenes* to address this problem requires more in-depth research.

To date *A. rhizogenes* has only been used to improve rooting of transplants of a small number of plant species. Accelerated growth of almonds was observed by STROBEL and NACHMIAS (1985) after *A. rhizogenes* inoculation. Inoculation of apples with *A. rhizogenes* resulted in resistance to limited soil water availability (MOORE *et al.*, 1979) and improved rooting (ANON, 1990;). LAMBERT and TEPFER (1991) showed that improved rooting of apple cuttings was dependent on the apple genotype, the physiological state of the plant tissue and the genotype of the bacterium and that under certain conditions *A. rhizogenes* can overcome the genotype determinant but not the physiological state determinant. *A. rhizogenes* strain A4 could be used to overcome the rooting deficiency of at least 3 year old M9b rooting stock when the layering technique was used. This bacterium therefore has potential in genetic grafting of apples (LAMBERT and TEPFER, 1991). In addition tomato transplants have been inoculated with *A. rhizogenes* to improve root mass and vigour, both of which are essential for the establishment and survival of transplanted tomatoes (ERICKSON *et al.*, 1990). These findings clearly suggest that *A. rhizogenes* has potential for improving rooting of plant species.

Another potential use of *A. rhizogenes* in plant improvement was shown by STROBEL *et al.* (1989). They found that nodulation in *Alfalfa* could be increased by introducing the Ri plasmid of *A. rhizogenes* into *Rhizobium meliloti*.

#### 1.4.6 Future Potential of the *A. rhizogenes* System in Plant Improvement

Although some progress has been made towards understanding *A. rhizogenes*-mediated gene transfer, there is a need for a more complete understanding of the physiological changes that are induced by the Ri T-DNA so that its ability to improve root cultures and produce chimeric plants with good root systems can be exploited in species that so far have proved to be recalcitrant. The Ri-induced transformed phenotype is unique and of basic biological interest. As a result of this TEPFER (1990) suggests that the future of gene transfer using *A. rhizogenes* is in the biology of the system, through root cultures (as a means of studying the root and its interactions) and through the effects of the Ri T-DNA on development, rather than as a vector for transferring foreign DNA into plant tissue. I agree, but feel that this organism has an additional role to play in plant improvement, namely, that of improving rooting during vegetative propagation of plants and at transplanting. The potential of *A. rhizogenes* strains to improve rooting of woody species, with *Eucalyptus* as the species of major interest, was investigated in this dissertation. It was therefore necessary to review briefly vegetative propagation and associated rooting problems.

## 1.5 VEGETATIVE PROPAGATION OF FOREST TREES

### 1.5.1 Introduction

Many plant species are routinely propagated by cuttings, including *Eucalyptus* species (MCCOMB and WROTH, 1986; WILSON, 1988; DENNISON and KIETSKA, 1993) and deciduous fruit varieties (LAMBERT and TEPFER, 1991), or by layering techniques, eg. apples (LAMBERT and TEPFER, 1991). While such techniques have the advantage of producing a clone from selected plants and stabilizing a genotype, they are limited by the inherent inability of the genotype to produce roots. For this reason rooting of woody plant cuttings is only feasible for a limited number of species and within a species some varieties/clones can only root with difficulty if at all. The increasing demand for high yielding, good quality *Eucalyptus* trees in South Africa has resulted in vegetative propagation of selected clones playing an increasingly important role in breeding programmes. This review will briefly cover clonal propagation of eucalypts and the importance of rooting in successful vegetative propagation.

Although South Africa is relatively poorly endowed with natural forests and timber resources it has grown into a world leader in plantation forestry. It began in 1886 with the establishment of a pioneering plantation in the Western Cape. The aim was

to supplement the sparse natural sources of fuel for the early railroads reaching into the interior. Today plantations cover 1.3 million hectares (1%) of the total surface area of the country. Every tree is planted individually in these man-made plantations and today they support a vibrant industry which employs 120 000 people and has become one of South Africa's largest exporters of manufactured goods. None the less a timber shortage has been projected for the turn of the century, not only in South Africa but throughout the world. In order to circumvent this shortage in South Africa, the planned strategy is to double the timber yield from 16 million to 32 million m<sup>3</sup> by 2010 (FORESTRY COUNCIL, 1992). Thus the demand is for trees with a superior growth potential. This can be achieved by tree breeding.

Tree improvement by breeding is, however, slow compared to other plant species, mainly due to the long intervals between generations and because trees are extremely heterozygous (LIBBY *et al.*, 1969). These constraints can be circumvented in the short term by vegetative propagation of selected superior trees. Vegetative propagation enables full transfer of non-additive genetic gains. This is particularly useful for rapid improvements of attributes such as volume, growth and cellulose yield, which are poorly heritable (ZOBEL and IKEMORI, 1983). As a result of this, vegetative propagation of eucalypts is playing an increasingly important role in tree improvement programmes. The limiting factor in successful commercial propagation of selected superior clones is the rooting ability of these clones.

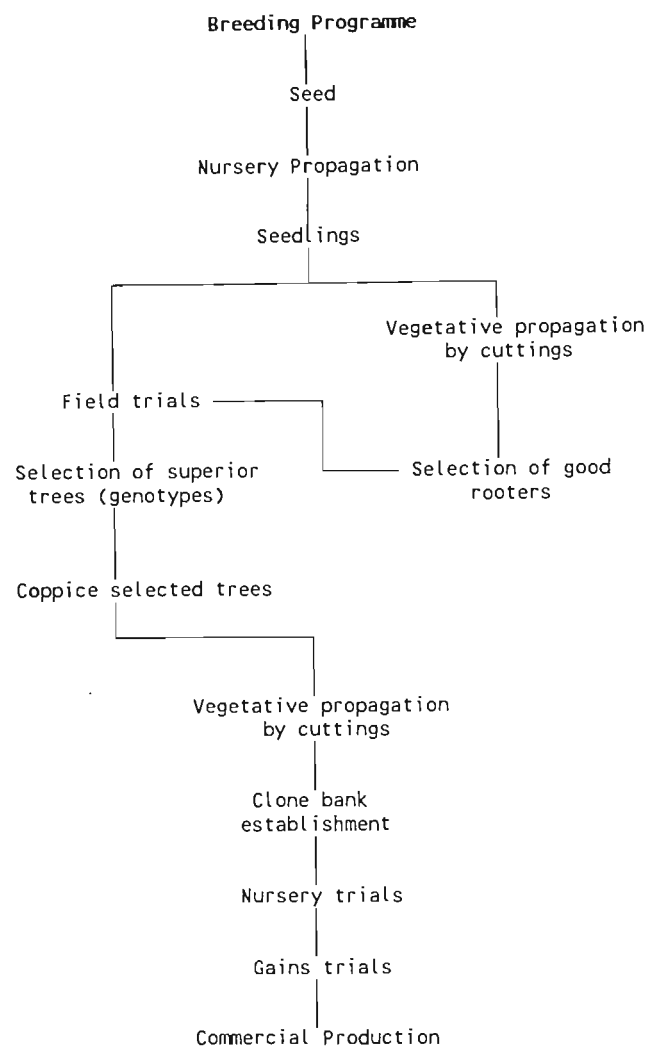
Clonal programmes in South Africa are confined mainly to subtropical *Eucalyptus* species, involving *E. grandis* and its hybrids with *E. camaldulensis*, *E. tereticornis* and *E. urophylla*. Research is well advanced in developing cold tolerant and temperate eucalypts such as *E. nitens* for operational planting. Clones are selected for good growth, form, tolerance to disease, good wood properties, drought and cold tolerance. The benefits of such clones has recently been seen in South Africa where the incidence of *Eucalyptus* diseases are increasing and the country has experienced severe drought conditions, especially during 1991/1992. Clonal propagation of *Eucalyptus* by stem cutting and through micropropagation will be briefly discussed.

### 1.5.2 Clonal Propagation by Stem Cuttings

A number of vegetative propagation techniques are available. The cheapest and easiest one for propagating forest trees vegetatively is by stem cuttings. Traditionally, only a few easy-to-root genera such as *Populus* were propagated routinely in this way. More recently, however, various attempts have been made to afforest on a commercial scale with *Eucalyptus* stem cuttings. Much research in this field has been done in Brazil and the Congo where careful selection and use of cuttings resulted in the establishment of clonal plantations of several species and hybrids (CAMPINHOS and IKEMORI, 1977; CHAPERON and QUILLET, 1977).

In 1979 a Brazilian company, Aracruz Florestal SA, began afforesting with rooted cuttings and by 1983 the company's annual planting of 12.5 million trees was almost exclusively of rooted cuttings (ZOBEL and IKEMORI, 1983). Clonal forestry research in South Africa was initiated about ten years ago and was modelled on the Aracruz Florestal operation. Since then commercial clonal propagation of *Eucalyptus* by this means has increased dramatically, with, for example, one of the South African forestry companies, Mondi, producing in excess of 10 million clonal plants annually (DENNISON and KIETZKA, 1993). France and Israel are also involved in research on propagation of tree species through cuttings from coppice and by utilising the 'rejuvenating' effect of grafting mature meristems onto seedling root stocks (DURAND-CRESSWELL *et al.*, 1982; HETH *et al.*, 1985; HETH *et al.*, 1986).

In clonal forestry one is dealing with biological processes where the age, quality and juvenility of the cuttings are critical elements in producing successful rooting and quality plants (DENNISON and KIETZKA, 1993). The complexity of successful cloning on an operational scale should therefore not be underestimated. The steps involved in clone development of *Eucalyptus* from either seedling material or coppice material are detailed in Figure 6.



**Figure 6.** Clone development under nursery conditions from coppice or seedling material.

To satisfy the growing demand for plants, initial clone selection was based on early performance and good rooting. This resulted in many incorrect choices during the developmental years of the clonal programmes. Clones with desirable wood properties were sometimes rejected on this basis (DENNISON and QUAILE, 1987; DENNISON and KIETZKA, 1993). The loss of rooting ability and poor response to auxins shown by cuttings with increasing age of the donor trees is the most serious obstacle to successful vegetative propagation and many commercially important selections are difficult to propagate vegetatively at the stage at which the selections are made. In the past 10-15 years the selection and cloning of superior trees from natural hybrids has had considerable impact on biomass production. However, the gene pool available for selection is restricted for commercial cloning by the requirement that at least 70% of the cuttings must root.

Rejuvenation of mature selected plant material generally results in improved rooting ability. However, in many instances the extent of rejuvenation through horticultural techniques is insufficient to permit commercial propagation by cuttings. The phenomena of juvenility, maturation and rejuvenation are still poorly understood (BONGA, 1982; FRANCKET and BOULAY, 1982; HARTMANN and KESTER, 1983; BONGA, 1987; PIERIK, 1990). Despite this problem the commercial propagation of *Eucalyptus* by stem cuttings, has been successful in many countries due mainly to the sound genetic base of their breeding programmes.

Much research has been focused on improving rooting of cuttings and plant quality by optimising through horticultural, physiological and environmental means, aspects such as stock plant (clone bank) management, nutrition, water quality and growing medium. Some success has been achieved (MCCOMB and WROTH, 1986; DENNISON and QUAILE, 1987; WILSON, 1988; DENNISON and KIETZKA, 1993).

The superiority of vegetative propagation by comparison with sexual regeneration lies in the possibility of transferring the whole genetic potential of a selected tree, the donor tree or ortet, to its asexually reproduced progeny (MONTEUUIS, 1988).

#### 1.5.3.1 Adventitious Rooting in Stem Cuttings of *Eucalyptus*

Although roots are central to the existence and survival of plants their formation is in some respects one of the most poorly understood phenomena of all plant functions. A number of factors are critical to successful rooting and propagation not only of *Eucalyptus* species, but all plants, from stem cuttings. These include:

##### I. Selection of cutting material

- \* Physiological condition of the stock plant

- \* Juvenility factor (age of the stock plant)
- \* Type of wood selected
- \* Presence of viruses
- \* Time of the year cuttings are taken

## II. Treatment of cuttings

- \* Growth regulators
- \* Mineral nutrients
- \* Fungicides
- \* Wounding

## III. Environmental conditions during rooting

- \* Water relations
- \* Temperature
- \* Light
  - a. Intensity
  - b. Day length
  - c. Light quality
- \* Rooting medium

(From HARTMANN and KESTER, 1983)

Research has focused on optimisation of these factors and has led to successful commercial propagation of a number of *Eucalyptus* clones. Mondi for example, implemented selective harvesting of cuttings from stock plants in their clone banks as opposed to total harvesting and this resulted in a 10 to 15 % increase in rooting. In addition optimisation of fertilizer application and the control of water pH to 5.4 - 5.8 further increased rooting of selected cuttings by 10 to 15% (DENNISON and KIETZKA, 1993).

WILSON (1988) studied the effect of physiological factors on adventitious rooting in stem cuttings of *Eucalyptus grandis* Hill ex Maid and found that stem cuttings contained a morphogen which operates as the 'switch' from stem to adventitious root. Although the leaves and buds of *E. grandis* did not appear to be the only source of this morphogen their activity was nevertheless good for rooting. It was found that in general the rate of efflux from the transporting tissue, the rate of attenuation of the morphogen after efflux and the number of potential sites for root initiation must interact on a small scale to determine rooting ability. As the relative prominence of these groups is likely to vary with circumstances, WILSON (1988) suggested that the traditional concept of a limiting morphogen ('rhizocaline') in its simplest form is not very helpful but does provide a starting point towards a more comprehensive view of adventitious rooting. This is required in order to predict and improve rooting ability. Further extensive research is however required. As a result

of the lack of understanding of the rooting phenomenon, clones with desirable wood properties but poor rooting are still being lost from clonal programmes. If these clones could be successfully rooted they would broaden the genetic base for future clonal development and this may contribute to preventing the pending timber shortage.

### 1.5.3 Clonal Propagation by Tissue Culture

Clonal propagation through tissue culture offers an alternative means of vegetative propagation to stem cuttings and has the potential to provide high multiplication rates of uniform genotypes resulting in short-term gains. Tissue culture results in rejuvenation of the plant material and thus rooting ability should improve (MCCOMB and WROTH, 1986). An extensive review of the development of *Eucalyptus* tissue culture has been written by LE ROUX and VAN STADEN (1991b).

In South Africa much of micropropagation research has been focused on *Eucalyptus grandis* (FURZE and CRESSWELL, 1985; MACRAE and VAN STADEN, 1990) because of its importance in the pulp and mining industries. Other important timber species that have been micropropagated in a number of countries throughout the

world include *E. gunnii*, *E. dalrympleana*, *E. maculata*, *E. resinifera*, *E. pauciflora*, *E. delagatensis*, *E. globulus*, *E. nitens*, *E. nova-angilica* and *E. viminalis* (LEE and DE FOSSARD, 1974; MCCOMB and BENNETT, 1982; MCCOMB and WROTH, 1986; LE ROUX and VAN STADEN, 1991b).

One of the benefits of mass micropropagation is that clones with selected properties can be rapidly propagated. Examples of this include the mass micropropagation of selected salt tolerant clones from species such as *E. camaldulensis* and *E. rudis*, which can provide trees for plantations in areas normally hostile to afforestation (ANON, 1981-1982; HARTNEY, 1982a; HARTNEY, 1982b; HARTNEY and KABAY, 1984). Similarly mass propagated clones of *E. citriodora* and *E. polybractea*, selected for high oil content, provide the potential for rapidly establishing high oil-yielding plantations.

#### 1.5.3.1 *In Vitro* Micropropagation of *Eucalyptus*

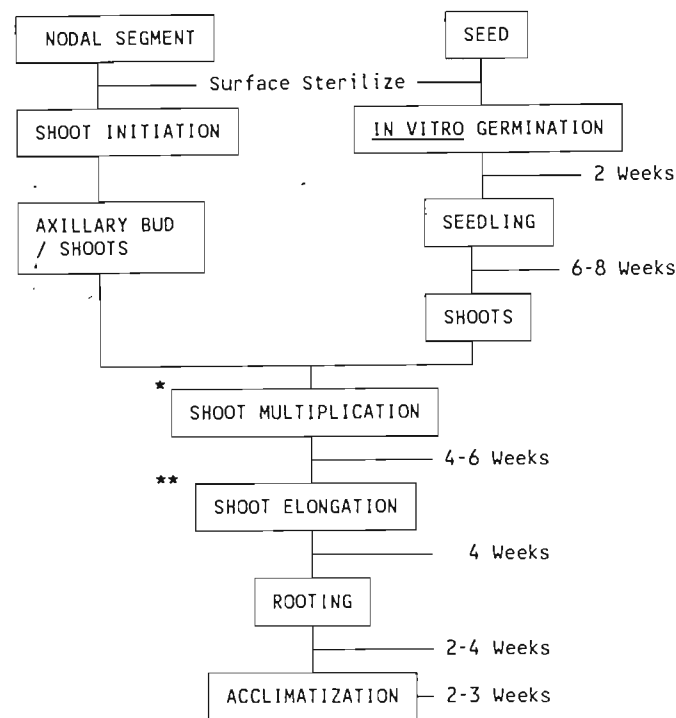
The ability of various eucalypt species to regenerate buds through *in vitro* culture is quite variable while callus formation is relatively easy to induce from various parts of juvenile and mature plants (CHRIQUI *et al.*, 1991). For a large number of species attempts to regenerate plantlets from callus cultures has, however, been

unsuccessful (HARTNEY, 1980).

Micropropagation of *Eucalyptus* species is therefore best achieved by axillary or adventitious shoot proliferation on nodal explants from seedlings (Figure 7) (DE FOSSARD and BOURNE, 1976; DE FOSSARD and BOURNE, 1977; HARTNEY and BARKER, 1980), mature trees (DE FOSSARD and BOURNE, 1976; DE FOSSARD and BOURNE, 1977; GUPTA *et al.*, 1983; MASCARENHAS *et al.*, 1982; RAO and VENKATESWARA, 1985), coppice (BADIA, 1982; HARTNEY, 1982a; HARTNEY, 1982b) or mature shoots grafted onto seedling rootstock (Figure 6; BOULAY, 1983; DURAND-CRESSWELL, *et al.*, 1982; FRANCKET and BOULAY, 1982). Once sufficient shoot growth has been achieved rooting can be attempted. As for stem cuttings of many eucalypt species (HARTNEY, 1980), rooting is a critical step in micropropagated explants. Poor rooting could possibly be due to rooting inhibitors (PATON *et al.*, 1970).

Dormancy and senescence of explants are obstacles to the establishment of cultures *in vitro*. Sterilisation procedures may elicit a wound response in addition to that caused by excision. In woody plants phenolic compounds are produced in response to wounding and these can be detrimental to growth *in vitro* (GEORGE and SHERRINGTON, 1987). The oxidation of phenolic compounds in the tissue and explant exudates is evident by browning of the medium, or the explant or both

(CRESSWELL and NITSCH, 1975; DE FOSSARD, 1974; DURAND-CRESSWELL and NITSCH, 1977; GONCALVES, 1980; FRANCKET and BOULAY, 1882). A number of precautions can, however, be taken to reduce phenolic compounds in *Eucalyptus* explants (LE ROUX and VAN STADEN, 1991a).



**Figure 7.** Diagrammatic representation of steps involved in the micropropagation of *Eucalyptus* seedlings and axillary buds.

- \* The shoot multiplication step is repeated until the required number of shoots are obtained and this is dependent on the multiplication rate of the plant material.
- \*\* This step is included only if necessary, that is if natural elongation does not occur.

GRATTAPAGLIA *et al.* (1990) listed three key factors for the successful large-scale micropropagation of *Eucalyptus* species : 1. The intrinsic propagation potential of the clone; 2. The level of juvenility of the initial explant; and 3. Strict observance of a maximum subculture of four weeks. Although a number of field trials have been established in many countries with *in vitro* propagated clones of *Eucalyptus* species, eucalypts have still not been successfully propagated on a commercial scale using this technique.

#### 1.5.4. *Agrobacterium* and its Use in *Eucalyptus* Propagation

Although no *Eucalyptus* species has been reported in the host range of either *Agrobacterium tumefaciens* or *A. rhizogenes* strains (DE CLEENE and DE LEY, 1976 and 1981), research has recently shown that both species are able to genetically transform *Eucalyptus* species *in vitro* (ADAMS, 1987; CHRIQUI *et al.*, 1991; MACRAE, 1991). As for other plant species, assessment of host susceptibility to the bacterial strain and the ability to regenerate plant material are critical to successful creation of transgenic eucalypts. The novel use of *A. rhizogenes* to improve adventitious rooting of *in vitro* propagated *Eucalyptus* explants and nursery propagated cuttings will be reported in this dissertation.

## CHAPTER 2

### BIOLOGICAL CONTROL BY *AGROBACTERIUM* SPECIES

#### 2.1 INTRODUCTION

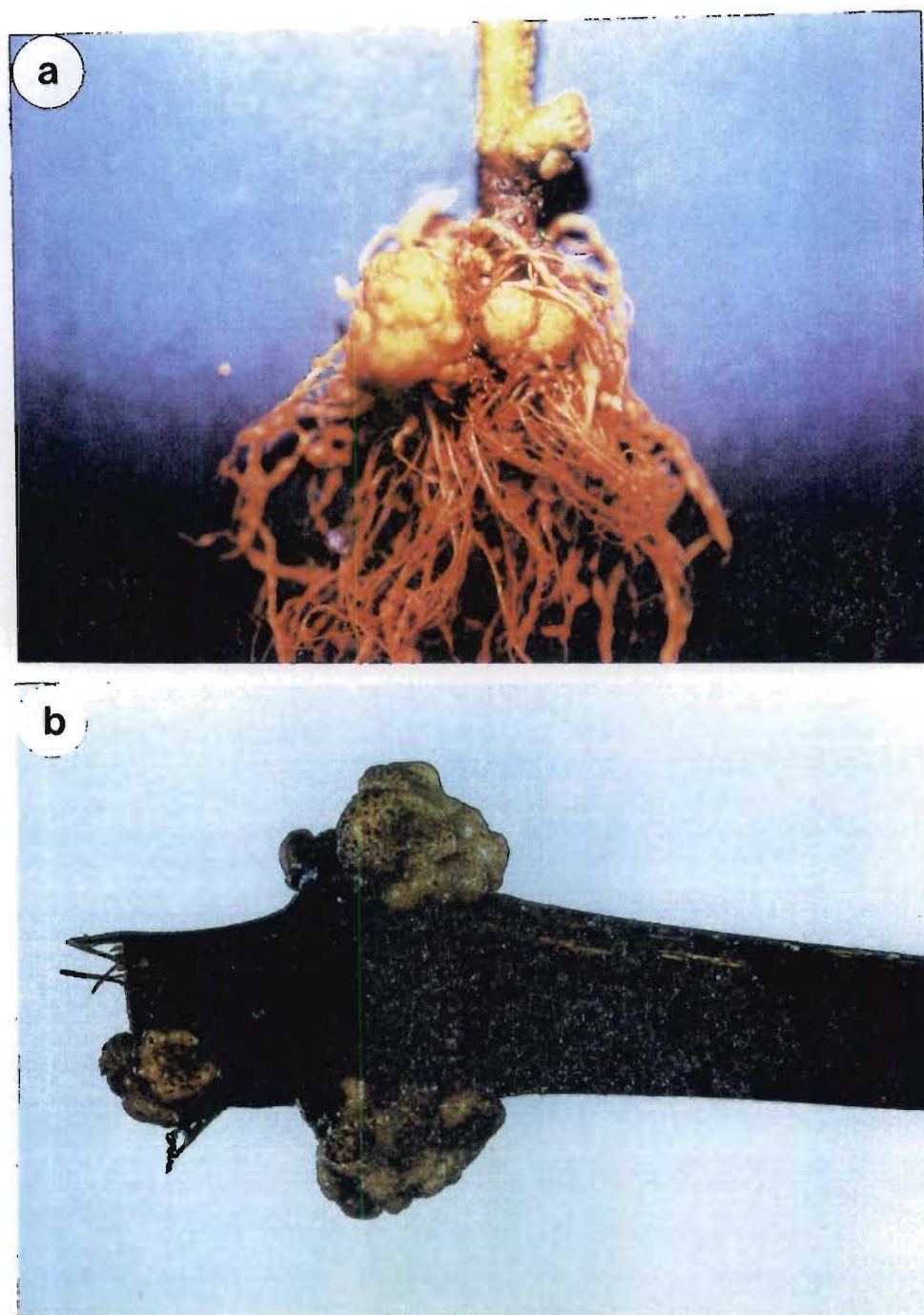
Pathogenic strains of *A. tumefaciens* cause crown gall disease on a variety of plant species (Figure 8). This disease can potentially be controlled by non-pathogenic *A. radiobacter* and *A. tumefaciens* strains. It is now generally accepted that a biological control agent must have two properties (see section 1.3.3) :-

- \* it must be able to produce sufficient amounts of an effective antibiotic substance, and
- \* it must be able to colonise the plants efficiently.

The difficulty lies in finding a new biological control agent that combines both these requirements.

Successful biological control of crown gall disease of plants by means of the non-pathogenic *A. radiobacter* strain K84 has been reported for a variety of susceptible plants in numerous localities (KERR, 1980; section 1.3.3). Its success is primarily due to its ability to produce an agrocin which is toxic to *A. tumefaciens* biotype 1 and 2 pathogens carrying an agrocinopine type Ti plasmid (ENGLER *et al.*, 1975).

However, its effectiveness is also enhanced by its ability to colonise a variety of plants efficiently (ELLIS *et al.*, 1979).



**Figure 8.** Crown gall disease on (a) a tomato plant and (b) a grapevine.

Good colonisation ability results in physical exclusion of a pathogen by blockage of infection sites. This mechanism of pathogen control was well demonstrated by COOKSEY and MOORE (1982a) when they found that if an agrocin<sup>r</sup> mutant of strain K84 was applied 24 hours before the pathogen it reduced infection by agrocin-sensitive strains on tomatoes. However, if the mutant was applied concomitantly with the pathogen a break-down in control was observed. Thus as the mutant did not produce agrocin these results implied that control was due to the strain's ability to bind to the wound site blocking pathogen attachment to infection sites. Physical blockage is therefore suggested as another mechanism by which strain K84 prevents infection (COOKSEY and MOORE, 1982a).

In support of this theory several authors have noted that strain K84 is effective in controlling agrocin-resistant pathogens (MOORE, 1977; COOKSEY and MOORE, 1980; VAN ZYL *et al.*, 1986). However, it has also been shown that the blockage of infection sites can be host dependent. MOORE (1979), for example, found that agrocin-resistant pathogens were controlled by K84 on pear seedlings but not apple seedlings. DU PLESSIS *et al.* (1985) confirmed these findings when they found that the pathogenic strain K28SA, which is resistant to agrocin 84, was completely inhibited by strain K84 on tomatoes but not on Chrysanthemum.

Evidence exists to suggest that the ability of bacterial strains to colonise roots is a

function of the chromosomal background. OPEL and KERR (1987) swapped the Ti plasmids from a biotype 2 to a biotype 3 strain and vice versa by conjugative transfer methods and found that both transconjugants maintained populations on almond roots and stems which were virtually identical to the wild-type background strain. Similarly, SHIM *et al.* (1987) found that a biotype 1 strain, a derivative of C58 harbouring the agrocin 84 plasmid, was less efficient at controlling crown gall on almonds than the parental strain. Its poor colonisation ability was suggested as the reason for its decreased biocontrolling ability and the strain was labelled ecologically inefficient.

Biological control of grapevine crown gall by K84 has not been very successful. This is probably due to a variety of reasons including the fact that most *A. tumefaciens* strains isolated from galls on vines are of the narrow host range, biotype 3 class (PANAGOPOULOS *et al.*, 1978) and usually carry limited host range Ti plasmids of the octopine type (THOMASHOW *et al.*, 1980). Strains harbouring octopine-type Ti plasmids are resistant to agrocin 84 (PERRY and KADO, 1982). Thus, in view of the fairly narrow spectrum of *A. tumefaciens* sensitivity to K84, and its inability to control crown gall disease caused by biotype 3 strains, it was decided to search for agrocin-producing strains with wider host ranges.

The research in this chapter formed part of a programme in which the strategy was

to genetically engineer an all purpose biocontrol strain that would control crown gall disease on a variety of hosts. Of the potential broad host range biocontrol strains isolated in South Africa, D286 (HENDSON *et.al.*, 1983, 1986) and J73 (WEBSTER *et al.*, 1986) were selected for this study. As crown gall disease on table grape vines is a disease of economic importance in South Africa, two narrow host range biotype 3 isolates, H8 and H6, were also selected for this study.

*A. tumefaciens* D286 is a biotype 2 strain isolated from *Eucalyptus* which spontaneously lost its pathogenicity. This strain affords some measure of protection against pathogenic strains carrying nopaline, octopine and agropine type Ti plasmids. Agrocin D286 was shown to inhibit DNA, RNA and protein synthesis (HENDSON *et al.*, 1983). As with K84, D286 was unable to effectively protect plants from infection by biotype 3 strains which have a narrow host range restricted to grapevines (KERR and PANAGOPOULOS, 1977; HENDSON and THOMSON, 1986).

*A. tumefaciens* J73 is a biotype 2 strain isolated from *Prunus salicina*. It synthesises an agrocin which is active *in vitro* against strains from all three biotypes of *A. tumefaciens* regardless of whether they carry a Ti plasmid or not (WEBSTER and THOMSON, 1988) and therefore has potential as a biocontrol agent. Although J73

was originally pathogenic, it was cured of its Ti plasmid by plasmid incompatibility using the plasmid pMP90 and pDP35::pJS4159K1 (pK1) (WEBSTER and THOMSON, 1988). Preliminary greenhouse trials were performed to determine its ability to protect plants from crown gall disease and results indicated that in those cases where strains of *A. tumefaciens* are sensitive to both K84 and J73, K84 was the more effective biocontrol agent. As both strains produce comparable levels of agrocin *in vitro* (WEBSTER and THOMSON, 1988) it was postulated that differences in biological control may be accounted for by different plant colonisation efficiencies. In order to increase the biological control potential of this bacterium, a transposon-marked agrocin plasmid from K84, pAg84::Tn5 was introduced into J73 by WEBSTER (1990). The resultant strain J73(pMP90)(pAg84) produced both agrocin J73 and agrocin 84.

*A. tumefaciens* H6 is a biotype 3 non-pathogenic grapevine isolate that produces an antibiotic effective against biotype 3 pathogens and successfully reduced crown gall formation on grapevines in a greenhouse experiment (STAPHORST *et al.*, 1985). Crown gall biocontrol strains could conceivably become pathogenic were they to receive a Ti plasmid either by transformation or conjugation *in planta* (VAN LAREBEKE *et al.*, 1975; see section 1.3.3.1.4). To ensure that this did not occur a selectable plasmid (pDP35::pJS4159K1) carrying the origin of replication of a Ti plasmid was introduced into H6. A spontaneous mutant H6D17, which produced

four times more agrocin in a slightly rich medium, was also isolated (J. A. Thomson, personal communication).

*A. tumefaciens* H8 has as yet not been characterised but is a pathogen that produces an agrocin effective *in vitro* against biotype 3 pathogens.

The ability of D286, H8 and J73 and its derivatives J73(pK1), J73(pMP90), J73(pMP90)(pAg84) and H6 and its derivatives, H6(pK1) and H6D17, to colonise tomatoes and grapevines and afford protection against crown gall disease was studied and compared to K84 with the aims of determining the role of colonisation in biological control and selecting an effective biological control agent for grapevines. This involved assessing *in vitro* and *in vivo* colonisation efficiencies and biological control abilities of the potential biocontrol strains.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Bacteria, Media and Growth Conditions

The strains used are listed in Table 3. *A. tumefaciens* K84, D286 and J73 and its derivatives were cultured on GT medium (DOUGLAS *et al.*, 1974) at 25°C. H8, H6 and its derivatives were grown on YMA medium (PILACINSKI and SCHMIDT, 1981) at 25°C. Pathogenic *A. tumefaciens* strains (Table 4) were grown on LB medium (Miller, 1972) at 28 °C. When required media were supplemented with 100  $\mu\text{g ml}^{-1}$  of nalidixic acid (Nx), neomycin (Nm), rifampicin (Rm), spectinomycin (Sp) and 40  $\mu\text{g ml}^{-1}$  gentamycin (Gm).

### 2.2.2 Development of Antibiotic Resistant Bacterial Strains

Spontaneous antibiotic-resistant mutants were isolated to facilitate selection ; K84Nx<sup>R</sup><sub>100</sub> - Nx resistant; J73Rm<sup>R</sup><sub>100</sub> - Rm resistant; J73(pK1)Sp<sup>R</sup><sub>100</sub>Nm<sup>R</sup><sub>50</sub> - Sp and Nm resistant; J73Gm<sup>R</sup><sub>40</sub>(pMP90) - Gm resistant; J73Gm<sup>R</sup><sub>40</sub>Nm<sup>R</sup><sub>100</sub>(pMP90)(pAg84) - Gm and Nm resistant; H8Rm<sup>R</sup><sub>100</sub> - Rm resistant; H6Sp<sup>R</sup><sub>100</sub> - Sp resistant; H6D17Nm<sup>R</sup><sub>10</sub>Nx<sup>R</sup><sub>100</sub> - Nm and Nx resistant. (Figures in subscript represents mic in  $\mu\text{g ml}^{-1}$ ).

**Table 3.** Biological Control *Agrobacterium radiobacter* (K84) and *A. tumefaciens* strains used in this study.

Strain	Relevant Characteristics	Source
K84	pCrypAg84 pAt844bAgr <sup>+</sup> Biotype 2	KERR, Australia
J73	pTiJ73 pAgJ73 pCryAgr <sup>+</sup> Biotype 2, pathogenic	Webster <i>et al.</i> (1986)
J73(pK1)	pAgJ73 pDP35::pJS4159K1 (or pK1)Agr <sup>+</sup> Sp <sup>r</sup> . Cured of pTiJ73 by plasmid incompatibility. Biotype 2	Webster (1990)
J73(pMP90)	pAgJ73 pMP90Agr <sup>+</sup> Sp <sup>r</sup> . Cured of TiJ73 by plasmid incompatibility Biotype 2	Webster (1990)
J73(pMP90)(pAg84)	pAgJ73 pAg84 Agr <sup>+</sup> Sp <sup>r</sup> . Produces both agrocin J73 and K84. Biotype 2	Webster (1990)
H6	Plasmid not identified, Agr <sup>+</sup> Biotype 3	Thomson, S. A.
H6(pK1)	pDP35::pJS4159K1(or pK1) Biotype 3	Thomson, S. A.
H6D17	H6::Tn5 Biotype 3	Thomson, S. A.
D286	pAgD286 Biotype 2	Hendson et al. (1986)
H8	Agr <sup>+</sup> Biotype 3 pathogen	Thomson, S.A.

**Table 4.** Pathogenic *Agrobacterium tumefaciens* strains used in this study.

Strain	Biotype	Relevant Characteristics	Source or Reference
45d	1	pTi 45d (Marguerite)	PPRI, South Africa
2086a	1	pTi2086a (Willow)	PPRI, South Africa
1895	1	pTi1895 (Peach)	PPRI, South Africa
K21	1	pTiK21 (Chrysanthemum)	PPRI, South Africa
B6	1	pTiB6 (Apple)	Kerstens <i>et al.</i> (1973)
C58	1	pTiC58 pCrynoc <sup>+</sup>	M. Van Montagu
2207	2	pTi2207 (Flowering Quince)	PPRI, South Africa
46	2	pTi46 (Cherry)	PPRI, South Africa
41b	2	pTi41b (Peach)	PPRI, South Africa
At2	3	pTiAt2 (Grapevine)	PPRI, South Africa
G2/4	3	pTiG2/4 (Grapevine)	PPRI, South Africa
A2/1	3	pTiA2/1 (Grapevine)	PPRI, South Africa
D2/3	3	pTiD2/3 (Grapevine)	PPRI, South Africa

**PPRI - Plant Protection Research Institute, South Africa**

### 2.2.3 Plant Material

*Lycopersicon esculentum* Mill cv Money Maker (tomato) seed and seedlings and grapevine cuttings (Richter 99) were used in this study.

### 2.2.4 *In Vivo* Colonisation of Tomato Plants

Tomato seeds or seedlings were inoculated with the antibiotic-resistant strains by incubation for 20 minutes at room temperature with late-logarithmic-phase bacterial suspensions adjusted turbidometrically to densities of approximately  $10^8$  cfu ml<sup>-1</sup>. The seeds and seedlings were removed from the bacterial suspensions without rinsing and planted in non-sterile soil. At various time intervals the seedlings were dug up and loosely adhering soil was removed. The roots were cut off at the point of seed attachment and the mass of roots and closely adhering soil was determined. The colonisation efficiencies of the stem, rhizosphere (zone of soil closely associated with the roots which is influenced by root exudate), and rhizoplane (root surface) were determined. The roots and closely adhering soil were washed, placed in 10 ml of quarter strength Ringer solution (full strength Ringer solution comprises 2.25 g NaCl, 0.105 g KCl, 0.06 g CaCl<sub>2</sub>, 0.05 g NaHCO<sub>3</sub> per litre) and shaken vigorously for five seconds. The roots were removed, dried between filter paper, and weighed.

This gave the fresh mass of the roots. The mass of the rhizosphere soil was obtained by determining the difference between root plus soil mass and root mass measurements as detailed above. The rhizosphere bacteria were enumerated by plating on appropriate antibiotic-containing GT medium, 100  $\mu\text{g}$  of nalidixic acid per ml for K84; 100  $\mu\text{g}$  of rifampicin per ml for J73; 100  $\mu\text{g}$  of spectinomycin and 50  $\mu\text{g}$  of neomycin per ml for J73(pK1); 40  $\mu\text{g}$  of gentamycin per ml for J73(pMP90); 40  $\mu\text{g}$  of gentamycin and 100  $\mu\text{g}$  of neomycin per ml for J73(pMP90)(pAg84); 100  $\mu\text{g}$  of rifampicin per ml for H8; 100  $\mu\text{g}$  of spectinomycin per ml for H6; and 10  $\mu\text{g}$  of naladixic acid and 100  $\mu\text{g}$  of neomycin per ml for H6D17. Data were expressed as colony forming units (cfu) per gram of rhizosphere soil. The roots were then placed in 10 ml of a quarter strength Ringer solution in vessels containing four glass beads and shaken vigorously for 15 minutes on a wrist action shaker, and the released rhizoplane bacteria were enumerated as described above. Data were expressed as cfu  $\text{g}^{-1}$  of fresh mass of root. Bacterial colonisation of the stem was determined as for the rhizoplane by using 40 mm stem segments taken from the point of seed attachment. The fresh mass was determined and data were expressed as cfu  $\text{g}^{-1}$ . In all cases six replicates were run for each treatment.

### 2.2.5 *In Vitro* Colonisation of Tomato Plants

Two methods were used to assay *in vitro* colonisation. In the first, surface-sterilised seed (sterilised by suspension in 3.5 % [wt/vol] sodium hypochlorite for 10 minutes, followed by three washes in sterile distilled water) or sterile seedlings (grown aseptically in petri dishes from sterile seed on sterile filter paper pre-moistened with distilled water) were aseptically inoculated with the test strains described earlier. These were planted in sterile test tubes containing sterile soil moistened with half-strength Hoagland solution (HOAGLAND and ARNON, 1939) and incubated in a growth room with a 16 hours light / eight hours dark cycle at 22 °C. At various time intervals, rhizosphere, rhizoplane and stem colonisation efficiencies were determined as described. In the second method the *in vitro* colonisation of tomato roots in liquid culture (FINNIE and VAN STADEN, 1985) was determined by aseptically excising 15 mm root tip segments from tomato root stocks, inoculating with test strains by dipping and incubating at 25 °C in seven ml Miller's medium (MILLER, 1965) in the light with agitation (80 rpm). At intervals, the root segments were aseptically removed and the rhizoplane colonisation efficiency was determined. Six replicates were run for each treatment.

### 2.2.6 *In Vivo* Colonisation Assays of Grapevines

Rooted grapevine cuttings were inoculated with the antibiotic-resistant biocontrol strains by dipping in late-logarithmic phase bacterial suspensions adjusted turbidometrically to densities of approximately  $10^8$  cfu ml<sup>-1</sup> and then planted in non-sterile soil. Three rooted cuttings were used per treatment. At various time intervals three root samples per plant were taken and the root area determined using a leaf area meter. The colonisation efficiency of the strains in the rhizosphere was then determined by placing the roots and closely adhering soil into 10 ml of quarter strength Ringer solution and shaking vigorously for 15 minutes on a wrist action shaker. The rhizosphere bacteria were enumerated by plating on the appropriate antibiotic-containing medium. Data were expressed as cfu cm<sup>-2</sup>. In all cases nine replicates were performed for each treatment.

### 2.2.7 Scanning Electron Microscopy

Root material was fixed overnight in 3 % (wt/vol) gluteraldehyde in 0.05 M cacodylate buffer (pH 7.2), washed twice for 30 minutes durations in 0.05 M cacodylate buffer, fixed in 2 % (wt/vol) osmium tetroxide for two hours, and washed in 0.05 M cacodylate buffer for 30 minutes. The root material was

dehydrated through an ethanol gradient series prior to critical-point drying under amyl acetate. The dried material was then mounted on scanning electron micrograph stubs which were sputter coated with gold-palladium prior to viewing.

### 2.2.8 Hydrophobicity Assay

The hexadecane assay based on the assay of ROSENBERG *et al.* (1980) was used. The bacteria were grown in GT medium at 25 °C to late log phase ( $10^7$  to  $10^8$  cfu ml<sup>-1</sup>), harvested, washed twice in PUM buffer (16.95 g K<sub>2</sub>HPO<sub>4</sub>, 7.26 g KH<sub>2</sub>PO<sub>4</sub>, 1.8 g urea, 0.2 g MgSO<sub>4</sub> per litre, pH7.2) and suspended in buffer to an A<sub>436</sub> of 0.6. Samples (three ml) of bacterial suspensions were dispensed into test tubes and 100 µl aliquots of hexadecane were added. The tubes were vigorously agitated for one minute on a vortex mixer and allowed to stand for 15 minutes. The aqueous phase was removed and the A<sub>436</sub> was determined. Six replicate samples and controls containing no hexadecane were prepared for each bacterial sample and the test was repeated three times. Hydrophobicity was expressed as the percentage of cells bound to hexadecane.

## 2.2.9 Biological Control Assays

### 2.2.9.1 *In Vitro* Biological Control Plate Assay

Successful and potential agrocin-producing biocontrol stains K84 and J73 and its derivatives were spotted onto appropriate agar medium (WEBSTER *et al.*, 1986) and grown for four days at 26°C. Colonies were killed with chloroform and overlaid with 3 ml of 0.6 % agar medium containing 200  $\mu$ l of 48 hours pathogen culture (Table 2) and incubated for three days at 26°C. Strains were classified as sensitive if a clearly defined zone of inhibition was recorded. Tests were performed in triplicate. The antibiotics produced by strains H6, H6(pK1) and H6D17 were detected by growing them on HP agar (HYNES *et al.*, 1985) at 28 °C for two days, killing with chloroform and overlaying with a 0.7 % molten solution of agar seeded with a stationary phase culture of the sensitive (pathogenic) *A. tumefaciens* strains (Table 2).

### 2.2.9.2 *In Vivo* Biological Control Assay

**For tomato plants:** Unsterilised soil was placed in pots and inoculated with the pathogen to give approximately  $10^6$  cfu g<sup>-1</sup> of soil. Two days later tomato seedlings

were dipped in suspensions of the biocontrol stains (approximately  $10^7$  cfu ml<sup>-1</sup>) and planted in the inoculated soil. Controls included cuttings inoculated with biocontrol strains and planted in uninoculated soil and uninoculated cuttings planted in pathogen-inoculated soil. Three replicates were performed for each treatment and tumour formation was assessed after three months. The gall index being the number of plants of each treatment with galls x the average gall size was determined. Gall size scored from 1 to 4: 1 = 1-5 mm diameter; 2 = 5-10 mm diameter; 3 = 10-15 mm diameter; 4 = > 15 mm diameter.

**For grapevines:** Unsterilised soil was placed in pots and inoculated with the pathogen to give approximately  $10^6$  cfu g<sup>-1</sup> of soil. Two days later rooted grapevine cuttings were dipped in suspensions of the biocontrol stains (approximately  $10^7$  cfu ml<sup>-1</sup>) and planted in the inoculated soil. Controls included cuttings inoculated with biocontrol strains and planted in uninoculated soil and uninoculated cuttings planted in pathogen-inoculated soil. Three replicates were performed for each treatment and tumour formation was assessed after eight months as detailed above.

#### 2.2.9.3 Plasmid Screening

Strains were screened for the presence of plasmid by the method of KADO and LIU (1981).

### 2.2.10 Statistical Analysis of Data

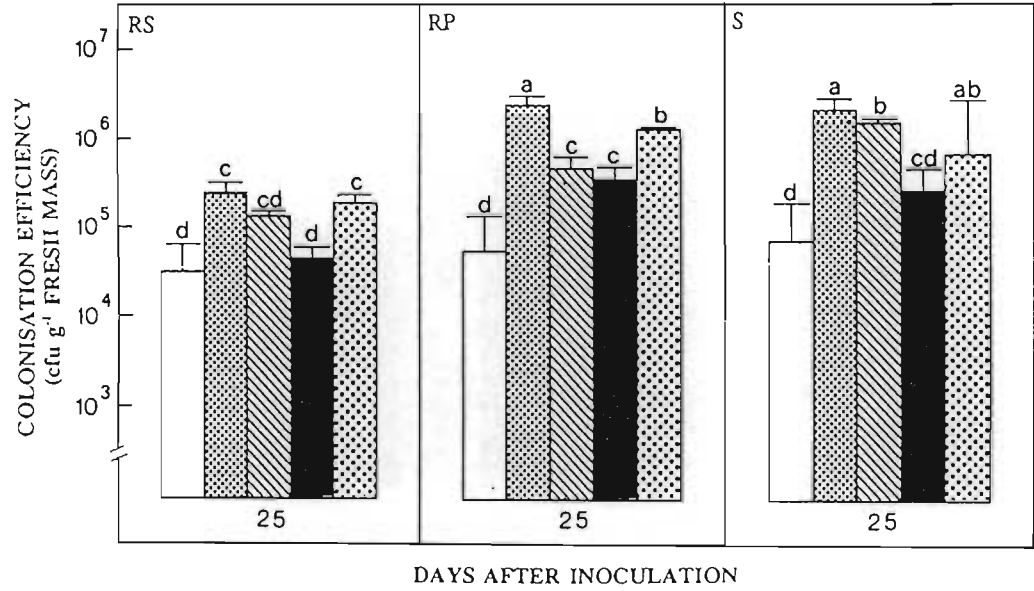
Analysis of variance of data collected from the various tests was carried out using the StatGraphics computer programme. Together with this the Duncan multiple range test (DUNCAN, 1955) was used to determine whether there were statistically significant differences in the data obtained.

## 2.3 RESULTS

### 2.3.1 *In vivo* Colonisation of Tomato Plants

Preliminary *in vivo* colonisation of tomato seedlings by K84, J73, D286, H8 and H6 showed colonisation of the rhizoplane of tomatoes by strains K84, D286, H8 and H6 was significantly better than colonisation by strain J73 (Figure 9). The colonisation efficiency of K84, D286 and H6 in the rhizosphere of tomato seedlings 25 days after seed inoculation, was significantly better than that of J73 and H8, while on stems colonisation by K84 was best. *In vivo* colonisation of tomato seedlings inoculated with J73, D286, H8 and H6 confirmed that 28 days after inoculation D286, H8 and H6 were more efficient colonisers of the rhizosphere, rhizoplane and stem than J73 (Figure 10). Twenty five day-colonisation results from seed inoculations confirmed

these findings (Figure 11). During preliminary colonisation tests uncured potential biocontrol strains J73 and H8 induced tumours on tomato plants (Figure 12) and as expected no sign of tumour formation occurred with cured D286 and non-pathogenic H6.



**Figure 9.** *In vivo* colonisation of tomato seedlings grown from seed inoculated with J73 (□), K84 (▨), D286 (▩), H8 (■) and H6 (▤) after twenty five days. The bars above the histograms indicate standard deviations, and the letters indicate statistically significant differences; bars bearing the same letter indicate that there is no statistical difference between those values, based on the Duncan Multiple Range test at the P= 0.1 level. RS, Rhizosphere; RP, Rhizoplane; S, Stem.

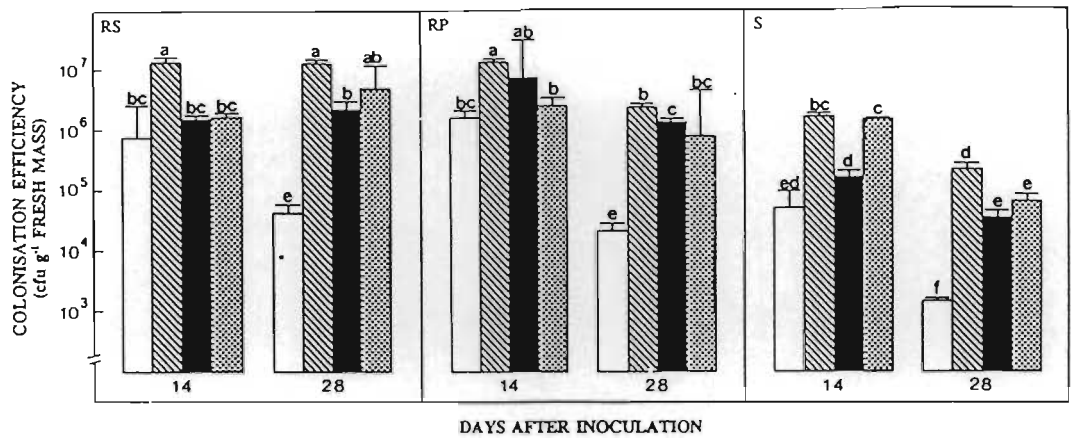


Figure 10. *In vivo* colonisation of tomato seedlings with J73 (□), D286 (▨), H8 (■) and H6 (▤) 14 and 28 days after inoculation. The bars above the histograms and the abbreviations are as for Figure 8.

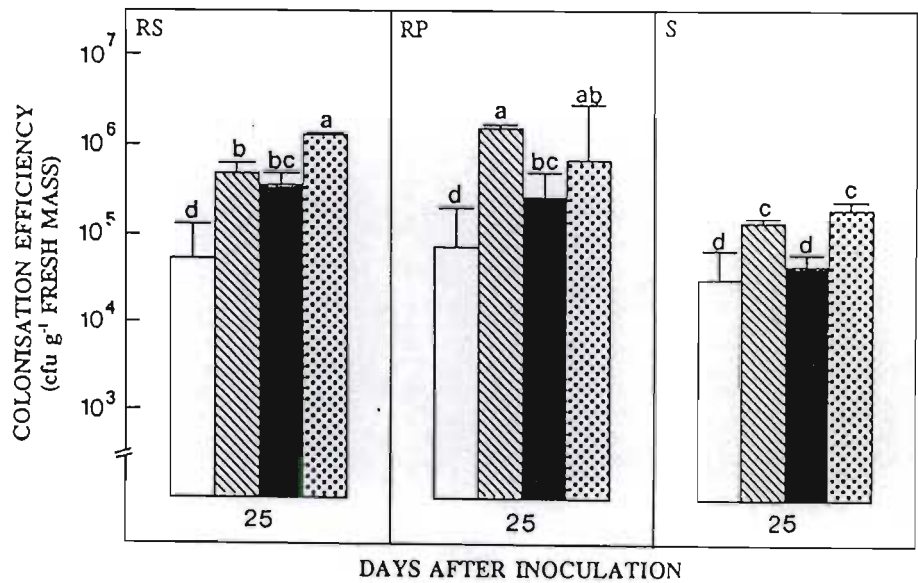
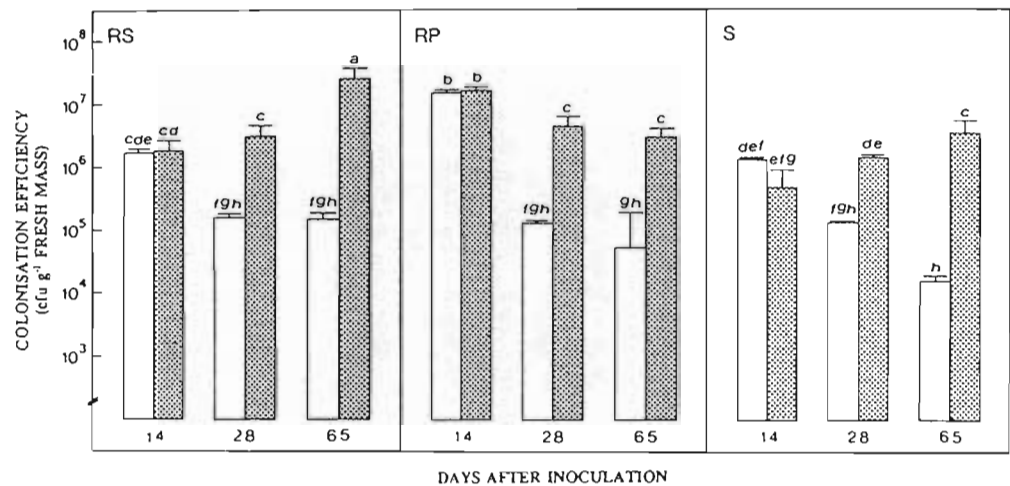


Figure 11. *In vivo* colonisation of tomato seedlings grown from seed inoculated with J73 (□), D286 (▨), H8 (■) and H6 (▤), 25 days after inoculation. The bars above the histograms and the abbreviations are as for Figure 8.

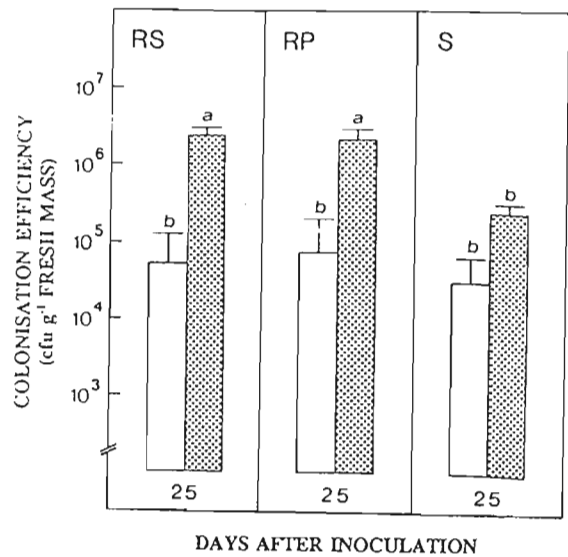


**Figure 12.** Galls induced by uncured potential biocontrol strains J73 and H8 during *in vivo* colonisation studies.

A comprehensive colonisation study was completed comparing J73 and K84, the potential and successful biocontrol bacteria. *In vivo* colonisation of tomato seedlings by K84 and J73 was tested on a daily basis for 14 days after inoculation. Both strains colonised with similar efficiencies during this period. However by 28 and 65 days after inoculation the numbers of K84 had either remained constant or increased slightly, while those of J73 had decreased by up to two orders of magnitude (Figure 13). There was no statistically significant difference in the K84 population levels between 14 and 28 days. However, there was a significant decrease in all J73 populations between 14 and 28 days. Colonisation of seedlings grown from inoculated seed (Figure 14) confirmed the relative inefficiency of J73 at 25 days after inoculation.



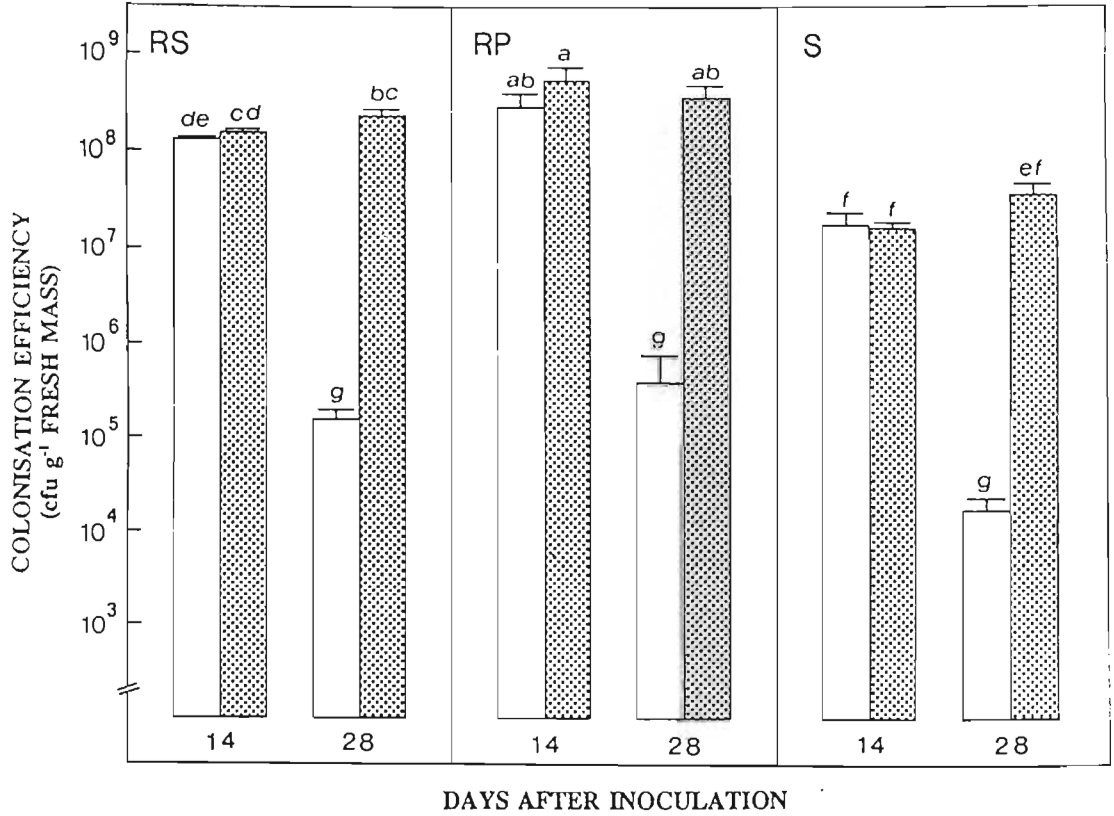
**Figure 13.** *In vivo* colonisation of tomato seedlings with J73 (□) and K84 (▨), 14, 28 and 65 days after inoculation. The bars above the histograms and the abbreviations are as for Figure 8.



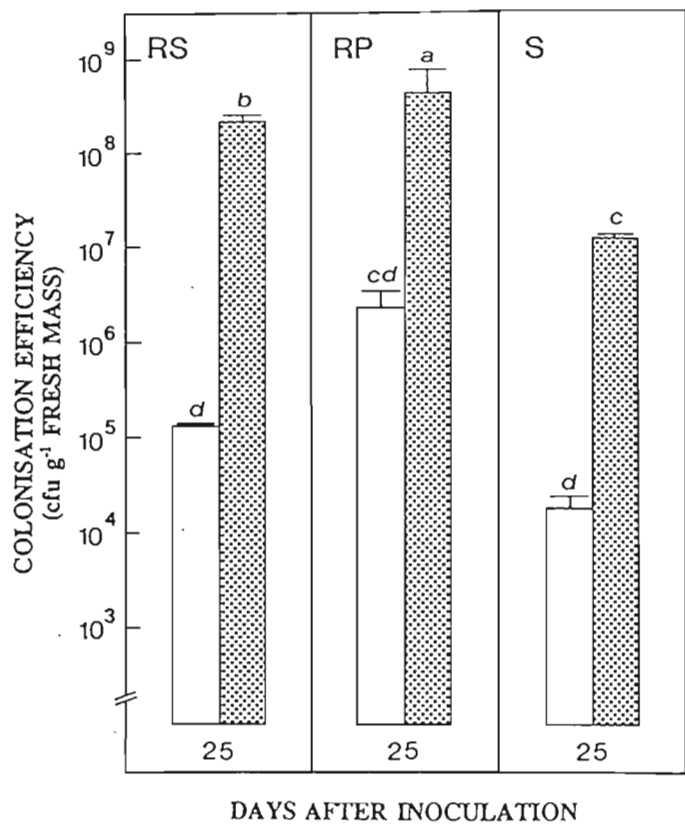
**Figure 14.** *In vivo* colonisation of tomato seedlings grown from seed inoculated with J73 (□) and K84 (▨), 25 days after inoculation. The bars above the histograms and the abbreviations are as for Figure 8.

2.3.2 *In Vitro* Colonisation of Tomato Plants

*In vitro* assays in sterile soil again showed that J73 could colonise efficiently in the early stages but by 28 days numbers had declined by three to four orders of magnitude (Figure 15). Colonisation of seedlings grown from inoculated seeds confirmed the relative inefficiency of J73 at 25 days after inoculation (Figure 16).



**Figure 15.** *In vitro* colonisation of tomato seedlings grown in sterile soil under sterile conditions with J73 (□) and K84 (▨), 14 and 28 days after inoculation. The bars above the histograms and abbreviations are as for Figure 8.

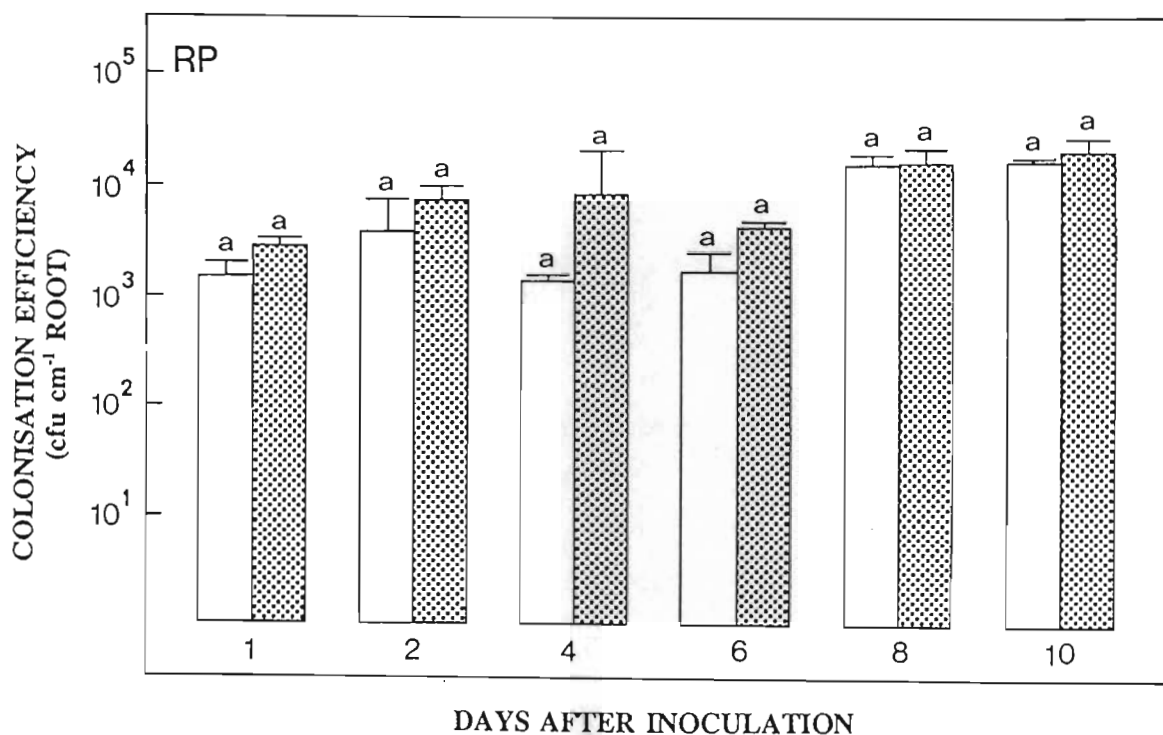


**Figure 16.** *In vitro* colonisation of tomato seedlings grown in sterile soil under sterile conditions from seed inoculated with J73 (□) and K84 (▨), 25 days after inoculation. The bars above the histograms and the abbreviations are as for Figure 8.

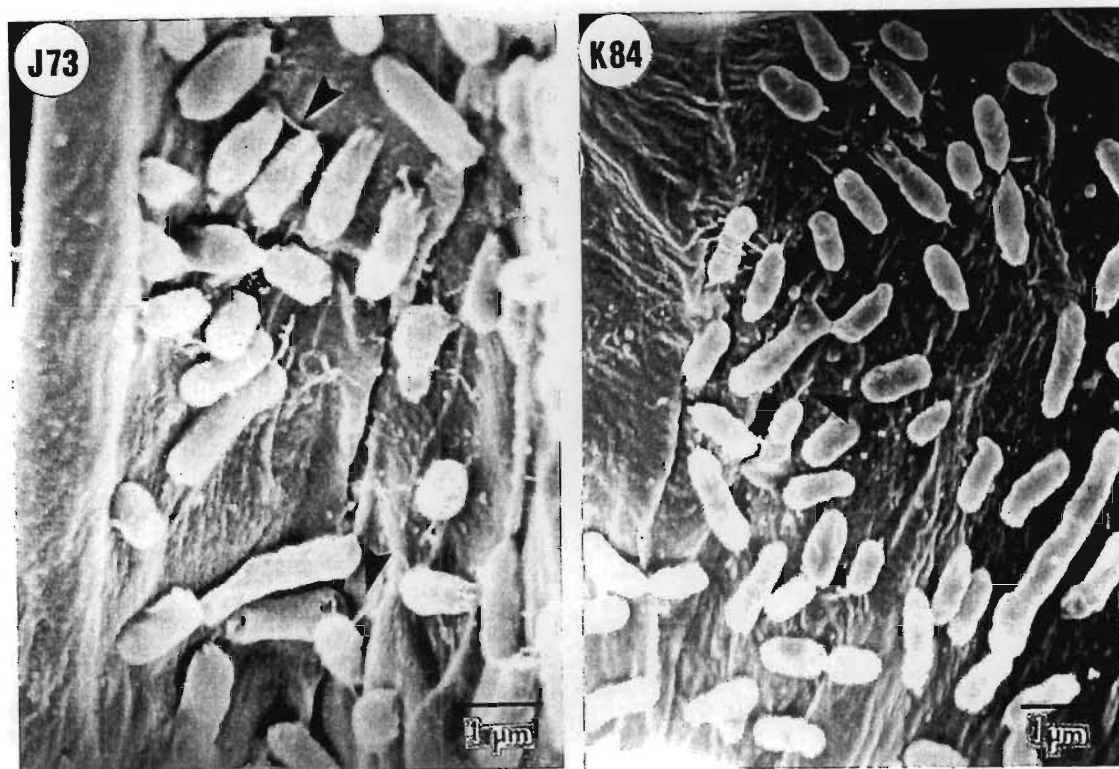
### 2.3.3 *In Vitro* Colonisation of Roots in Liquid Culture

To determine whether the poor colonising ability of J73 was due to its inability to adhere to the tomato seedlings, *in vitro* colonisation in liquid culture was studied. It was only possible to maintain the cultures for 10 days, but during this time there was no significant difference between the two strains (Figure 17). The number of

bacteria colonising the rhizoplane in liquid culture rose from approximately  $2 \times 10^3$  to  $4 \times 10^3$  cfu cm<sup>-1</sup> of root after 1 day to approximately  $2 \times 10^4$  after 10 days, an increase of five- to 10-fold. Scanning electron micrographs showed the presence of attachment strands from both J73 and K84 onto the surface of tomato roots (Figure 18). These were first observed 48 h after inoculation. The bacteria attached mainly in and around the root hair region, covering approximately 10 % of the surface.



**Figure 17.** *In vitro* colonisation of tomato roots grown in liquid medium with J73 (□) and K84 (▨) over a 10 day period. The bars above the histograms and the abbreviations are as for Figure 8.



**Figure 18.** Scanning electron micrographs showing attachment between bacteria and tomato roots.

### 2.3.5 Hydrophobicity Assay

A number of studies have suggested a relationship between bacterial hydrophobicity and adherence (WEIS *et al.*, 1982). However the hexadecane binding assay indicated that there was no significant difference between K84 and J73 (Table 5).

**Table 5.** Hydrophobicity of *A. radiobacter* and *A. tumefaciens* strains.

Strain	cfu ml <sup>-1</sup>	% Hydrophobicity
K84	1.0 x 10 <sup>8</sup>	66.4
J73	1.3 x 10 <sup>8</sup>	62.6
D286	6.5 x 10 <sup>7</sup>	1.7
H6	2.6 x 10 <sup>7</sup>	7.9

### 2.3.6 Biological Control of Crown Gall Disease on Tomatoes

One of the six biotype 1 (2086a) and two of the three biotype 2 (46 and 41b) pathogens tested were found to be sensitive *in vitro* to agrocin 84 (Table 6). However, only pathogenic *A. tumefaciens* B6 was found to be sensitive to agrocin J73. *A. tumefaciens* (pMP90;pAg84) producing both agrocons J73 and 84 was able to control four of the pathogens tested, 2086a, B6, C58 and 2077 under *in vitro* conditions.

Under *in vivo* conditions K84 was not able to effectively control crown gall disease caused by any of the pathogenic strains tested (Table 7). Cured J73 strains, J73(pK1) and J73(pMP90) were only effective in controlling crown gall caused by B6, a

biotype 1 strain. J73(pMP90)(pAg84) was only able to control two pathogenic biotype 2 strains, 2077 and 41b, under *in vivo* conditions.

### 2.3.6 *In Vivo* Colonisation of Six *Agrobacterium* Strains on Grapevines

K84 and J73 and its derivatives were found to be poor long term colonisers of the grapevine rhizosphere, whereas H6 and its derivatives were good long term colonisers with up to 100-fold more bacteria per cm<sup>2</sup> of root after 248 days. Colonisation by H6D17 was significantly better than that of H6 and H6(pK1) after this time (Figure 19).

### 2.3.7 Biological Control of Crown Gall Disease on Grapevines

Agrocin 84 was ineffective against the biotype 3 pathogens tested under *in vitro* conditions, however these pathogens were sensitive to agrocin J73 (Table 6; Figure 20). H6, H6(pK1) and H6D17 were all effective in controlling biotype 3 pathogens *in vitro*. Under *in vivo* conditions only H6 and its derivatives were effective in controlling crown gall on grapevines (Table 8).

**Table 6.** *In vitro* sensitivity of biotype 1, 2 and 3 pathogenic strains to agrocin-producing strains.

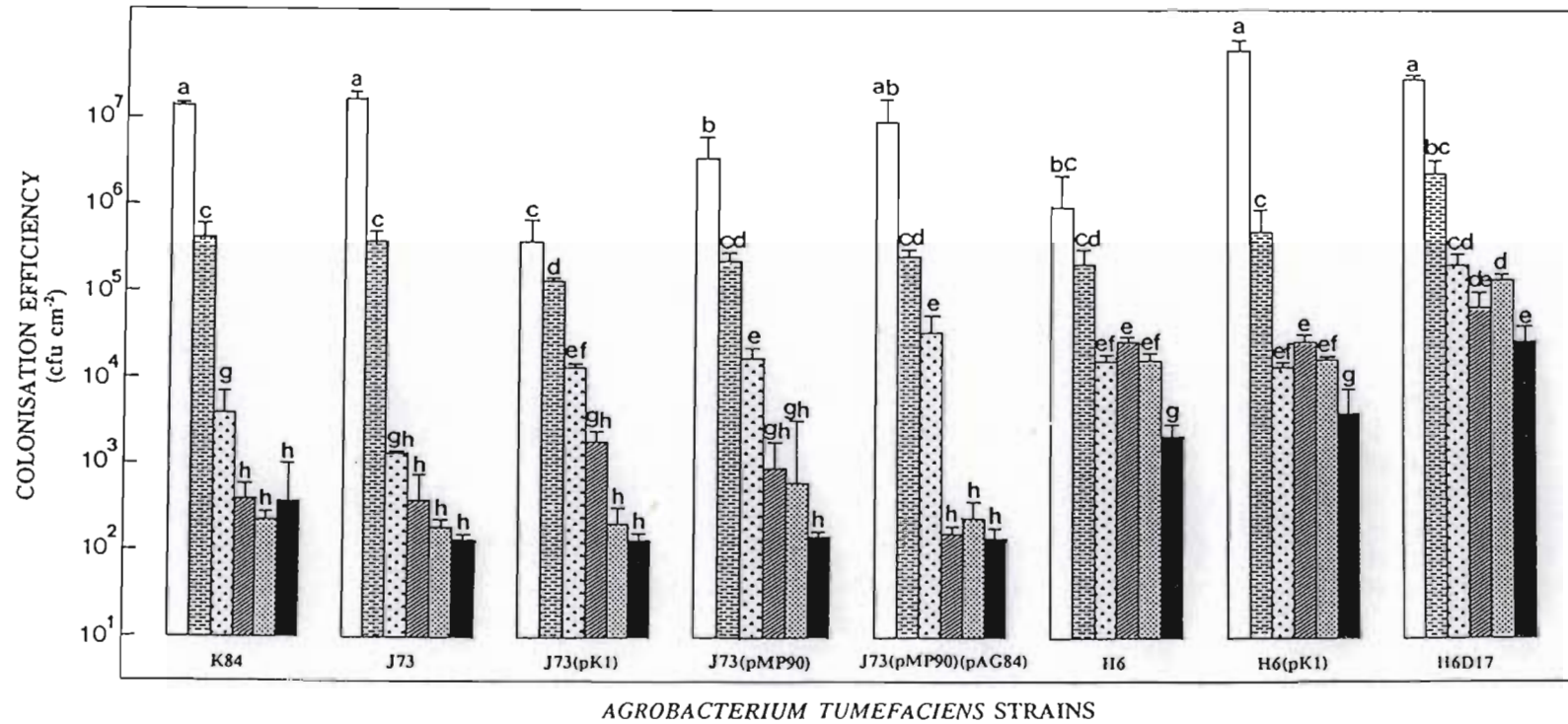
Pathogens	Biotype	Agrocin-Producing Strains							
		K84	J73	J73(pK1)	J73(pMP90)	J73(pMP90)(pAg84)	H6	H6(pK1)	H6D17
45d	1	R	R	R	R	R			
2086a	1	S	R	R	R	S			
1895	1	R	R	R	R	R			
K21	1	R	R	R	R	R			
B6	1	R	S	S	S	S			
C58	1	R	R	R	R	S			
2077	2	R	R	R	R	S			
46	2	S	R	R	R	R			
41b	2	S	R	R	R	R			
AT2	3	R	S	S	S	R	S	S	S
G2/4	3	R	S	S	S	R	S	S	S
A2/1	3	R	S	S	S	R	S	S	S

**Table 7.** Number of tomato plants<sup>a</sup> with galls (and gall index<sup>b</sup>) twelve weeks after simultaneous inoculation with pathogenic and agrocin-producing strains.

Pathogenic strains	Biotype	Uninoculated	Agrocin-Producing Strains				
			K84	J73	J73(pK1)	J73(pMP90)	J73(pMP90)(pAg84)
Uninoculated		0	0	3( 6)	0	0	0
45d	1	3( 6)	2( 4)	3( 6)	3( 6)	3( 6)	3( 6)
2086a	1	3( 6)	3( 6)	3( 6)	3( 9)	3( 9)	3( 6)
1895	1	3( 6)	3( 6)	3( 6)	3( 6)	3( 6)	3( 9)
K21	1	3( 6)	3( 6)	3( 6)	3( 6)	3( 3)	3( 6)
B6	1	3( 6)	3( 6)	0	0	0	3( 6)
C58	1	3( 6)	3( 6)	3( 6)	3( 6)	3( 6)	3( 6)
2077	2	3( 6)	3( 6)	3( 6)	3( 6)	3( 6)	0
46	2	1( 1)	3( 6)	3( 6)	3( 9)	3( 9)	3( 6)
41b	2	3( 6)	3( 6)	3( 9)	3( 6)	3( 6)	0
<b>Total</b>		25(49)	26(52)	27(57)	24(54)	24(51)	21(49)

<sup>a</sup> Three tomato plants per treatment

<sup>b</sup> Gall index = number of plants of each treatment with galls x average gall size. Gall size scored from 1 to 4: 1 = 1-5mm diameter; 2 = 5-10 mm diameter; 3 = 10-15 mm diameter; 4 = > 15 mm diameter.



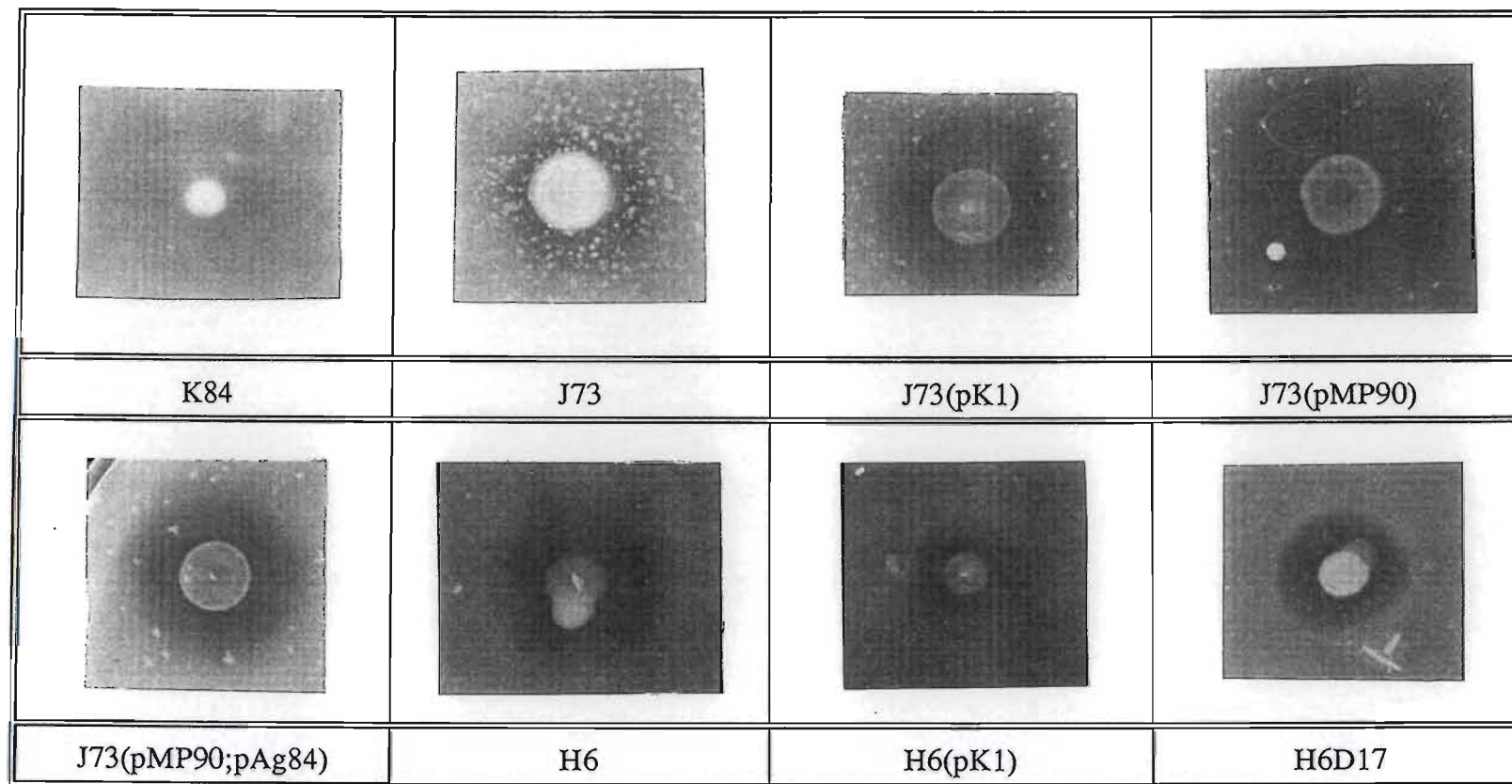
**Figure 19.** Colonisation of grapevine rhizosphere by agrocin-producing strains of *A. tumefaciens* K84, J73, J73(pK1), J73(pMP90), J73(pMP90)(pAg84), H6, H6(pK1) and H6D17, after 0 (□), 30 (▨), 64 (▩), 120 (▤), 191 (▦) and 248 (■) days. The bars above the histograms and the abbreviations are as for Figure 8.

**Table 8.** Number of grapevines<sup>a</sup> with galls (and gall index<sup>b</sup>) eight months after the vines were inoculated with agrocin-producing strains and planted in pathogen inoculated soil.

Agrocin-producing strains	Uninoculated	Biotype 3 pathogens			Total
		AT2	A2/1	G2/4	
Uninoculated	0	3(12)	3(12)	2( 8)	8(32)
K84	0	2( 6)	2( 6)	2( 8)	6(20)
J73	0	3(12)	3( 9)	3( 6)	9(27)
J73(pK1)	0	3( 9)	3( 9)	3(12)	9(30)
J73(pMP90)	0	3( 9)	3( 6)	3(12)	9(27)
J73(pMP90)(pAg84)	0	3( 6)	3( 9)	3(12)	9(27)
H6	0	0	0	0	0
H6(pK1)	0	0	0	0	0
H6D17	0	0	0	0	0

<sup>a</sup> Three grapevines per treatment

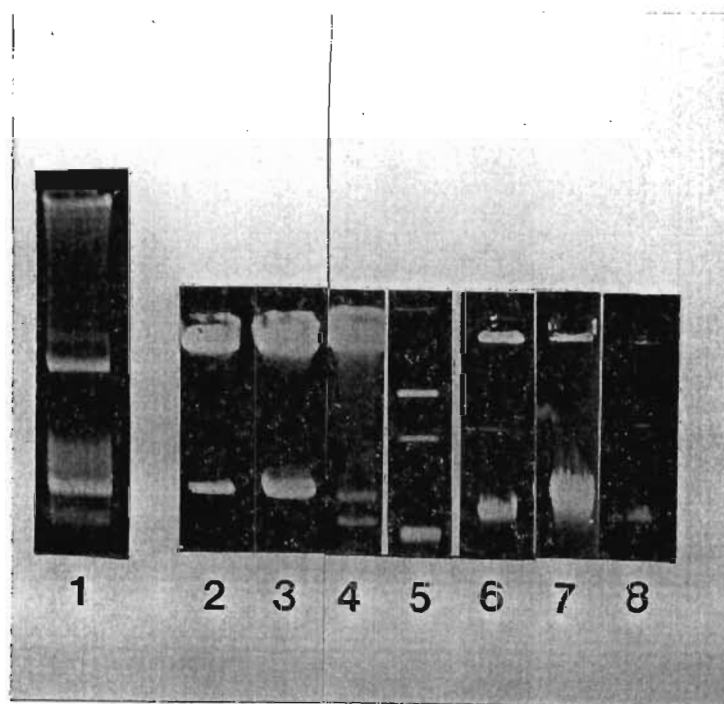
<sup>b</sup> Gall index = number of plants of each treatment with galls x average gall size. Gall size scored from 1 to 4: 1 = 1-5mm diameter; 2 = 5-10 mm diameter; 3 = 10-15 mm diameter; 4 = > 15 mm diameter.



**Figure 20.** Zones of inhibition showing biotype 3 strain, AT2, sensitivity to agrocin-producing strains.

#### 2.4.8 Plasmid Screens of *Agrobacterium tumefaciens*

The plasmid content of the potential biological control bacteria reisolated from the grapevine rhizosphere 248 days after inoculation, was determined and the results (Figure 21) confirmed that they did loose or gain a plasmid during colonisation by *in planta* transformation or conjugation with other bacteria.



**Figure 21.** Plasmid screens of *A. radiobacter* strain K84 (1) and *A. tumefaciens* strains J73Sp<sup>r</sup> (2), J73(pMP90) (3), J73Sp<sup>r</sup>(pK1) (4), J73(pMP90)(pAg84) (5), H6 (6), H6D17 (7) and H6(pK1) (8), reisolated from the rhizosphere 248 days after inoculation.

## 2.4 DISCUSSION

*A. tumefaciens* D286 was originally identified as a potential biocontrol strain by HENDSON *et al.* (1983), based on its ability to produce an agrocin with a broader host range than K84. Results from preliminary colonisation trials shown here indicated that although D286 is a good long term coloniser, it did not effectively control crown gall disease on grapevine (results not shown). This substantiated the finding of HENDSON *et al.* (1986). D286 was therefore eliminated from this programme. Similarly, although H8 was found to be a good long term coloniser, it has a narrow host range for biotype 3 pathogens and is a pathogen which could not be cured. For this reason it was also eliminated from the programme.

Colonisation of the rhizosphere, rhizoplane and stems of tomato plants by J73 was significantly lower than that for K84, D286 and H6 confirming the suggestion made by WEBSTER (1990) that this strain may be a poor coloniser. The colonisation ability of J73 was therefore compared to that of K84, the successful biocontrol strain, under various conditions with the aim of establishing the reason for its poor colonisation ability.

The first step in the colonisation process is site-specific attachment of bacteria to the plant. Cellulose fibrils produced by *A. tumefaciens* and *Rhizobium leguminosarum*

have been implicated in aspects of this process (MATTHYSSE, 1983; MATTHYSSE, 1987a and b). The lack of these fibrils does not render bacteria non-infective. The extracellular polysaccharide products of chromosomal genes *chv* and *exoC* (CANGELOSI *et al.*, 1987; DOUGLAS *et al.*, 1985), as well as some unidentified surface polypeptides (MATTHYSSE, 1987a), are required for these processes. However, cellulose fibrils may be important in colonisation by biocontrolling bacteria.

The results reported here from both *in vivo* and *in vitro* colonisation of tomato seedlings, whether by seed or seedling inoculation, showed that in all cases J73 was significantly less efficient than K84. Up to 14 days after inoculation, however, there was no significant difference between the two strains. Scanning electron microscopy showed the presence of attachment strands from the bacteria onto the tomato root surface over a 10 day period. This suggests that J73, while attaching efficiently with the aid of fibrils, is unable to maintain this initial colonisation. Thus, while fibrils may be important in initial attachment of bacteria to plants, their presence is not sufficient to ensure long term colonisation. The fact that both *in vivo* and *in vitro* assays gave similar results indicates that the poor colonisation by J73 in non-sterile soil was not due simply to competition with indigenous bacteria but was due to some property inherent in this strain.

The 5- to 10-fold increase in K84 and J73 numbers in the rhizoplane of tomato roots grown in liquid culture after a 10-day period is not surprising, as JAMES *et al.* (1985) found that a number of rhizobacteria, including *A. tumefaciens*, adhered very rapidly to roots. They showed that the rhizobacteria reached half-maximum by five minutes, and after 25 days observed an increase in colonisation of 67-fold.

Contradictory results exist for the role of hydrophobic properties of cell surfaces in bacterial adhesion (RUTTER and VINCENT, 1980; JAMES *et al.*, 1985). One school of thought is that hydrophobic interactions may stabilise the adhesive reaction and in some instances loss of hydrophobicity has been associated with the loss of the ability to adhere. The other school of thought is that hydrophobic properties do not play a role in the colonisation process (JAMES *et al.*, 1985). J73 has a similar hydrophobicity to K84, which is very much higher than those of D286, H6 and H8 and yet only J73 is a poor long term coloniser. This clearly indicates that there is no correlation between the hydrophobic properties of these strains and their colonisation efficiency, ruling out any major hydrophobic interactions in the colonisation process. These results support the findings of JAMES *et al.* (1985).

A comparison of the *in vitro* agrocin sensitivity and *in vivo* biocontrol results show that if a pathogen is sensitive to an agrocin *in vitro* it does not necessarily mean that it will be effective under *in vivo* conditions. For example, four biotype 1 pathogenic

strains, 2086a, B6, C58 and 2077, were sensitive to agrocin J73 and agrocin 84 produced by strains J73(pMP90)(pAg84). However, this strain was only effective in controlling pathogens 2077 and 41b on tomato seedlings. Similarly, although pathogens 2086a, 46 and 41b were sensitive to agrocin 84 *in vitro*, strain K84 was unable to effectively protect tomatoes from crown gall disease induced *in vivo* by these pathogens. The importance of both *in vitro* agrocin sensitivity and *in vivo* biocontrol tests in assessing the potential of a new biocontrol strain is emphasised by these findings. The inability of J73 and its derivatives, J73(pK1); J73(pMP90); J73(pMP90)(pAg84) to efficiently control crown gall disease on tomato seedlings, even though they produce an effective agrocin or agrocin in the latter case, can be attributed to their poor long term colonisation ability.

The ability of J73 and H6 and their derivatives to colonise grapevines and control crown gall disease was studied because bacterial colonisation efficiencies have been shown to vary from plant to plant in some instances. The *in vivo* colonisation efficiencies of K84 and J73 and its derivatives were compared to those of H6 and its derivatives. The results clearly indicate that only H6, H6pK1 and H6D17 (an agrocin H6 super producer) are efficient long term colonisers of the grapevine rhizosphere. They could be isolated from the grapevine rhizosphere at titres between  $1 \times 10^6$  and  $8 \times 10^7$  cfu cm<sup>-2</sup> initially, and after 248 days their colonisation efficiencies were between  $3,5 \times 10^3$  and  $4,5 \times 10^4$  cfu cm<sup>-2</sup>. The other potential

biocontrol strains tested, J73, J73(pK1), J73(pMP90) and J73(pMP90)(pAg84) were found to be poor long term colonisers of the grapevine, as was the case for tomatoes. The initial titres of J73 and its derivatives were between  $6 \times 10^5$  and  $1 \times 10^7$  cfu cm<sup>-2</sup>, however, after 248 days their colonisation efficiencies were down to *ca.*  $2 \times 10^2$  cfu cm<sup>-2</sup>. Similarly, the colonisation efficiency of K84 had decreased significantly by 248 days and it was not significantly different from J73, indicating that it was not able to efficiently colonise grapevines.

No significant difference was found in the long term colonisation efficiency of J73(pK1) compared to J73 on grapevines, suggesting that pK1 does not enhance colonisation ability. This differs from the finding of WEBSTER (1990) who observed a significant enhancement in colonisation as a result of the introduction of plasmid pK1. Similarly, the insertion of pK1 into H6 did not significantly improve colonisation of H6 on grapevines, substantiating the finding that pK1 does not improve a strain's ability to colonise grapevines efficiently.

*In vitro* biocontrol studies revealed that strains J73 and H6 and their derivatives produced agrocins that were effective against the three grapevine pathogens AT2, A2/1 and G2/4, tested. However, under *in vivo* conditions, although in some instances J73 and its derivatives were able to reduce gall formation by the pathogens on grapevines, but none of the strains completely prevented the disease from

occurring. This strain is therefore ineffective as a biocontrol agent against three biotype 3 pathogens. When H6 and its derivatives were used as biocontrol agents, gall formation by all three pathogens was completely inhibited, confirming the results of STAPHORST *et al.* (1985) who found that H6 was able to inhibit gall formation by 12 biotype 3 pathogens. H6 therefore has great potential as a biocontrol strain for grapevine pathogens. Further nursery and field trials with this strain are necessary to access its commercial potential.

Although J73 may have little value as a biocontrol agent it is possible that agrocin J73 may be of value if it could be produced by strains such as K84 and H6, both of which are efficient colonisers, but produce agrocin with narrow host ranges. WEBSTER (1990) showed that pAgJ73 and pAg84 are compatible and that both agrocin J73 and 84 can be produced by the same strain. Thus, as suggested by her, it may be worthwhile continuing the research on J73 with the aim of transferring pAgJ73 to strain K84 and H6 or another potential biocontrol strain which can colonise plants efficiently.

In conclusion, the results presented in this chapter provide additional evidence that future searches for biological control strains should not be restricted at the initial stage to the ability to produce a potent agrocin. The approach should rather be one in which the agrocin activity spectrum of a potential strain is determined

concomitantly with its colonisation efficiency. The strategy of the biocontrol program, of which this work formed a part, was to genetically engineer an all-purpose strain that would control crown gall on a variety of hosts. Research results obtained in this study suggest that this is unlikely to succeed unless strains can be isolated that can colonise several different hosts efficiently. Future strategies for biological control of crown gall disease should perhaps focus on the isolation of potential biocontrol strains that are host specific.

### CHAPTER 3

## MICROPROPAGATION OF COMMERCIAL FORESTRY TREES

### (HARDWOODS)

#### 3.1 INTRODUCTION

*Eucalyptus* species are evergreen hardwood trees which are regarded as one of the most productive forest crops in short rotation plantations because of their fast growth, wide adaptability and useful products (TURNBULL and BOLAND, 1984; WARRAG *et al.*, 1989). In South Africa *E. grandis* is the major *Eucalyptus* species with a plantation area of 394 006 hectares, representing 30.4 % of the total afforested land. The plantation area of all the other *Eucalyptus* species planted is 137 003 hectares, which represents 11 % of the total afforested land (FORESTRY COUNCIL, 1992). The demand for forest products in South Africa is however, increasing rapidly and a massive timber shortage has been forecast for the turn of the century. To circumvent this it is essential to find methods for increasing productivity, especially as the land for timber production is becoming scarce (VAN WYK, 1985).

Natural regeneration of *Eucalyptus* stands is mainly by coppice or by seed, but

breeding is a slow process because of the length of the juvenile phase before flowering. Vegetative propagation through rooted cuttings has been successful with large genetic gains being obtained in different parts of the world, including South Africa (ZOBEL and IKEMORI, 1983; CAMPINHOS and IKEMORI, 1985; DENNISON and QUAILE, 1987; ROCKWOOD *et al.*, 1988; ADENDORFF and SCHÖN, 1991; DENNISON and KIETZKA, 1993). However, vegetative propagation can also be difficult because by the time *Eucalyptus* trees reach an age where they can be evaluated by foresters and geneticists, they have usually passed the stage at which they can be easily propagated vegetatively. Vegetative propagation of adult material by cuttings is therefore not always very successful (DURAND-CRESSWELL, 1982; BOULAY and FRANCKET, 1982). Rejuvenation of mature material (see 1.5.2) can be achieved by grafting, hedging or cytokinin application, which results in improved rooting and hence propagation potential. Although grafting has been successful it is not used extensively in forestry. Rooting is the limiting factor for successful vegetative propagation of a clone by cuttings, with a rooting ability of at least 70 % being necessary (ADENDORFF and SCHÖN, 1991; DENNISON and KIETZKA, 1993).

The success and the difficulties experienced in vegetative propagation by cuttings and the increasing demand for timber has stimulated renewed and increased interest in *Eucalyptus* tissue culture as an alternative means of vegetative propagation. Tissue

culture research has focused on a number of species including *E. grandis*, a species of major importance in the pulp and mining industries, *E. nitens*, *E. gunnii*, *E. globulus*, *E. dalrympleana*, *E. pauciflora*, *E. delagatenis*, *E. nova-anglica* and *E. viminalis*, and certain *Eucalyptus* hybrids (FURZE and CRESSWELL, 1985; MEHRA-PALTA, 1982; MACRAE and VAN STADEN, 1990; GORDON, 1991; LE ROUX and VAN STADEN, 1991a and 1991b; MARQUES *et al.*, 1991; DENNISON and KIETZKA, 1993). This research has resulted in the direct micropropagation of many of these *Eucalyptus* species from nodal explants of coppice and seedling material (MCCOMB and BENNETT, 1986; LE ROUX and VAN STADEN, 1991b) and has shown that potentially large numbers of plants can be produced within a relatively short period of time (DE FOSSARD *et al.*, 1974; DURAND-CRESSWELL *et al.*, 1982). In addition micropropagation has been shown to improve rooting of selected material (FURZE and CRESSWELL, 1985; MCCOMB and BENNETT, 1986). Mass plantlet production of selected *Eucalyptus grandis* hybrids has also been developed through direct micropropagation methods (WARRAG *et al.*, 1989). Application of such systems on an operational level, however, depends on the post-culture performance of plantlets, especially their physiological adaptation to greenhouse- and subsequently field conditions.

WARRAG *et al.* (1989) compared the physiological adaptation process of tissue culture-derived plantlets from three superior five-year-old *E. grandis* Hill ex Maid

hybrids to their related half-sib seedlings under greenhouse conditions. Although seedlings initially had a higher net photosynthesis, chlorophyll and nitrogen concentration, the difference between plantlets and seedlings decreased and reached similar values over time. These results suggest that there are no changes in a plantlet's basic physiological responses to changing environmental conditions around the plantlet and seedlings. Thus under practical conditions deleterious physiological differences, which could influence future performance of *E. grandis* seedlings or tissue cultured plantlets, are not apparent. This points to the possible use of tissue culture for mass propagation of elite *Eucalyptus* trees for operational planting (WARRAG *et al.*, 1989). In support of this DENNISON and KIETZKA (1993) reported that in 10-month-old field trials tissue cultured plants showed greater uniformity than plants raised from rooted cuttings.

Clonal propagation of *Eucalyptus* is playing an increasingly important role in forestry in South Africa, however the difficulties experienced have led to an investigation into the use of direct micropropagation as an alternative means of propagation. In this study, the *in vitro* propagation of *E. grandis* from nodal segments of commercial seedling material was initially studied with the aim of developing a suitable culture medium and improving micropropagation success.

Agar is widely used as the gelling agent for tissue culture media of most plants

including *Eucalyptus*. Reports have indicated that growth-inhibitory substances may be present in the agar (HU and WANG, 1983). Thus unless purified agar is used, or charcoal is added to the medium, there is a potential danger of growth retardation or inhibition. Gelrite (gellan gum), formerly known as PS-60, produced by *Pseudomonas elodea* has been developed as a gelling agent (KANG *et al.*, 1982) and is used for bacteriological media (SHUNGU *et al.*, 1983). ICHI *et al.* (1986) found that callus growth and shoot production of a number of plants were greater on gelrite-containing medium than on agar-containing medium. The use of gelrite as the gelling agent for micropropagation of *E. grandis* was therefore investigated and compared with agar and agarose.

The fact that clonal propagation through tissue culture offers an alternative means of vegetative propagation which has the potential to provide high shoot multiplication rates of uniform genotypes, which root more easily, resulting in short-term gains, led to an investigation into the *in vitro* development of clones from seed of selected *E. grandis* genotypes. The novel approach of integrating this into a clonal cuttings programme to accelerate clone bank development is also proposed.

Finally, the development of an efficient *in vitro* propagation method was necessary in order to rapidly propagate the material required for an investigation into the potential use of *A. rhizogenes* to improve rooting in *Eucalyptus*.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Effect of Gelling Agent on Micropropagation of *E. grandis*

#### 3.2.1.1 Plant Material

Three-month-old *E. grandis* seedlings were obtained from a commercial nursery and kept under shade cloth. Seedlings were sprayed once a week with a fungicide (benomyl) for two weeks before tissue culture.

#### 3.2.2 Media

The medium of MURASHIGE and SKOOG (1962) (MS) was used routinely. For *E. grandis* the shoot initiation medium comprised the MS salts, 30 g l<sup>-1</sup> sucrose, 0.1 mg l<sup>-1</sup> BA and 0.01 mg l<sup>-1</sup> NAA at pH 5.8. Shoot multiplication was achieved on MS salts, 30 g l<sup>-1</sup> sucrose, 0.5 % PVP, 0.2 mg l<sup>-1</sup> BA and 0.01 mg l<sup>-1</sup> NAA at pH 5.8. The medium for shoot elongation was the MS salts, 30 g l<sup>-1</sup> sucrose, 0.5 % PVP, 0.1 mg l<sup>-1</sup> BA 0.01 mg l<sup>-1</sup> NAA and 0.1 mg l<sup>-1</sup> gibberellic acid at pH 5.8. Roots were initiated on MS salts, 15 g l<sup>-1</sup> sucrose, 0.5 % PVP and 1 mg l<sup>-1</sup> IBA at a pH of 5.8.

### 3.2.1.3 Culture Conditions

Explants and shoots were initially incubated in the dark for seven days at 26°C and then for three weeks at 26°C in a growth room with a 16 hour photoperiod. A light fluence of  $36 \mu\text{mol m}^{-2} \text{s}^{-1}$  was provided by cool white fluorescent tubes.

### 3.2.1.4 Micropropagation Method

Selected nodal explants as described by FURZE and CRESSWELL (1985) were surface-sterilized using 3.5 % sodium hypochlorite for 15 minutes, followed by three 15 minute washes in sterile distilled water. Leaf and stem edges were trimmed after sterilization and the explants placed vertically on shoot initiation medium solidified with 0.8 % agar (Oxoid Technical Agar No. 3). Newly formed shoots from axillary buds were excised and placed on shoot multiplication medium which was solidified with 0.8 % agar. Multiple shoots were divided into clusters of about 4 to 5 shoots and placed on shoot multiplication medium solidified with either 0.8 % agar, 0.2 % gelrite or 0.6 % agarose. Clusters of shoots were also placed on shoot elongation medium solidified in the same way. To induce rooting, shoots 1 to 2 cm in height, were placed on root initiation medium which was solidified with 0.8 % agar or 0.2 % gelrite. These tubes were incubated in the dark for 10 days at 26°C and then in the growth room for a further three weeks. For all tests there were ten replicates per

treatment and all experiments were repeated three times.

### 3.2.2 *E. grandis* Clone Development *In Vitro*

#### 3.2.2.1 Plant Material

Seeds from two *E. grandis* genotypes (clones), SGR481 and SGR047 (CSIR, FORESTEK) were placed into culture with the aim of developing clones *in vitro* based primarily on shoot multiplication rate.

#### 3.2.2.2 Media

The medium of MURASHIGE and SKOOG (1962) (MS) was used routinely. Shoot multiplication was achieved on MS salts, 30 g l<sup>-1</sup> sucrose, 0.2 mg l<sup>-1</sup> BA and 0.01 mg l<sup>-1</sup> NAA at pH 5.8, solidified with 0.8 % agar. Roots were initiated on half-strength MS salts, 15 g l<sup>-1</sup> sucrose and 1 mg l<sup>-1</sup> IBA at a pH of 5.8, solidified with 0.8 % agar.

### 3.2.2.3 Micropropagation Method

Seeds from the two *E. grandis* genotypes (SGR481 and SGR047) were soaked in hydrogen peroxide (100 volume; 30 % m/v) : absolute ethanol (1:1) for 10 minutes, washed once in sterile distilled water for 10 minutes and then twice in 0.01 % Benlate. They were germinated on hormone-free MS medium in the dark for five days. The seedlings were then grown for 6 to 8 weeks at 22°C in a growth room with a 16 hour photoperiod and a light fluence of 36  $\mu\text{mol m}^{-2} \text{s}^{-1}$  provided by cool white fluorescent tubes. The roots were then aseptically excised from the seedlings, the shoots were transferred to multiplication medium and incubated under the conditions described above for 4 to 6 weeks. The shoots from each clone were subcultured onto fresh shoot multiplication medium (by dividing the multiple shoots into small clusters of 4 to 5 shoots) and incubated for 4 to 6 weeks. This step was repeated until the desired number of shoots per clone were produced. The number of shoots that developed from each clone after each subculture period was recorded and shoot multiplication rates determined. If shoot elongation did not occur naturally, it was stimulated by the addition of 0.1 mg l<sup>-1</sup> gibberellic acid to the multiplication medium. Rooting *in vitro* was achieved by selecting elongated shoots, at least 20 mm in height, and aseptically transferring them to the root initiation medium and incubating at 22°C in the dark for 10 days. Thereafter they were transferred to the growth room with a 16 hour photoperiod as detailed above for a further 2 to 3 weeks. The alternative to rooting *in vitro* is rooting *in vivo* during

acclimatization and this has proved to be more efficient in some cases and was achieved by dipping the selected shoots or small shoot clusters in a 1 mg l<sup>-1</sup> IBA solution before aseptically transferring to a sterile substrate and acclimatizing. For all rooting tests there were 10 replicates per treatment and three blocks, thus a total of 30 explants.

#### 3.2.2.4 Acclimatization

The elongated shoots or rooted plantlets were aseptically transferred into a sterile substrate, vermiculite moistened with half strength MS medium (2:1 v/v), in Speedling trays which were placed into large plastic bags to maintain the relative humidity above 70 %. These trays were then maintained for seven days at 25°C under fluorescent light of 36  $\mu\text{mol m}^{-2} \text{s}^{-1}$  intensity in a growth room with a 16 hour photoperiod. Thereafter a gradual decrease in humidity was achieved by opening the bag daily for increasingly longer periods of time over a further 7 day period. During this time plantlets were sprayed with a Benlate (0.01 %) / Kelpak (0.1 %) (an organic fertiliser) solution every alternate day. The plastic was then removed and the plantlets placed in the greenhouse under mist irrigation. Six to 8 weeks later they were repotted into large containers filled with a soil:sand (2:1) mixture.

### 3.3 RESULTS

#### 3.3.1 Effect of Gelling Agent on Micropropagation of *E. grandis*

The excised axillary buds formed multiple shoots on all media containing  $0.1 \text{ mg l}^{-1}$  BA and  $0.01 \text{ mg l}^{-1}$  NAA. On the shoot multiplication medium containing gelrite the mean number of shoots increased seven-fold after four weeks whereas with agarose and agar this increased at 6- and 2-fold respectively (Table 9). The length of shoots was also greatly enhanced on the medium solidified with gelrite (Table 10). Shoot number was greatly enhanced on a gelrite compared to a agar containing medium (Figure 22). On shoot elongation medium containing  $0.1 \text{ mg l}^{-1}$  BA,  $0.01 \text{ mg l}^{-1}$  NAA and  $0.1 \text{ mg l}^{-1}$  gibberellic acid shoot length increased 2.5-fold with gelrite whereas with agarose and agar the increase was only 1.9 and 1.5-fold respectively (Table 10, Figure 23). Rooting was better with a gelrite-containing medium than with one containing agar (Figure 24).

**Table 9.** Effect of gelling agent on shoot multiplication of *E. grandis*<sup>a</sup>.

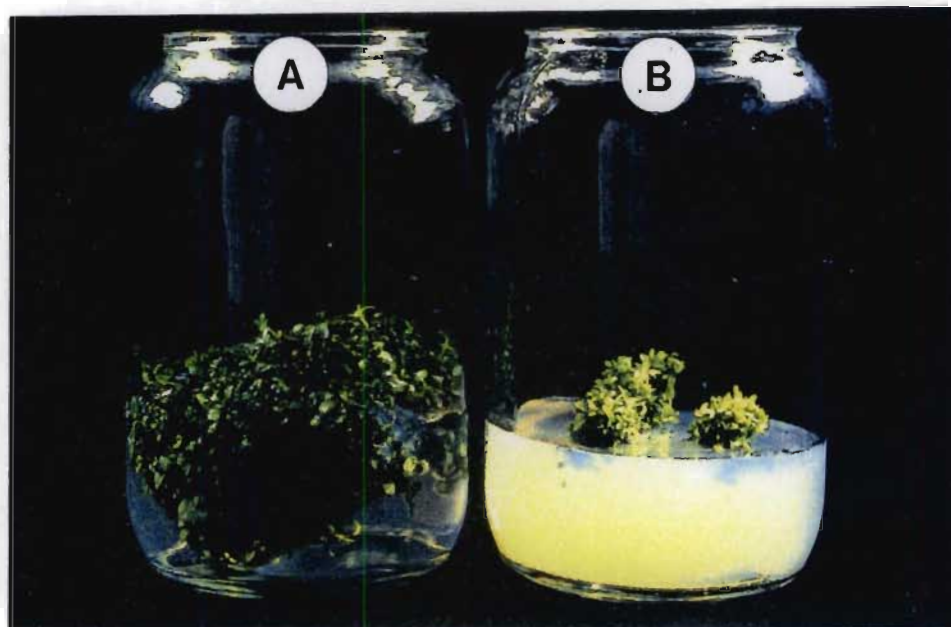
Gelling agent	No. of Shoots	Shoot height (mm)
Gelrite	28.9 (4.5)	14.6 (1.5)
Agarose	25.1 (4.1)	11.3 (1.9)
Agar	9.1 (2.2)	6.3 (0.9)

<sup>a</sup> Multiple shoot development from a small cluster of about four shoots on MS medium containing 0.2 mg l<sup>-1</sup> BA and 0.01 mg l<sup>-1</sup> NAA was assessed after a four week culture period. Values in brackets represent standard errors.

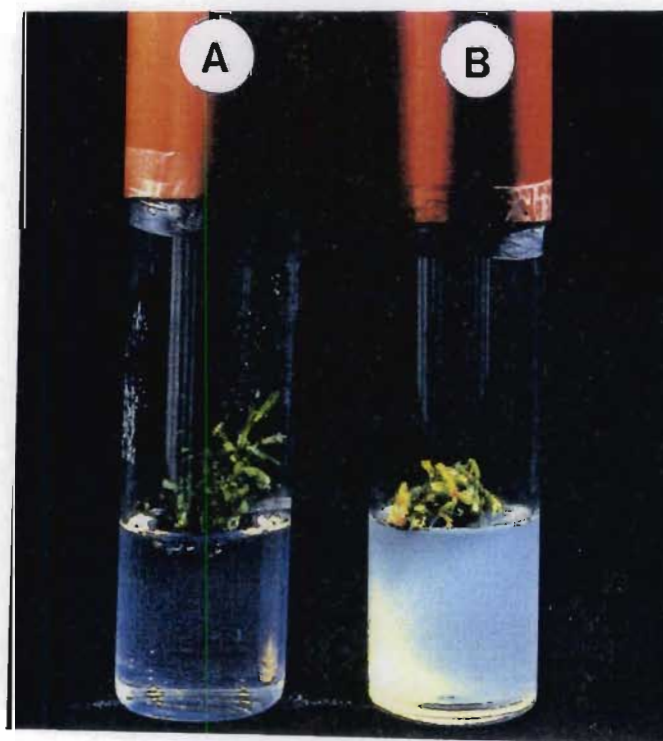
**Table 10.** Effect of gelling agent on shoot elongation of *E. grandis*<sup>a</sup>.

Gelling agent	No. of Shoots	Shoot height (mm)
Gelrite	14.1 (5.0)	10.3 (1.5)
Agarose	9.5 (2.2)	7.7 (0.6)
Agar	6.4 (0.9)	6.1 (0.6)

<sup>a</sup> Elongation of small clusters of shoots approximately five mm in height on MS medium containing 0.1 mg l<sup>-1</sup> BA, 0.01 mg l<sup>-1</sup> NAA and 0.1 mg l<sup>-1</sup> gibberellic acid was assessed after a four week culture period. Values in brackets represent standard errors.



**Figure 22.** Shoot multiplication of *E. grandis* on a medium solidified with gelrite (A) and agar (B).



**Figure 23.** Elongation of *E. grandis* shoots on a medium solidified with gelrite (A) and agar (B).

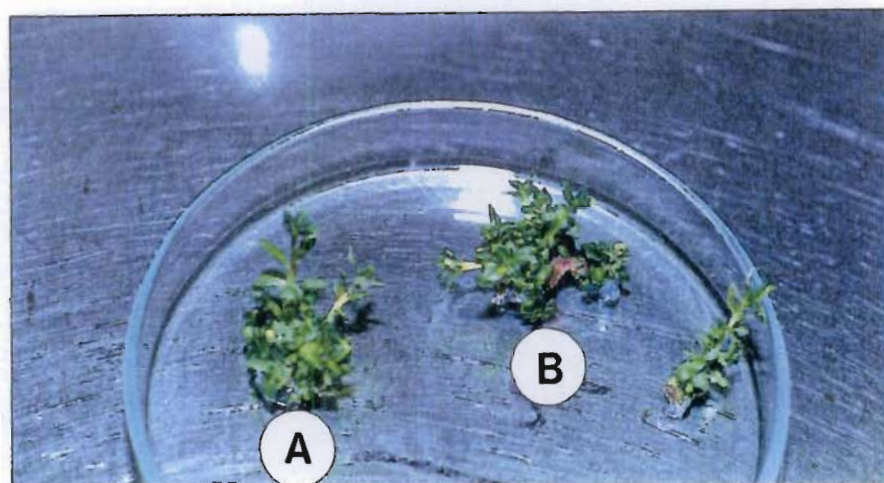


**Figure 24.** Rooting of *E. grandis* shoots on a medium solidified with gelrite (A) and agar (B).

### 3.3.2 *E. grandis* Clone Development *In Vitro*

Seeds from two *E. grandis* genotypes (SGR481 and SGR047) were placed into culture with the aim of developing clones *in vitro* based primarily on shoot multiplication rate. Twenty percent germination rates were achieved for both *E. grandis* SGR481 and SGR047. One hundred healthy seedlings were selected, their roots aseptically excised and the shoots placed onto shoot multiplication medium. Each individual shoot was now regarded as a clone and classified as, for example,

SGR481/4, where 4 represents fourth clone of genotype SGR481. The shoot multiplication rates of the 200 clones initiated varied greatly from clone to clone within a genotype and between genotypes, ranged from 3- to 10-fold in the 4 to 6 week culture period (Figure 25). Based on multiplication rates, 37 clones were selected from the two *E. grandis* genotypes, 21 from SGR481 and 16 from SGR 047.



**Figure 25.** Multiple shoots of *Eucalyptus grandis* clones SGR481/33 (A) and SGR047/39 (B).

Rooting of the selected clones also varied from clone to clone and between the genotypes. Of the 21 SGR481 clones selected only 12 rooted and their rooting ability ranged from 25 - 100% (Table 11). Similarly, for the 16 SGR047 clones only 9 rooted. Their rooting abilities ranging from 17 to 100 %. With the aim of improving the rooting of these clones *in vitro*, the potential use of *A. rhizogenes*-mediated rooting was investigated and the results are presented in Chapter 4.

**Table 11.** *In vitro* rooting of selected *E. grandis* clones.

CLONE	Rooting Ability %	CLONE	Rooting Ability %
SGR481/9	100	SGR047/8	17
SGR481/12	33	SGR047/9	50
SGR481/18	66	SGR047/13	25
SGR481/26	50	SGR047/18	75
SGR481/28	100	SGR047/24	50
SGR481/30	100	SGR047/29	66
SGR481/32	25	SGR047/37	66
SGR481/33	100	SGR047/39	100
SGR481/44	100	SGR047/41	50
SGR481/48	60		
SGR481/72	66		
SGR481/50	50		

Losses during acclimatization ranged from zero to 10 %. Acclimatised plants were uniform and of good quality, growing rapidly under greenhouse conditions. Good branching was obtained on acclimatised *in vitro* propagated *Eucalyptus* clones. These plantlets were repotted, four months later shoots were cut back to stimulated further shoot development. Two months after pruning, the potted plants were suitable for use as stock/mother plants (Figure 26) for cuttings trials.

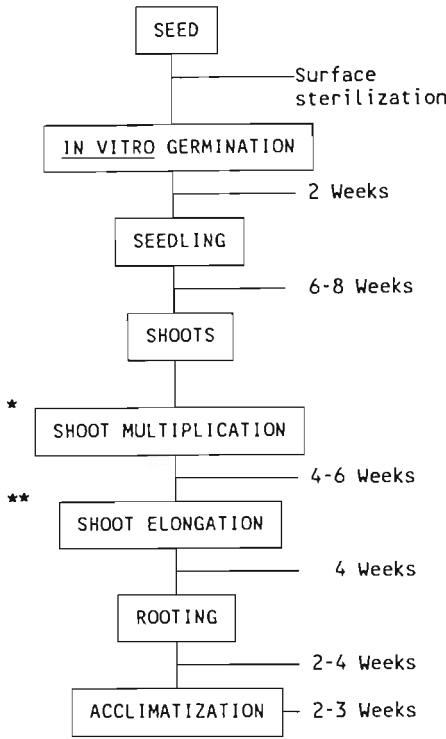


**Figure 26.** *E. grandis* clones six months after acclimatization. This plant was cut back at four months to stimulate further branching.

### 3.4 DISCUSSION

The steps involved in *in vitro* propagation of *Eucalyptus* from seedling material are detailed in Figure 27. Modification of the medium to optimise shoot multiplication for each selected clone is not economically feasible on an operational scale. As a result an MS-based medium (MURASHIGE and SKOOG, 1962) was developed and used successfully to propagate a large number of clones. The addition of PVP to this medium reduced phenolics in the medium which resulted in healthier explants. In order to improve the medium further, the effect of gelling agents on micropropagation of *E. grandis* was investigated.

The results presented indicate that shoot propagation was greatly enhanced on a medium solidified with gelrite, compared to those with agarose and agar. This, together with the production of a greater number of roots of superior morphology, indicated that gelrite-containing media should be the medium of choice for tissue culture of *Eucalyptus grandis*. This is in agreement with the results for *in vitro* culture of a number of herbaceous plants (ICHI *et al.*, 1986). The nature of the putative inhibitory constituents in agar (HU and WANG, 1983) awaits further elucidation. Although agarose was shown to be superior to agar its unacceptably high cost precluded its use as an alternative for routine use.



**Figure 27.** Diagrammatic representation of steps involved in micropropagation of *E. globulus* seedlings.

\* The shoot multiplication step is repeated until the required number of shoots are obtained. This is dependent on the multiplication rate of the plant material.

\*\* This step is included only if necessary, that is, if natural elongation does not occur.

This modified medium has been used to micropropagate a number of *Eucalyptus* species, clones and hybrids, such as *E. grandis*, *E. nitens* and *grandis/nitens* hybrids (results not shown). The intrinsic propagation potential of a species/clone under both *in vitro* and *in vivo* conditions is dependent on a number of factors. These included the condition (health) of the stock material (undoubtedly one of the most important factors), physiological and environmental conditions (see section 1.5.2.1). However, rooting is ultimately under genetic control and this has been emphasised by the fact that certain species are more difficult to root than others. For example, the cold tolerant species, *E. nitens*, is more difficult to root than *E. grandis* (see Chapter 4). In addition, the clonal variation in rooting under apparently optimal conditions supports this.

Micropropagation of selected *Eucalyptus* clones from coppice- and nursery-grown seedlings using the axillary bud technique is well documented (FURZE and CRESSWELL, 1985, MCCOMB and BENNETT, 1986, LE ROUX and VAN STADEN, 1991a and b), although there are no reports of the successful use of this technique on a commercial scale. High shoot multiplication rates have been reported suggesting that large numbers of shoots, eg. 350 000, can potentially be produced from a single nodal explant in a six month period (FURZE and CRESSWELL, 1985). In practice however, the actual number of plantlets produced in this time will be far less due to losses during rooting and acclimatization. In addition, not all

shoots produced are of uniform size and elongation may be necessary before rooting can occur. This would increase the time required to produce shoots suitable for rooting. The *in vitro* propagated clones can either be used to establish clone banks which would serve as a source of cuttings for vegetative propagation or can be used for plantation establishment.

The development of clones from seedling material, eg. seed from controlled crosses selected from a breeding program, under nursery conditions is currently being undertaken in many countries (P. J. Wilson and S. Verry, personal communication). This can take about 2 years depending on the rooting ability and the number of plantlets required for the clone bank. Similarly, from the time a clone has been approved it takes approximately 2 years using the conventional cutting system to raise 10 000 to 15 000 ramets required as a source of cuttings for commercial production (DENNISON and KIETZKA, 1993). DENNISON and KIETZKA (1993) suggest that micropropagation offers a means of reducing this developmental time by one year.

The *in vitro* development of clones from two *in vitro* propagated *E. grandis* genotypes was investigated. As phenolics were not a problem in *in vitro* propagation from seedling material the media were modified by excluding PVP. In addition, the gelrite concentration was increased to 3 g l<sup>-1</sup>. This resulted in a better quality of

microcuttings and a lower incidence of vitrification.

The viability of seeds from clones SGR481 and SGR047 germinated *in vitro* was extremely poor (20%) and this was confirmed by the fact that *in vivo* germination rates were similar, suggesting poor fertilization. The results from this study show that shoot multiplication rates and rooting ability of clones developed *in vitro* from selected seed varied from clone to clone and between genotypes. Clones were therefore selected based on their multiplication rates and rooting ability. As in conventional propagation by cuttings, rooting ability was found to be the limiting factor in *in vitro* propagation. The clonal variation in rooting substantiated the fact that rooting is under genetic control as rooting of juvenile material should have been more successful. However, it must be borne in mind that, as under *in vivo* propagation conditions (ADENDORFF and SCHÖN, 1991), a number of other factors influence rooting under *in vitro* conditions (see sections 1.5.2.1 and 1.5.3.1). These may have influenced the rooting percentages obtained in this study.

The selection of clones with high multiplication rates and good rooting ability implies that plants could be produced more rapidly. Theoretically for example, in six months 15 625 explants could be produced from a clone with a multiplication rate of 5 and 1 million explants from a clone with a multiplication rate of 10. In practice, however, as already mentioned, a smaller number of explants will be

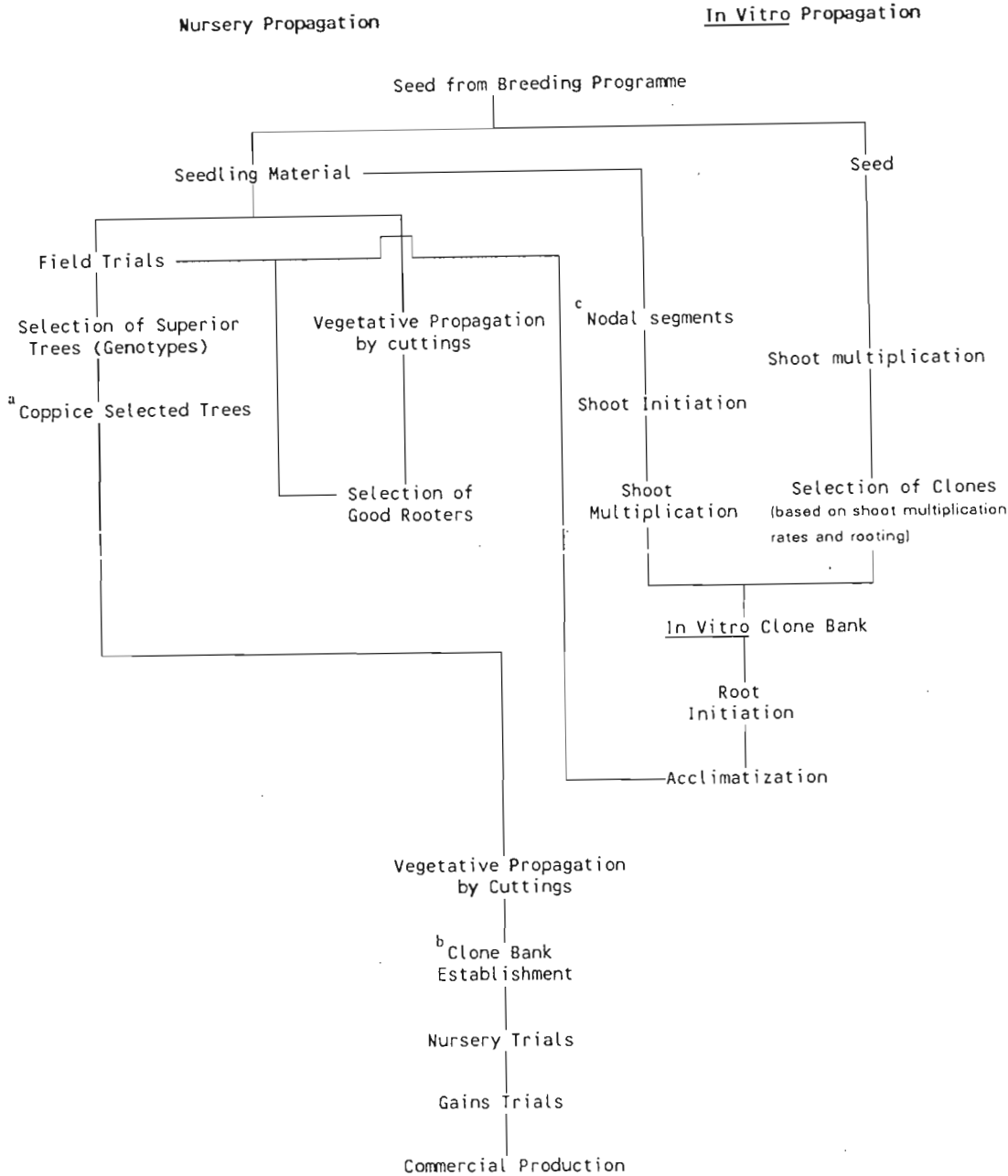
produced. However, this suggests that the 10 000 - 15 000 plants required for clone bank establishment is quite attainable in a 12 month period, implying that a 50 % reduction in the time required to develop a clone from selected seedlings is possible using this method.

Acclimatised *in vitro* propagated plantlets tended to be more branched than rooted cuttings which suggests that more cuttings per plantlet could be taken from this material than from the stock plants developed in the nursery. Rooting of small shoot clusters, of 4 to 6 shoots, during acclimatization has also been successful. This may result in an increase in the number of cuttings that could be taken from each plantlet (cluster) compared to that from an individual shoot that has been rooted and acclimatised. The value of these results in clonal propagation still has to be determined.

In conclusion the results from this investigation suggest that *in vitro* propagation has the potential to accelerate clone bank establishment, in that sufficient numbers of stock (mother) plants can be developed more rapidly than by conventional cutting techniques. The integration of this method into existing vegetative propagation programmes is proposed (Figure 28). *In vitro* propagation of coppice and seedling material using the axillary bud technique has also been included. This could also contribute to reducing the time required to bulk up a selected clone from coppice-

or nursery grown seedling material for clone bank establishment. Further work is necessary to prove the value of this proposed procedure in clonal forestry. For example, the rooting ability of cuttings taken from the acclimatised *in vitro* propagated stock plants will have to be established and compared to stock plants developed from cuttings under nursery conditions. The potential use of the bacterium *A. rhizogenes* to improve rooting of the clones developed *in vitro* is detailed later (Chapter 4).

Finally, vegetative propagation techniques will never surpass breeding as it is essential to create new genotypes which are adapted to changing environments. Vegetative propagation by cuttings and tissue culture does however, have an important role to play in multiplying these genotypes asexually, if the increasing demands for timber are to be met.



**Figure 28.** *Eucalyptus* clone development under nursery conditions from coppice or seedling material and under tissue culture conditions from seedling material. Material from <sup>a</sup> and <sup>b</sup> can be used as a source of material for <sup>c</sup>.

## CHAPTER 4

### *IN VITRO AGROBACTERIUM RHIZOGENES* TRANSFORMATION SYSTEM FOR IMPROVING ROOTING OF TREE SPECIES

#### 4.1 INTRODUCTION

Many plant species are routinely propagated by cuttings. While this technique has the advantage of producing clones from selected plants (see sections 1.5 and 3.1) and stabilising a genotype, it is limited by the inherent ability of the genotype to produce roots (see section 3.2). Thus the rooting of woody plant cuttings is only feasible for a limited number of species. The molecular approach to improving rooting using a natural genetic transformation system offers an alternative means of addressing the problem of rooting. This involves the use of *Agrobacterium rhizogenes* strains which can genetically transform many dicotyledonous plants by means of root inducing genes carried on their Ri plasmids, resulting in the production of numerous secondary functional roots (WHITEMAN RUNS HIM *et al.*, 1988; see section 1.2). Inoculation of bare-root almond and olive stock with *A. rhizogenes* resulted in an increase in both the number and weight of new roots and a significant increase in leaf number, stem diameter and shoot elongation during the first growing season after treatment (STROBEL and NACHMIAS, 1985; STROBEL *et al.*, 1988). One

of the most important factors affecting the success of *Eucalyptus* clonal nurseries is the rooting capacity of selected clones (see section 1.5.2). Research has show that rooting not only varies between families but also from clone to clone within a family. Optimisation of physiological and environmental conditions in the nursery can result in a marked increase in the rooting capacity of some *Eucalyptus* clones (ADENDORFF and SCHÖN, 1991; DENNISON and KIETZKA, 1993). However, it may not be possible to further increase rooting of certain clones by conventional means. *In vitro* propagation of *Eucalyptus* is also limited by the rooting ability of the material. The poor rooting capacity of a number of *Eucalyptus* clones exhibiting desirable wood properties led to a study of the use of *Agrobacterium rhizogenes*-mediated transformation for improving rooting of *Eucalyptus* species. Rooting of *in vitro* propagated explants of *Eucalyptus grandis*, *E. dunnii*, *E. nitens*, and *E. globulus* and of microcuttings of *in vitro* developed *E. grandis* clones and genotypes, and *E. globulus* clones was investigated.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Plant Material and Medium

Seeds of *E. grandis*, *E. dunnii*, *E. nitens* and *E. globulus* (Table 12) were surface-sterilised in hydrogen peroxide: absolute ethanol, 50:50, for 10 minutes, washed twice in sterile distilled water and once in 0.1 % benlate solution and germinated on the medium of MURASHIGE and SKOOG (1962)(MS) at 26°C in a growth room with a 16 hour photoperiod. Shoot multiplication of *E. grandis* was achieved using the method of MACRAE and VAN STADEN (1990). This method was modified for *E. globulus* as detailed in section 4.2.3.6. *In vitro* propagated *E. grandis* clones (SGR481 and SGR047) (section 3.4.2) and genotypes (section 4.3.2.5) and *E. globulus* clones (section 4.3.2.5) were also used.

**Table 12.** *Eucalyptus* species, clones and hybrid clones used in the *in vitro* transformation studies.

SPECIES/CLONE	TYPE	SOURCE
<i>E. grandis</i>	Seed	ICFR, Pietermaritzburg
<i>E. grandis</i>	Seed	CSIR, Forestek, Nelspruit
<i>E. nitens</i>	Seed	ICFR, Pietermaritzburg
<i>E. dunnii</i>	Seed	ICFR, Pietermaritzburg
<i>E. globulus</i> C59	Seed	CELBI, Portugal
<i>E. grandis</i> SGR038 (G1) SGR041 (G2) SGR042 (G3) SGR046 (G4) SGR047 (G5) SGR394 (G6) SGR481 (G7)	Seed	CSIR, FORESTEK, Nelspruit
SGR481 SGR047	Microcuttings	Chapter 3
<i>E. globulus</i> C33xC29 (G1) C29xC37 (G2) C33xC25 (G3) C37xC11 (G4) C18xC29/28 (G5) Mu10xC37/78 (G6)	Seed	Dr Paul Cotterill CELBI, Portugal

#### 4.2.2 Bacteria and Culture Conditions

The *Agrobacterium* strains used are listed in Table 13. Bacterial cultures were prepared by plating on yeast extract mannitol agar (YMA) (PILACINSKI and SCHMIDT, 1981) and incubating for 48 hours. The medium was supplemented with 50  $\mu\text{g l}^{-1}$  of Kanamycin (Km) if necessary.

**Table 13.** *A. rhizogenes* and *A. tumefaciens* strains used to induce rooting in this study.

Strain	Relevant Characteristic	Source
<i>A. rhizogenes</i>		
LBA9402	Agropine Ri plasmid	M.J.C. Rhodes, UK.
TR8,3	Ri plasmid	PPRI*
R1601	Ri plasmid	E. Nester, USA
A4R	Agropine Ri plasmid	D. Tepfer, France
<i>A. tumefaciens</i>		
LBA4404(pGA6403)	Km <sub>50</sub> <sup>R</sup> plasmid with an artificial T-DNA	D. Tepfer, France
LBA4404(pGA6403-12)	Km <sub>50</sub> <sup>R</sup> plasmid with ORF12 from the Ri TL-DNA cloned into pGA6403 behind the 35S promotor of CaMV	D. Tepfer, France

\* PPRI - Plant Protection Research Institute

**Bacterial cocktails:**

T1 comprised *A. rhizogenes* strains LBA9402, R1601 and TR8,3.

T2 comprised *A. rhizogenes* strains LBA9402, R1601 and TR8,3 and *A. tumefaciens* strains LBA4404(pGA643) and LBA4404(pGA643-12).

T1<sub>1</sub> comprised *A. rhizogenes* strains LBA9402, R1601 and TR8,3 supplemented with 1 mg l<sup>-1</sup> IBA.

T2<sub>1</sub> comprised *A. rhizogenes* strains LBA9402, R1601 and TR8,3.

Bacterial cocktails were prepared by re-suspending 48 hour cultures of these strains in quarter strength Ringer solution (see section 2.3.4) to give 10<sup>8</sup> cfu ml<sup>-1</sup> of each strain.

#### 4.2.3 Transformation of *In Vitro* Propagated Explants

In initial trials (results not shown), after inoculation with *A. rhizogenes* strains, explants were placed upright in half-strength MS for 48 hours and then transferred to hormone free MS containing 500 mg l<sup>-1</sup> Cefotaxime to kill the bacteria. With this method difficulty was experienced in curing the explants of the bacteria owing to excessive bacterial growth. Subsequently, an inverted explant technique was developed and was used routinely.

#### 4.2.3.1 *In Vitro* Rooting of Three *Eucalyptus* Species by Three *A. rhizogenes* Strains

Three *in vitro* trials were set up using two-week-old *in vitro* propagated *E. grandis*, *E. nitens* and *E. dunnii* seedlings (seed source ICFR). The roots were aseptically removed from the seedlings and the shoot explants placed in an inverted position on half-strength MS. *A. rhizogenes* LBA9402, R1601 and TR8,3 cultures were applied to the cut surface using a sterile inoculating needle. Inoculated plantlets were then incubated for 7 days at 26°C in a growth room with a 16 hour photoperiod. A light fluence of  $36 \mu\text{mol m}^{-2} \text{s}^{-1}$  was provided by cool white fluorescent tubes. Explants were then placed upright in hormone-free MS containing 500 mg l<sup>-1</sup> Cefotaxime (Rousell) to kill bacteria and incubated for 7 days. Thereafter they were transferred to hormone-free MS for a further 4 weeks and then hardened off. Two controls were set up. One set of control plants was transferred to hormone-free MS and the other to MS supplemented with 1 mg l<sup>-1</sup> IBA and both were incubated for four weeks before hardening off. Hardening off was achieved by transferring the rooted plantlets to autoclaved vermiculite moistened with half strength MS in Unigro Speedling trays. These were placed in large plastic bags to maintain the relative humidity above 70 %. Trays were kept under fluorescent lights of intensity  $36 \mu\text{mol m}^{-2} \text{s}^{-1}$  in a growth room with a 16 hour photoperiod. After 7 days, a gradual decrease in humidity was achieved by opening the bags daily for increasingly longer periods of

time over a further seven day period. During this time plantlets were sprayed with a Benlate (0.01%) / Kelpak (0.1%) solution every alternate day. The trays were then removed from the plastic bags and placed under mist irrigation (10 seconds on / 15 minutes off during the day and 10 seconds on / 20 minutes off during the night) in a shade tunnel for two weeks. The plantlets were transferred to a potting medium in 25 mm plastic pots. Twenty explants were inoculated per treatment in the three trials. Rooting was assessed at 7 days and again at 4 weeks after inoculation and expressed as a percentage of the total number of inoculated explants. Root quality was assessed in terms of number of roots per plantlet and the distribution of roots around the base of the cutting. The data presented are the average rooting percentages for the three trials.

#### 4.2.3.2 *In Vitro* Rooting of *E. grandis* and *E. nitens* by Six *A. rhizogenes* Strains

Three *in vitro* trials were set up using three-week-old *in vitro* propagated *E. grandis* and *E. nitens* seedlings (seed source: ICFR), and the six *Agrobacterium* strains listed in Table 13. The roots were aseptically removed from the seedlings and the explants placed in an inverted position on 1 % water agar, inoculated and incubated as described in section 4.2.3.1, with the exception that the control plants were transferred to half-strength MS supplemented with 1 mg l<sup>-1</sup> IBA only. Rooting was

assessed at 6 weeks after inoculation and expressed as a percentage of the total number of inoculated explants. Root quality was assessed in terms of the number of roots per plantlet and the distribution of roots around the base of the cutting. The data presented are the average for the three trials.

#### 4.2.3.3 *In Vitro* Rooting of *E. globulus* by *A. rhizogenes* Strain LBA9402

Sixty, three-week-old, *in vitro* propagated *E. globulus* C59 seedlings were inoculated with *A. rhizogenes* LBA9402 as described in section 4.2.3.1 to determine the susceptibility of *E. globulus* to infection by *A. rhizogenes*. Sixty control plants were placed on MS medium supplemented with 1 mg l<sup>-1</sup> IBA.

#### 4.2.3.4 *In Vitro* Rooting of *In Vitro* Developed Clones of *E. grandis* SGR481 and SGR047 Using *A. rhizogenes* Cocktails

A limited number of microcuttings of the *in vitro* developed and propagated *E. grandis* clones (see section 3.2.2) were available for this study, so only one *in vitro* trial was set up. Two weeks prior to setting up this trial the *in vitro* propagated *Eucalyptus* clones were transferred from shoot multiplication medium to hormone-

free MS medium containing 1 % activated charcoal. Two bacterial cocktails, T1 and T2, were prepared as described in section 4.2.2. The lower leaves were removed from the microcuttings and the freshly cut bases were inoculated by dipping them into the various inocula. They were then placed in an inverted position on 1 % water agar for 4 to 5 days. Control explants (T3) were not inoculated. After inoculation plantlets were incubated as described in section 4.2.3.1, with the exception that the cuttings were reinverted onto hormone-free half-strength MS medium containing 15 g l<sup>-1</sup> sucrose and 500 mg l<sup>-1</sup> Cefotaxime at pH5.8.

Twenty explants were inoculated per treatment. Rooting was assessed 6 weeks after inoculation and expressed as a percentage of the total number of inoculated explants.

#### 4.2.3.5 *In Vitro* Rooting of Seven *In Vitro* Propagated *E. grandis* Genotypes Using *A. rhizogenes* Cocktails

Seeds from each of seven *E. grandis* genotypes (Table 12) were surface sterilized, germinated, and cultured using the method developed in section 3.2.2. Germination rates of the seeds from the seven *E. grandis* genotypes was poor ranging from 7.6 to 20.8 %. This was probably due to poor fertilization and/or poor storage. As a result the number of clones developed for each genotype differed, ranging from 8

to 22 clones (Table 14). The shoot multiplication rates of these clones varied from clone to clone within a genotype and were low, ranging from 3 to 5 times in a 4 to 6 week propagation period. The clones from these seven genotypes did not generally respond well to the *in vitro* culture conditions. A lack of shoot elongation was observed and they were extremely sensitive to *in vitro* environmental stresses. Callusing and necrosis of shoot clusters was observed on a number of the clones, hence the numbers of microcuttings available for transformation were low. Only healthy and stress-free microcuttings of appropriate size (20 mm +), were selected from each of the clones developed for each genotype for the *A. rhizogenes*-mediated rooting trials. The number of microcuttings of each clone that were inoculated with each treatment is listed in Table 14.

Two bacterial cocktails, T1 and T2 were prepared as described in section 4.2.3.3. The lower leaves were removed from the microcuttings and the freshly cut bases were inoculated by dipping them into the bacterial cocktails or water (control treatment T3). They were then placed in an inverted position on 1 % water agar for 4 to 5 days. Thereafter, the microcuttings were reinverted onto half-strength MS medium containing 15 g l<sup>-1</sup> sucrose and 500 mg l<sup>-1</sup> Cefotaxime (pH 5.8). The control cuttings were inverted onto rooting medium (RM) comprising half-strength MS medium containing 15 g l<sup>-1</sup> sucrose and 1 mg l<sup>-1</sup> IBA (pH 5.8). Treatments were randomised.

**Table 14.** Number of microcuttings (M) per treatment per clone developed from the *E. grandis* genotypes SGR038, SGR041, SGR042, SGR046, SGR047, SGR394 and SGR481.

[illegible]

The percentage survival and rooting (expressed as a percentage of the total number of inoculated surviving microcuttings) were assessed for each clone, 6 weeks after inoculation, as were the number of roots, root length and shoot height. The data presented are the averages for each parameter measured for each genotype.

#### 4.2.3.6 *In Vitro* Rooting of Twelve *In Vitro* Developed Clones of *E. globulus* Using *A. rhizogenes* Cocktails

With the aim of developing *E. globulus* clones *in vitro* for *Agrobacterium*-mediated rooting experiments, seeds from each of six *E. globulus* families (Table 12) were surface sterilized, germinated, and cultured, as described in section 3.2.2 on the following media:- Shoot multiplication medium (SMMg) comprising MS medium supplemented with 0.28 mg l<sup>-1</sup> BA, 0.47 mg l<sup>-1</sup> NAA and 20 g l<sup>-1</sup> sucrose, and solidified with 2.5 g l<sup>-1</sup> gelrite and 2.5 g l<sup>-1</sup> agar at pH 5.5; shoot lengthening medium (SLMg) comprising SMMg supplemented with 0.53 mg l<sup>-1</sup> kinetin and rooting medium (RMg) comprising half-strength MS medium supplemented with 10 g l<sup>-1</sup> sucrose and 1 mg l<sup>-1</sup> IBA and solidified with 2.5 g l<sup>-1</sup> gelrite and 2.5 g l<sup>-1</sup> agar at pH 5.6. The germination rates of seeds from these families were good ranging from 82 to 94 %. Numerous clones were developed from each of the six *E. globulus* families. From these, two clones (Table 15) were selected from each of the

six *E. globulus* families, based on their *in vitro* condition and shoot multiplication rate, for the *Agrobacterium*-mediated rooting trial .

A transformation trial was set up in a randomised block design with three replications comprising twelve *E. globulus* clones (Table 15), 10 microcuttings per row plot and three treatments (two bacterial cocktails T1 and T2 (see section 4.2.2) and a control, T3, which involved dipping bases of the cuttings in water).

Ninety healthy microcuttings, 20 to 30 mm in height, were selected from the 12 clones, inoculated and incubated as described in section 4.2.3.5. Five days after inoculation all the microcuttings were reinverted onto rooting medium (RMg) supplemented with 500 mg l<sup>-1</sup> Cefotaxime and incubated in a growth room for 6 weeks. After this period the parameters measured were as detailed in section 4.2.3.5, with the exception that the data presented were the averages for each parameter measured for each clone.

**Table 15.** List of *E. globulus* clones selected for *in vitro* *Agrobacterium*-mediated rooting experiments.

<i>E. globulus</i> Family	Clone	
C33xC29	C33xC29/17	(C1)
	C33xC29/84	(C2)
C29xC37	C29xC37/8	(C3)
	C29xC37/34	(C4)
C33xC25	C33xC25/61	(C5)
	C33xC25/69	(C6)
C37xC11	C37xC11/40	(C7)
	C37xC11/24	(C8)
C18xC29	C18xC29/28	(C9)
	C18xC29/7	(C10)
Mu10xC37	Mu10xC37/78	(C11)
	Mu10xC37/63	(C12)

#### 4.2.3.7 *In Vitro* Rooting of *E. grandis* and *E. globulus* using *A. rhizogenes* Cocktails

The concept of using bacterial cocktails was further developed by setting up three *in vitro* trials using three-week-old *in vitro* propagated *E. grandis* and *E. globulus* C59 seedlings (seed source CSIR and CELBI, respectively, Table 12) to test the use of bacterial cocktail supplemented with auxin. Two bacterial cocktails, T1 and T2, were prepared as described in section 4.2.2. The control treatment comprised a 1

mg l<sup>-1</sup> IBA solution. The roots were aseptically removed from the seedlings and the explants dipped into the various treatments and placed in an inverted position on 1 % water agar. Inoculated plantlets were then incubated as described in section 4.2.3.1, with the exception that the reinversion medium comprised half-strength MS supplemented with 15 g l<sup>-1</sup> sucrose, 1mg l<sup>-1</sup> IBA and 500 mg l<sup>-1</sup> Cefotaxime, pH5.8 and solidified with 3 g l<sup>-1</sup> gelrite.

Twenty explants were inoculated per treatment in the three trials. Rooting was assessed 8 weeks after inoculation and expressed as a percentage of the total number of inoculated explants. Root quality was assessed in terms of number of roots per plantlet and the distribution of roots around the base of the cutting. Root length, shoot height and root and shoot wet and dry masses were also determined. The data presented are the averages for the three trials.

#### 4.2.4 Statistical Analysis of Data

Rooting data were subjected to arcsine transformation prior to statistical analysis. Data presented in the figures are untransformed means. Each entry is the mean of three repetitions of 20 explants unless otherwise stated. For each treatment any two means having a common letter are not significantly different at the P=0.1 level,

based on the Scheffé test (SCHEFFÉ, 1959). For small data sets, the least conservative multiple comparison procedure, Least Significant Difference (LSD) test (Anon., 1987) was performed at the  $P=0.05$  or  $P=0.1$  levels.

#### 4.2.5 Opine Detection

Standard procedures for the detection of mannopine and arginine were used (DRAPER *et al.*, 1988).

#### 4.2.6 Production of Root Cultures

Root samples from control and transformed roots of *E. grandis* and *E. globulus* were placed in liquid culture (hormone-free, full and half strength MS) and incubated in the dark at 24°C on an orbital shaker at 56 rpm. Roots were subcultured or the medium replaced at 2 to 3 week intervals.

#### 4.2.7 DNA Isolation

**Plant DNA:** A modified CTAB method (DOYLE and DOYLE, 1990) was used to isolate DNA from the roots of control and transformed roots.

Five to 7.5 ml CTAB isolation buffer (2 % (w/v) CTAB, 1.4 M NaCl, 0.2 % (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, 1 % (w/v) PVP-40, pH 8.0) in a 30 ml centrifuge tube, was preheated to 60 °C in a water bath. A half to one gram root tissue sample was ground to a fine powder in liquid nitrogen using a chilled mortar and pestle. The powder was scraped directly into the preheated buffer, gently swirled and incubated at 60 °C for 30 minutes with occasional swirling (optional). The sample was then extracted by adding an equal volume of chloroform-isoamyl alcohol (24:1) and mixed gently but thoroughly. It was then centrifuged (1600 g) for 10 minutes at room temperature to concentrate the phases. The aqueous phase was removed with a wide bore pipette (200  $\mu$ l at a time) and transferred to a clean, centrifuge tube. Two to 3 volumes of cold iso-propanol was added and mixed gently to precipitate the nucleic acids. If no evidence of DNA precipitation was observed, the sample was left at room temperature for several hours to overnight. If DNA was flocculent, the sample was centrifuged immediately at 500 g for 1 to 2 minutes. Thereafter, as much of the supernatant as possible was gently poured off and 10 to 20 ml of wash buffer (76 % ethanol and 10 mM ammonium acetate) was

added directly to the pellet, swirled gently to re-suspend and washed for a minimum of 20 minutes. (Samples could be left in the wash solution at room temperature for at least two days if necessary). The nucleic acids were then pelleted by centrifuging at 1600g for 10 minutes. The supernatant was poured off and the pellet was allowed to air dry briefly at room temperature before re-suspending it in 1 ml TE buffer (10 mM Tris-HCl (pH 7.4); 1 mM EDTA). RNase was added to a final concentration of 10  $\mu\text{g ml}^{-1}$  and the sample was incubated at 37°C for 30 minutes. Thereafter it was diluted with two volumes TE buffer (2 ml). Ammonium acetate (7.5M, pH 7.7) was added to a final concentration of 2.5 M (0.5 ml  $\text{ml}^{-1}$ ) and 2.5 volumes of cold ethanol (i.e. 2.5 volumes of the final volume in the tube, 11.25 ml) was added and gently mixed to precipitate the DNA. The DNA was then pelleted by centrifugation at 10 000 g for 10 minutes in a refrigerated centrifuge set at 4 °C. Thereafter the DNA was dried in Savant speedvac and finally re-suspend in an appropriate volume of TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA pH 8.0)(200 - 500  $\mu\text{l}$ ). A phenol / chloroform extraction was then performed on the re-suspended DNA sample. The aqueous phase was removed and placed in a clean eppendorff tube and another ammonium acetate precipitation was carried out (250  $\mu\text{l}$  aqueous phase + 125  $\mu\text{l}$  ammonium acetate + 937.5  $\mu\text{l}$  ethanol). The resultant DNA pellet was dried in a Savant speedvac and then re-suspended in TE buffer (100 - 200  $\mu\text{l}$ ).

DNA was isolated from roots of 2 month old *in vitro* propagated acclimatised control *E. grandis*(gc<sub>1</sub><sup>a</sup>) and *E. globulus* (gbc<sub>1</sub><sup>a</sup>) and chimeric *E. grandis* (gl<sub>1</sub><sup>a</sup>, gl<sub>2</sub><sup>a</sup>) and *E. globulus* (gbl<sub>1</sub><sup>a</sup>, gbl<sub>2</sub><sup>a</sup>) plants. In addition DNA was isolated from two *E. grandis* (gl<sub>1</sub><sup>r</sup>, gl<sub>2</sub><sup>r</sup>) and two *E. globulus* (gbl<sub>1</sub><sup>r</sup>, gbl<sub>2</sub><sup>r</sup>) root culture lines. Transformation was achieved using *A. rhizogenes* LBA9402.

**Plasmid DNA:** Large scale isolations of the Ri plasmids of *A. rhizogenes* strains were performed using the alkaline lysis method described in MANIATIS *et al.* (1982).

#### 4.2.8 DNA Restriction Digestion

Restriction digests were carried out according to the method of MANIATIS *et al.* (1982).

#### 4.2.9 Southern Hybridization

Plant DNA samples separated on agarose gels (0.8 %) were denatured and transferred to nylon filters by the technique of SOUTHERN (1975). Hybridization was carried out using DIG, a non-radioactive, DNA Labelling and Detection Kit according to manufacturer's (Boehringer Mannheim) instructions. The probe was prepared using 44 ng Bam-HI digested LBA9402 plasmid DNA. The data were recorded by photocopying the nylon filters.

### 4.3 RESULTS

#### 4.3.1 *In Vitro* Rooting of Three *Eucalyptus* Species by Three *A. rhizogenes* Strains

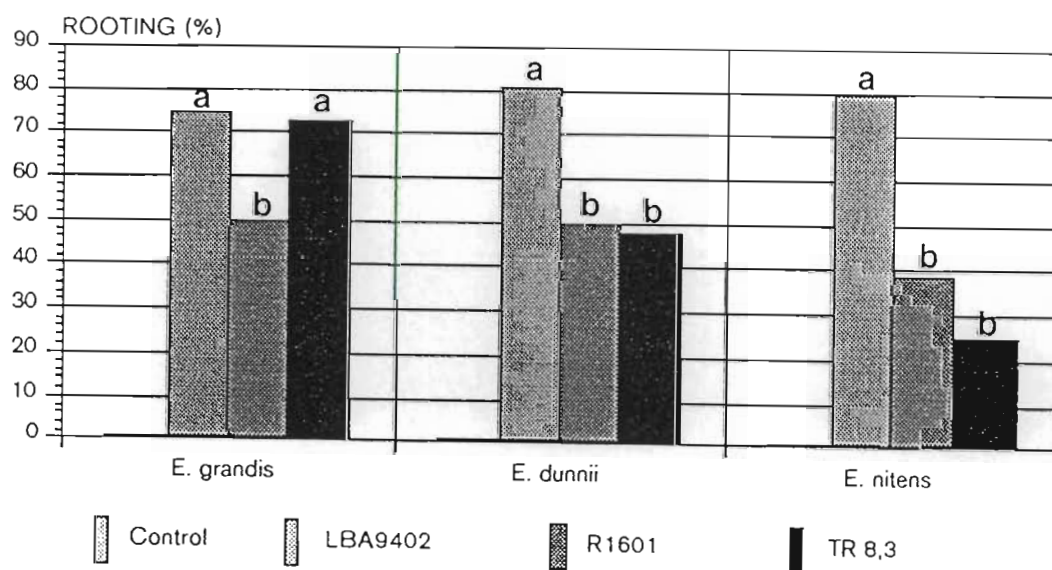
Inoculation of *Eucalyptus grandis* with *A. rhizogenes* LBA9402 resulted in root development at the wound site (Figure 29).



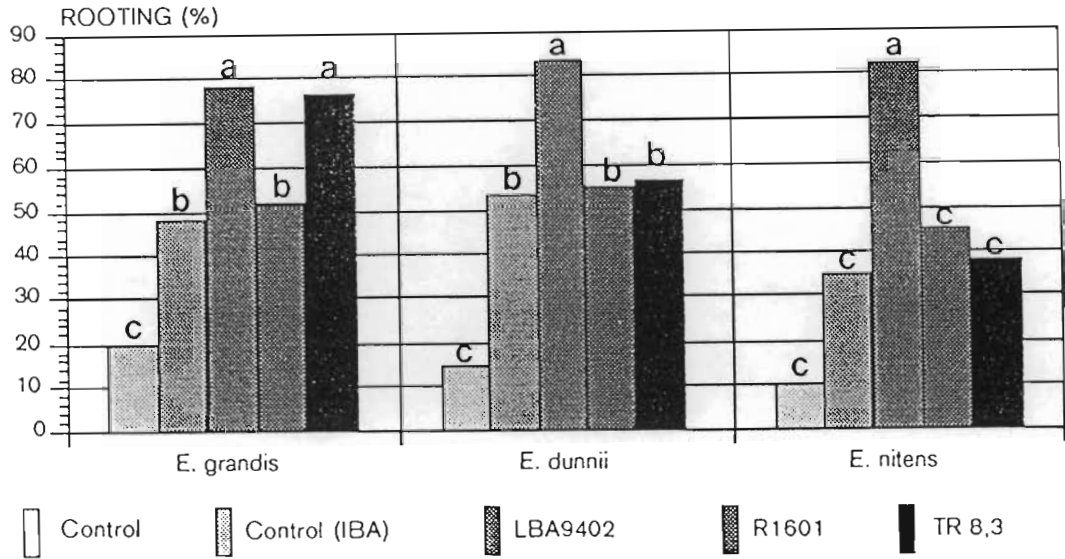
**Figure 29.** Root development on *in vitro* propagated *E. grandis* explants occurred at the point of inoculation with *A. rhizogenes* LBA9402.

*A. rhizogenes* LBA9402 and two other wild type strains, R1601 and TR8,3 were found to induce rooting in *in vitro* propagated explants of not only *E. grandis* but also of *E. dunnii* and *E. nitens*. Transformation of the bases of *in vitro* propagated *Eucalyptus* explants was achieved by inoculating the cut surface with the bacteria. Root initiation occurred within the first 2 to 4 days after inoculation of *Eucalyptus* species with *A. rhizogenes* strains. All three *A. rhizogenes* strains were able to induce rooting in the three *Eucalyptus* species tested (Figure 30).

Four weeks after re-inverting the explants spontaneous rooting of uninoculated control explants was detected in hormone-free medium (Figure 31). Rooting of control plants was increased to approximately 50 percent in all species by the addition of 1 mg l<sup>-1</sup> IBA to the medium. *A. rhizogenes* LBA9402 induced 80 % rooting in all three *Eucalyptus* species and strain TR 8,3 induced 76 % rooting in *E. grandis* only. Root induction by R1601 was of the same order as that achieved by the addition of IBA for the three *Eucalyptus* species.

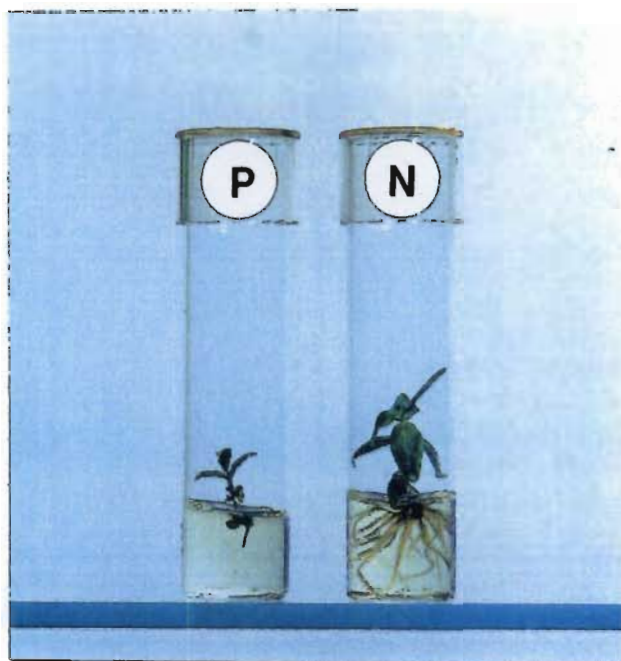


**Figure 30.** Rooting of three *Eucalyptus* species *in vitro* seven days after inoculation with three *A. rhizogenes* strains. Controls did not root. Based on the Scheffé test any two means having a common letter are not significantly different at the  $P=0.1$  level.



**Figure 31.** *In vitro* rooting of three *Eucalyptus* species inoculated with three *A. rhizogenes* strains four weeks after inversion onto MS media. Based on the Scheffé test any two means having a common letter are not significantly different at the  $P=0.1$  level.

A comparison of root quality of control plants and chimeric plants six weeks after inoculation is presented in Figure 32 and clearly demonstrates the improved root quality in chimeric plantlets. *In vitro* propagated *Eucalyptus* plantlets were hardened off and extensive lateral root development occurred (Figure 33). No morphological abnormalities were observed in chimeric plantlets (plantlets with transformed roots) (Figure 34), suggesting that this procedure could be used to improve rooting in difficult-to-root *Eucalyptus* species.



**Figure 32.** Rooting of *E. nitens* explants *in vitro* six weeks after treatment. P, control treated with IBA; N, inoculation with *A. rhizogenes* LBA9402 (no IBA treatment).



**Figure 33.** Transformed roots of *in vitro* propagated *E. grandis* plantlets two months after hardening off.

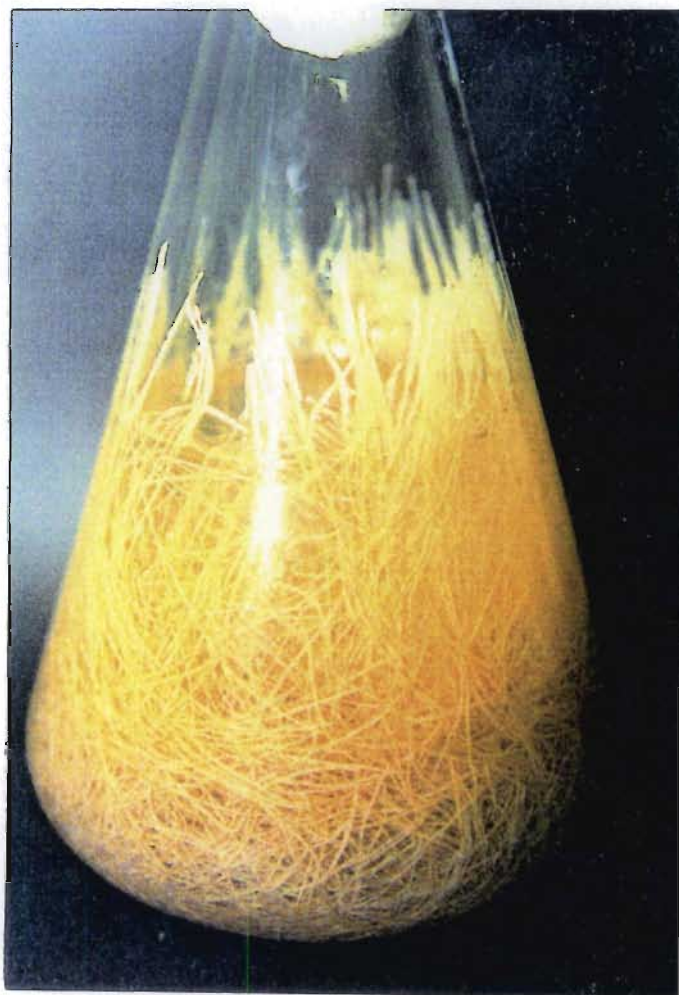


**Figure 34.** Three month old chimeric *in vitro* propagated, acclimatized, *E. grandis* plantlets.

#### 4.3.2 Root Cultures

Before hardening off *in vitro* propagated plantlets, root samples from transformed *E. grandis* roots induced by *A. rhizogenes* LBA9402 and control roots were placed into liquid culture. Control roots could not be cultured in this manner. The addition of IBA at 1 mg l<sup>-1</sup> to the medium to induce root growth of untransformed roots was unsuccessful. Transformed roots however, grew rapidly in hormone-free half strength MS (Figure 35). These roots displayed the typical hairy root phenotype

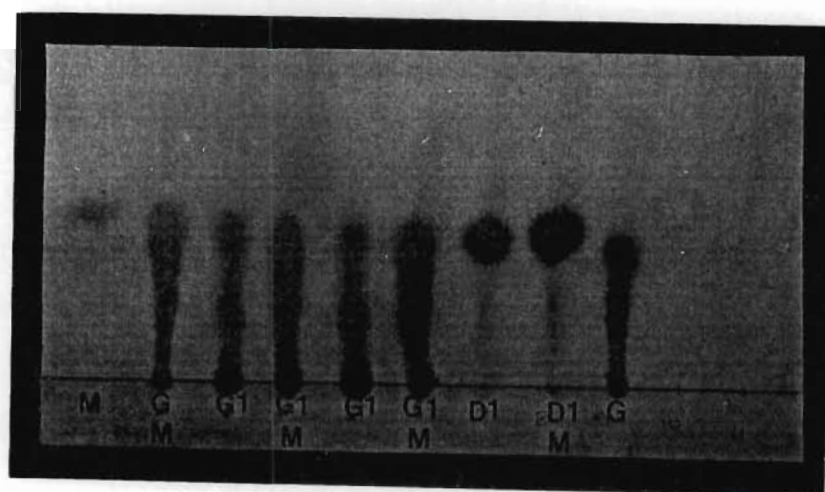
similar to that described for other species (TEPFER, 1983a; PHELEP *et al.*, 1991). They showed a high growth rate, extensive lateral branching and a lack of geotropism. Transformed roots on plantlets grown in the soil did not display the typical hairy root phenotype but grew normally (Figure 32); good secondary root development occurred and a firm root plug developed.



**Figure 35.** Transformed *E. grandis* root cultures grown in half strength MS in the dark at 24°C on an orbital shaker at 56 rpm.

### 4.3.3 Opine Detection

Genetic transformation was also assessed by the presence of opines. Although opines are a sensitive positive indicator of transformation, their absence does not prove that the tissue is normal. Mannopine was observed in the root tissue of some plants transformed by LBA9402, R1601 and TR 8,3 (Figure 36).



**Figure 36.** Mannopine (M) production in roots of *E. grandis* transformed by *A. rhizogenes* LBA9402 (G1), *E. dunnii* transformed by *A. rhizogenes* R1601 (D1) and non-transformed *E. grandis* (G). Mannopine (Sigma M5020) standard was 1mg ml<sup>-1</sup>. Mannopine was not detected in normal roots but was observed in roots of chimeric *Eucalyptus*.

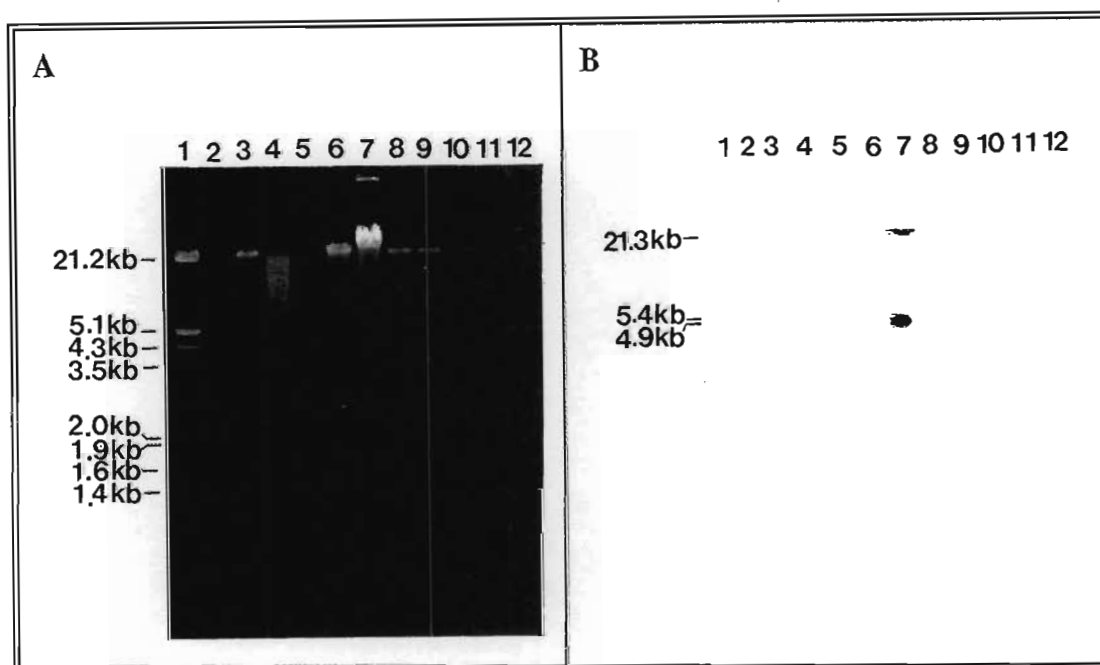
#### 4.3.4 DNA Hybridization

Although LAMBERT and TEPFER (1991) did not use molecular hybridization with *in vitro* inoculated microcuttings as there was a risk of residual bacterial contamination, this method was investigated in this study.

DNA yields from roots samples of *in vitro* propagated "transformed" *Eucalyptus* root cultures, *in vitro* developed acclimatized chimeric eucalypts and *in vitro* propagated control eucalypts were low, ranging from 40 ng g<sup>-1</sup> to 80 ng g<sup>-1</sup>. For this reason only small quantities of DNA were available for molecular hybridization testing. Bam HI-digestion of the *Eucalyptus* root DNA samples was incomplete after the 24 hour digestion period (Figure 37 A). Although this period was increased up to 72 hours (results not shown) the digestion was not markedly improved. It is thought that this may be due to the presence of inhibitory compounds, such as phenolics or polysaccharides in the DNA samples.

Using the DIG technique hybridisation of the LBA9402 plasmid probe was detected at only two sites on the DNA isolated from *E. globulus* gb1<sub>2</sub>' (Figure 37 B - lane 7); this confirms transformation of this root culture. The inability to detect homologous DNA fragments in restriction digests from the other "transformed" samples as well as from the restriction digested plasmid LBA9402, suggests that the sensitivity of the

DIG reaction was insufficient to detect low concentrations of DNA; only the highest concentration of 500 ng produced a result.

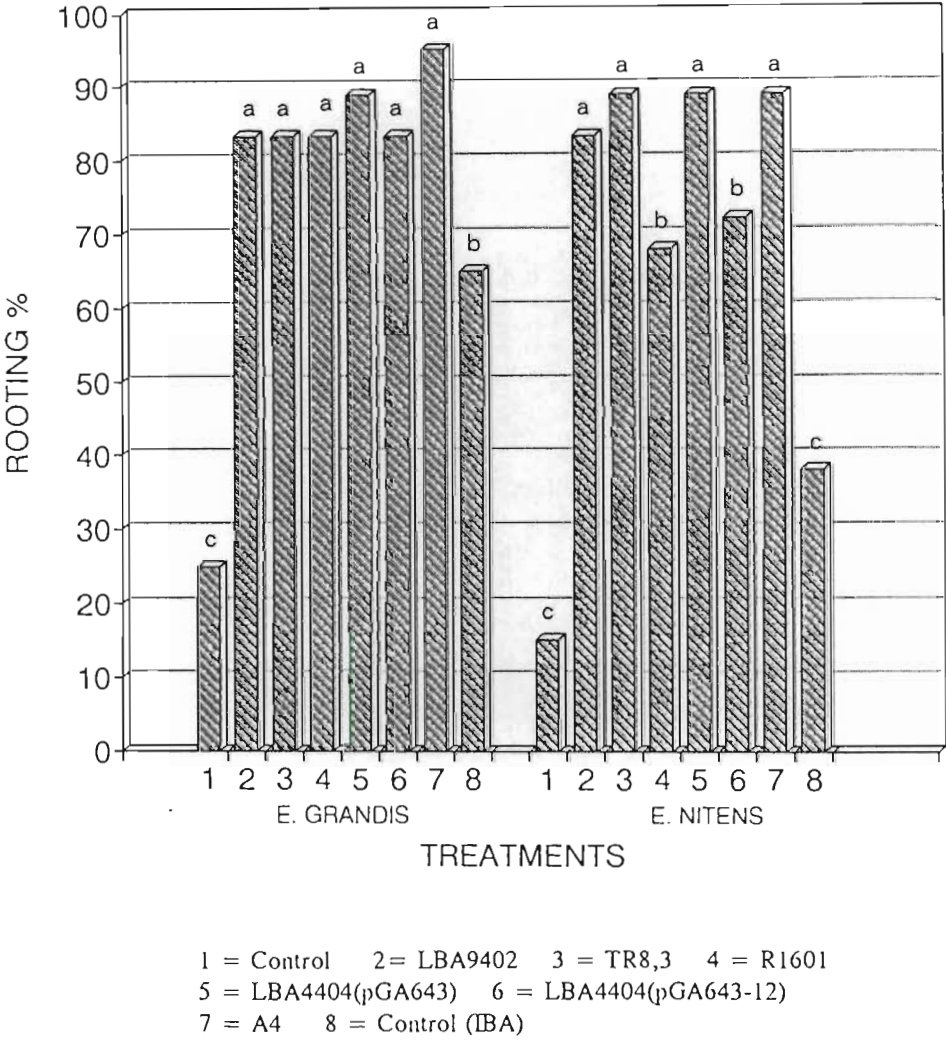


**Figure 37.** A: Eletrophoresis patterns of Boehringer Mannheim molecular weight marker III (lane 1); Bam HI-digested DNA from untransformed *E. grandis* ( $gc_1^a$  (21 ng) - lane 2), and *E. globulus* ( $gbc_1^a$  (37 ng) - lane 3) roots; transformed *E. grandis* ( $gl_1^r$  (200 ng) - lane 4 and  $gl_2^r$  (15 ng) - lane 5) and *E. globulus* ( $gb1_1^r$  (200 ng) - lane 6 and  $gb1_2^r$  (500 ng) - lane 7) root cultures; transformed roots from two month old acclimatized *in vitro* propagated *E. grandis* (lanes  $gl_1^a$  (44 ng) - lane 8 and  $gl_2^a$  (44 ng) - lane 9) and *E. globulus* ( $gb1_1^a$  (37 ng) - lane 10 and  $gb1_2^a$  (15 ng) - lane 11) plants; and Ri plasmid of *A. rhizogenes* LBA9402 (30 ng) (lane 12). B: Southern blot analysis of above mentioned Bam HI-digested DNA samples using a Bam HI-digested DIG labeled *A. rhizogenes* LBA9402 plasmid as a probe.

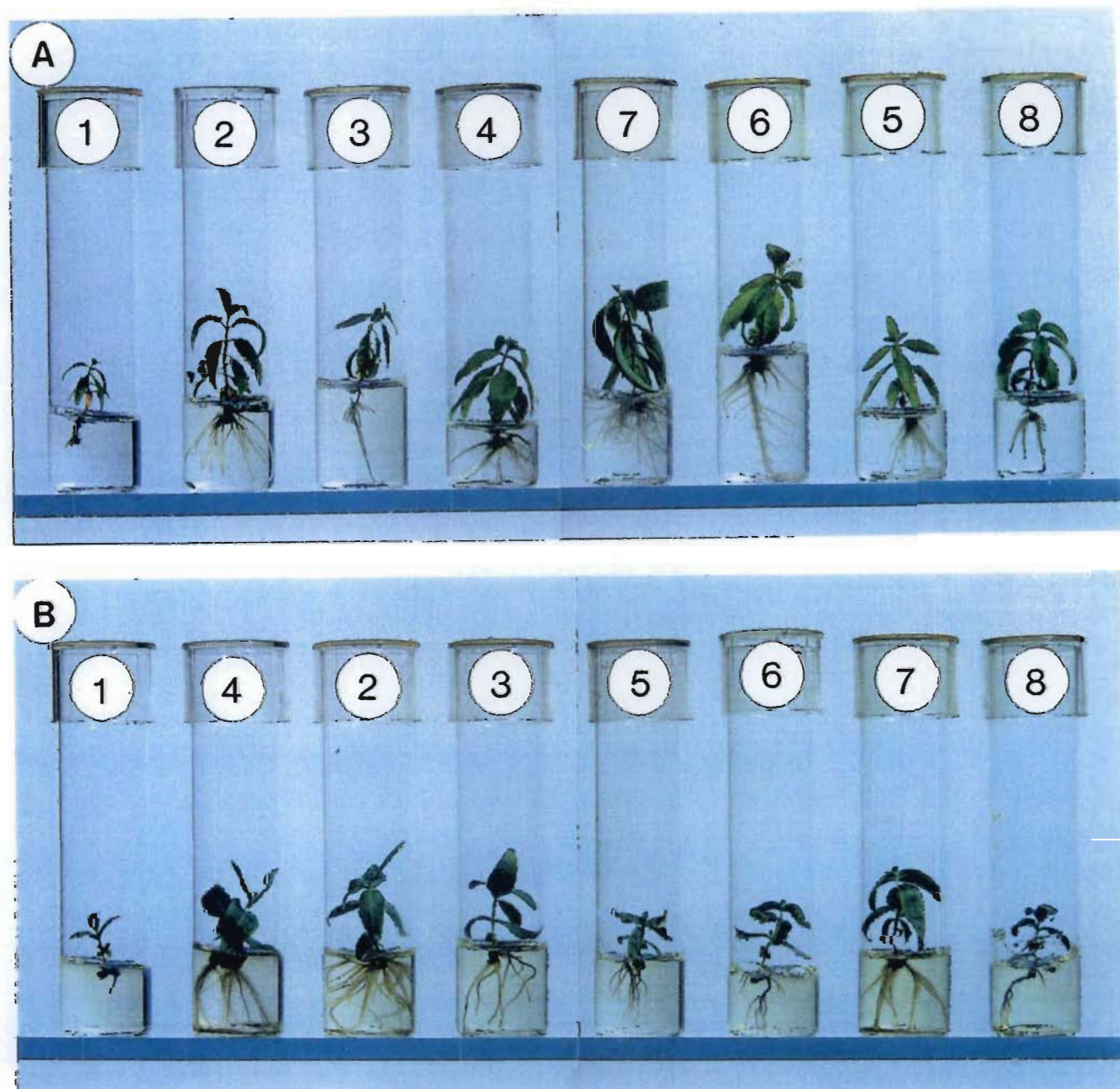
#### 4.3.5 *In Vitro* Rooting of *E. grandis* and *E. nitens* by Six *A. rhizogenes* Strains

The effectiveness of rooting *in vitro* propagated explants of *E. grandis* and *E. nitens* using wild-type *A. rhizogenes* strains, LBA9402, TR8,3, R1601 and A4 was compared to genetically modified *A. tumefaciens* strains LBA4404(pGA643) and LBA4404(pGA643-12). The latter strain carried a single gene (OFR12 or *rolC*) from the Ri TL-DNA, placed in an *A. tumefaciens* background (TEPFER, 1991). Controls were either allowed to root spontaneously or were treated with auxin. The inherent rooting abilities (1) of *E. grandis* and *E. nitens*, as determined on hormone-free medium, was 35% and 15% respectively. The relative ability of the two *Eucalyptus* species to respond to auxin (8) differed with *E. grandis* being more responsive (Figure 38). All *Agrobacterium* strains were found to induce rooting on *in vitro* propagated *E. grandis* and *E. nitens* explants. Although root induction on *E. grandis* by strain LBA4404(pAG643-12) (6) was marginally better than that obtained by the other strains, there was no significant difference between rooting of *E. grandis* induced by the six *Agrobacterium* strains. Root formation by these strains was however, significantly better than that induced by auxin. Root induction on *E. nitens* by *A. rhizogenes* R1601 (4) and *A. tumefaciens* LBA4404(pGA643) (5) was significantly poorer than that of the other *Agrobacterium* strains tested but was significantly better than that induced by auxin (8) (Figure 38).

All the *Agrobacterium* strains therefore appeared to be able to alter the inherent ability of the investigated *Eucalyptus* genotypes to root. The morphology of the resultant root systems was altered and the root quality of the chimeric plants six weeks after inoculation was better than that of the control plants (Figure 39).



**Figure 38.** *In vitro* rooting of *E. grandis* and *E. nitens* inoculated with four *A. rhizogenes* and two *A. tumefaciens* strains six weeks after inversion onto MS media. Based on the Scheffé test any two means having a common letter are not significantly different at the P=0.1 level.



**Figure 39.** Rooting of *E. grandis* (A) and *E. nitens* (B) explants *in vitro*, six weeks after inoculation with *A. rhizogenes* LBA9402 (2), TR8,3 (3), R1601 (4) and A4 (7), and *A. tumefaciens* LBA4404(pGA643) (5), LBA4404(pGA643-12) (6). One set of control explants was uninoculated (1) and the other was treated with 1 mg l<sup>-1</sup> IBA (8). All explants were rooted on hormone-free MS medium.

#### 4.3.6                    *In Vitro* Rooting of *E. globulus* by *A. rhizogenes* Strain LBA9402

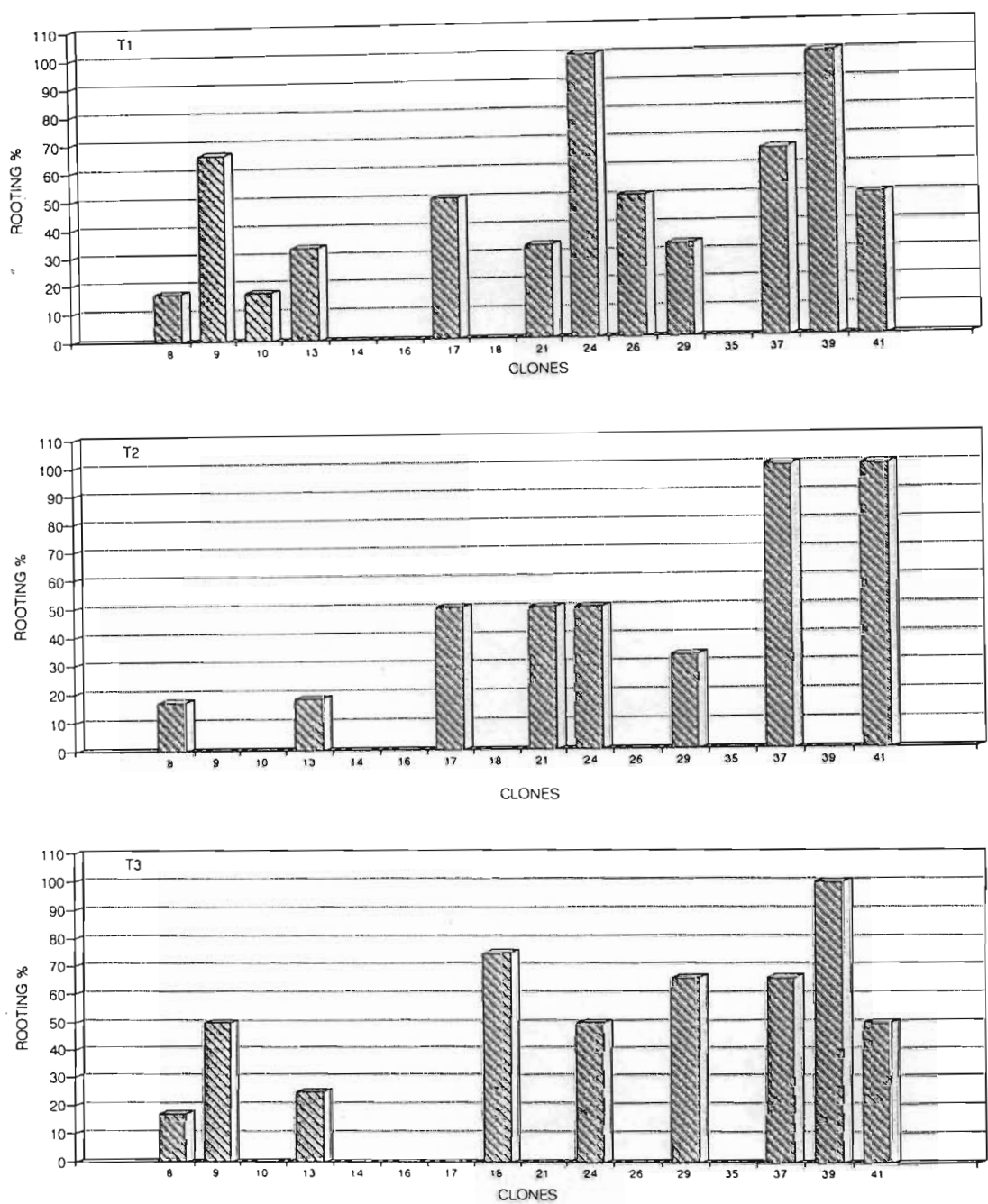
One hundred percent rooting of *in vitro* propagated *E. globulus* C59 seedlings was achieved with both *A. rhizogenes* LBA9402 and auxin treatments. The root quality of the chimeric plants was however, better than that of control plants. This showed that *E. globulus* was susceptible to root induction by *A. rhizogenes* and that this *E. globulus* genotype was an easy-to-root one.

#### 4.3.7                    *In Vitro* Rooting of *In Vitro* Developed Clones of *E. grandis* SGR481 and SGR047 Using *A. rhizogenes* Cocktails

The successful use of *A. rhizogenes* to transform microcuttings and induce rooting is dependent in part, on the genotype of the bacterium. The host range of *A. rhizogenes* strains, like *A. tumefaciens* strains, varies from strain to strain (see 1.2.4). The potential use of mixed *Agrobacterium* cultures (bacterial cocktails) to transform *in vitro* propagated *E. grandis* clones was therefore investigated with the aim of overcoming the host range limitation which occurs with *Agrobacterium* strains. The effectiveness of two *Agrobacterium* cocktails in rooting microcuttings of clones developed *in vitro* from two *E. grandis* genotypes was therefore compared

to that of auxin.

Only 58.3 % of the *E. grandis* SGR047 clones tested rooted on auxin-containing medium (T3) while 68.8 % and 50 % of the clones rooted as a result of *Agrobacterium* cocktails T1 and T2 respectively (Figure 40). The percentage of successful rooting of *E. grandis* SGR047 clones was found to vary from clone to clone for each of the treatments. For example, the rooting percentage induced by auxin on clone 8 was 17 % and this could not be improved by either *Agrobacterium* cocktail. Rooting of clone 17 however, could not be induced by auxin (T3) but 50 and 66 % rooting could be achieved using *Agrobacterium* cocktails T2 and T1 respectively. Certain clones such as 14, 16 and 35 could not be rooted by any of the treatments (Figure 40).

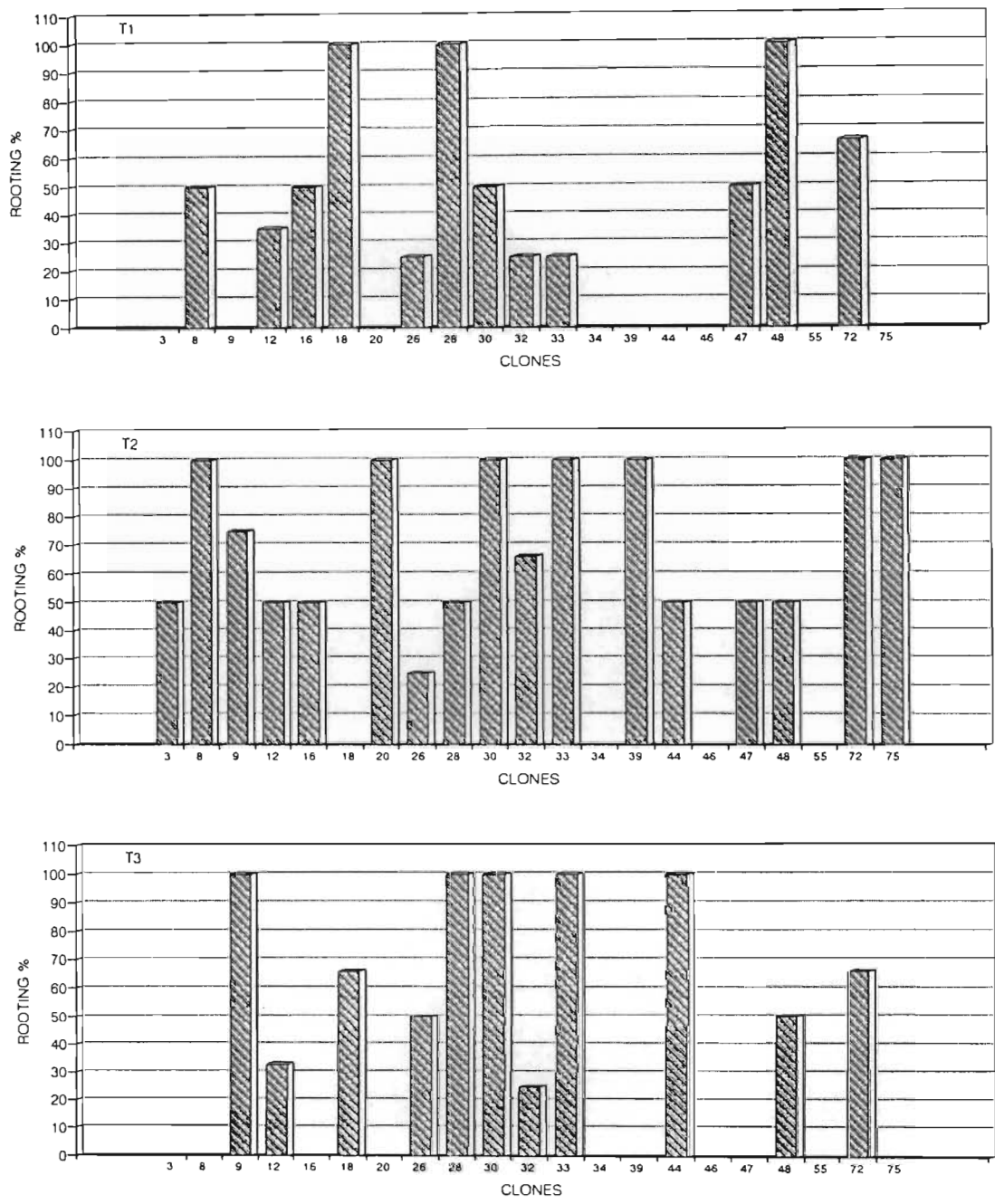


**Figure 40.** *In vitro* rooting of sixteen *in vitro*-developed *E. grandis* SGR047 clones inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and control treatment, T3 (uninoculated and placed on IBA containing medium), six weeks after inversion onto half-strength MS media.

The rooting trends of *E. grandis* SGR481 differed from that of SGR047 in that 63.2 % of the clones rooted on auxin-containing medium (T3) and as a result of *Agrobacterium* cocktail T1, while 79 % of the clones rooted as a result of *Agrobacterium* cocktail T2 (Figure 41). As was found for SGR047 clones, the percentage of successful rooting of *E. grandis* SGR481 clones varied from clone to clone for each of the treatments. For example, clones 8 and 47 did not root on medium containing auxin (T3) but 50 % rooting was achieved with both *Agrobacterium* cocktails (T1 and T2) for both these clones. Successful rooting of clone 20 could only be achieved with *Agrobacterium* cocktail T2, comprising five bacterial strains. Three clones, 34, 46 and 55, did not respond to any of the rooting treatments (Figure 41).

Although these results have not been statistically proven they do show that the bacterial cocktail treatments T1 (comprising *A. rhizogenes* LBA9402, TR8,3 and R1601) and T2 (comprising *A. rhizogenes* LBA9402, TR8,3 and R1601 and *A. tumefaciens* LB4404(pGA643) and LB4404(pGA643-12)) are able to induce roots on certain *in vitro* developed *E. grandis* clones.

Chimeric and control plants produced from *E. grandis* SGR047 and SGR481 clones were acclimatized.

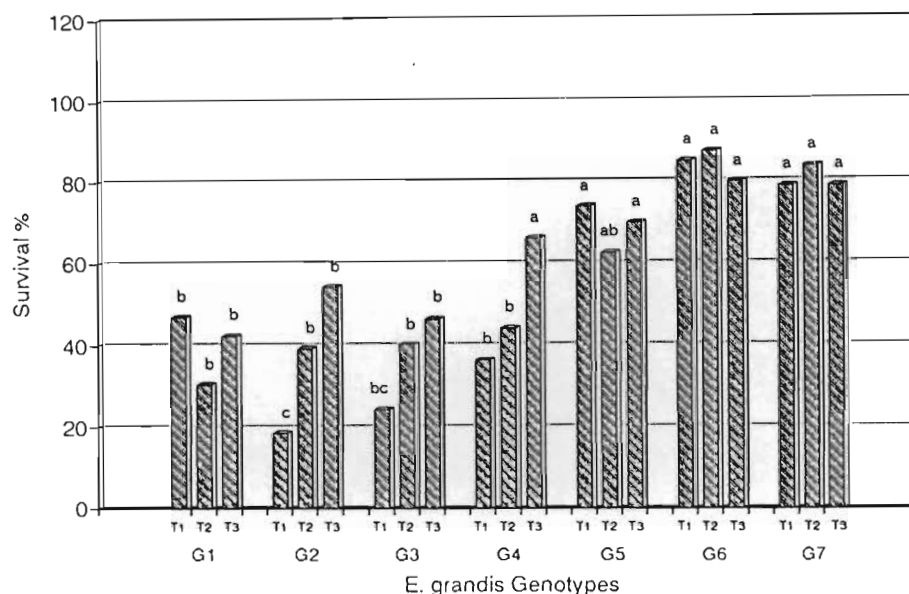


**Figure 41.** *In vitro* rooting of nineteen *in vitro*-developed *E. grandis* SGR481 clones inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and control treatment, T3 (uninoculated and placed on IBA containing medium), six weeks after inversion onto half-strength MS media.

#### 4.3.8 *In Vitro* Rooting of Seven *In Vitro* Propagated *E. grandis* Genotypes Using *A. rhizogenes* Cocktails

The auxin-induced rooting abilities of microcuttings from the 92 clones developed *in vitro*, from seven *E. grandis* (Table 14) genotypes, were compared to those induced by two bacterial cocktails (see section 4.3.2.4). Owing to the variable number of healthy microcuttings available per clone and the resultant relatively small data sets, the data from this trial were assessed statistically per genotype and not per clone within each genotype. The survival of *E. grandis* microcuttings in this trial ranged from 19 to 82 % (Figure 42). With the exception of genotypes G2 and G4, no significant effect of treatments on survival was evident. Survival of clone G2 was significantly lower after treatment with bacterial cocktail T1 (comprising three *A. rhizogenes* strains) than after treatment with T2 (comprising 3 *A. rhizogenes* and 2 genetically modified *A. tumefaciens* strains) or after the control treatment (T3). There was however, no significant difference between treatments T2 and T3. The survival of microcuttings of *E. grandis* genotype G4 after treatment with either of the two bacterial cocktails, T1 or T2, were significantly lower by up to 30 % than the untreated control, T3, microcuttings. This suggests that the bacterial treatments could have an adverse effect on survival of certain *E. grandis* genotypes. However, it must be borne in mind that other factors such as condition of the microcuttings could also have contributed to the poor survival of these clones. This possibility is supported by the fact that survival of a number of the genotypes, SGR038 (G1), SGR041 (G2), SGR042 (G3), and SGR046 (G4) was poor even in the absence of

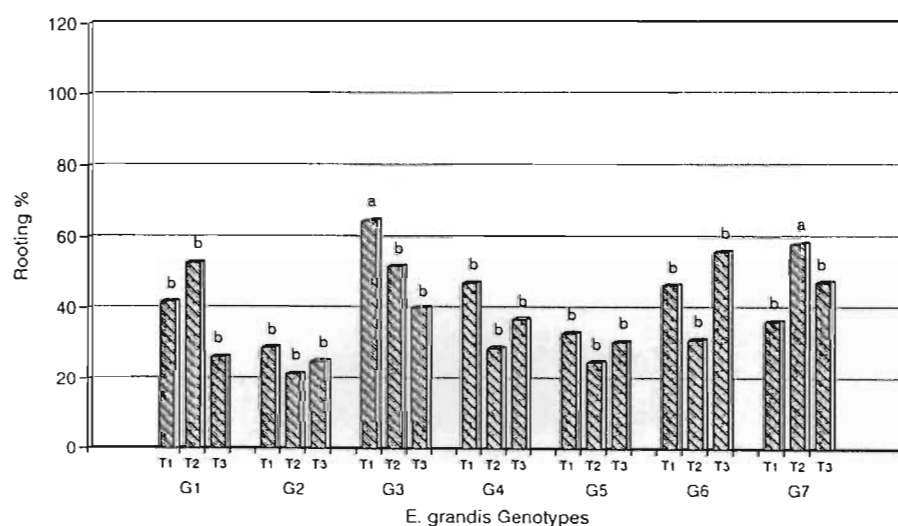
bacterial treatment (Figure 42).



**Figure 42.** *In vitro* survival (%) of microcuttings of seven *E. grandis* genotypes (see Table 2) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS media (see section 4.2.3.5). Based on the LSD Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

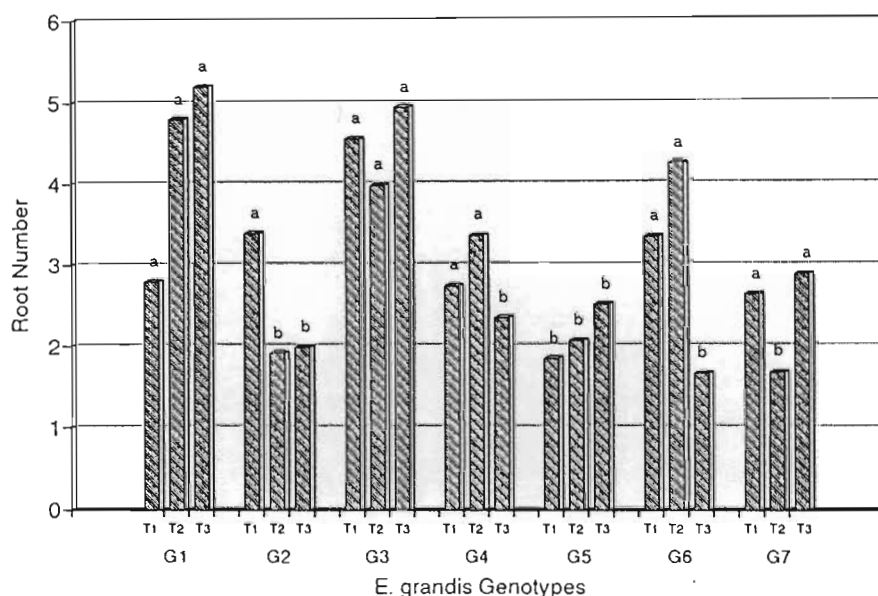
The rooting abilities of the seven *E. grandis* genotypes was generally poor ranging from 22 to 65 % depending on the genotype and treatment (Figure 43). Bacterial treatments, T1 and T2, improved the rooting ability of *E. grandis* SGR038 (G1) by 16 and 30 % respectively, compared to the control treatment, T3, in which microcuttings were rooted on auxin-containing medium. However, these difference were not significant. Similar trends in rooting induced by the three treatments were observed in genotypes SGR041 (G2), SGR046 (G4) and SGR047 (G5), namely, that treatment T1 resulted in a better average rooting percentage and treatment T2 a

lower average rooting percentage than treatment T3. These differences were however, not significant. In the case of genotype SGR394 (G6) treatment T3 produced the highest percentage rooting followed by bacterial cocktail treatments T1 and T2. The differences were however not significant. Rooting induced on genotype SGR042 (G3) by bacterial cocktail T1 was significantly better (19 %) than that achieved with the other two treatments, which did not differ significantly from each other. Treatment of microcuttings of genotype SGR481 (G7) with bacterial cocktail T2 significantly increased rooting ability of this genotype compared to rooting achieved with the other bacterial cocktail (T1) and with that obtained on auxin-containing medium (T3). The difference between rooting induced by treatments T1 and T3 for this clone was not significant (Figure 43).



**Figure 43.** *In vitro* rooting (%) of microcuttings of seven *E. grandis* genotypes (see Table 2) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS media (see section 4.2.3.5). Based on the LSD Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

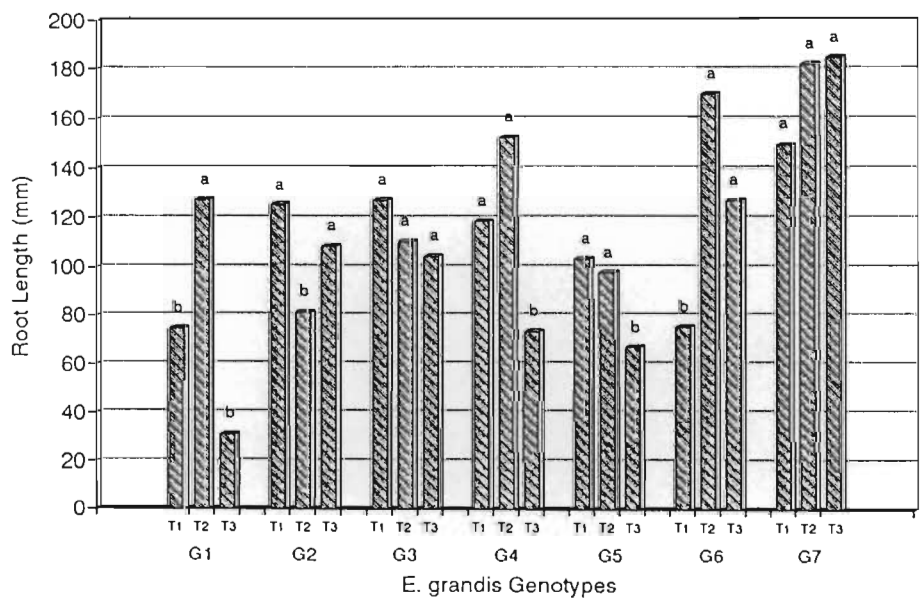
Although the average number of roots varied per genotype, they also varied with the treatment within a genotype (Figure 44). These variations were not significant for genotypes SGR038 (G1), SGR042 (G3) and SGR047 (G5). In the case of genotype G2, the average number of roots per rooted microcutting was significantly increased by up to 1.8 fold by bacterial treatment T1, comprising three *A. rhizogenes* strains, compared to the other two treatments, for which no significant difference was found. Both bacterial cocktails (T1 and T2) significantly increased average number of roots per rooted microcutting of genotypes SGR046 (G4) and SGR394 (G6), compared to control treatments (T3). The differences between treatments T1 and T2 for these clones were however, not significant. The average root number per rooted microcutting of SGR481 (G7) after inoculation with bacterial cocktail T1 was not significantly different from that obtained on auxin medium (T3). However, they were significantly better than that obtained after inoculation with bacterial cocktail T2. This implies that the bacterial cocktail comprising five *Agrobacterium* strains (T2) may have an adverse effect on rooting of certain *E. grandis* genotypes.



**Figure 44.** Average number of roots per rooted microcutting of the seven *E. grandis* genotypes (see Table 2) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS media (see section 4.2.3.5). Based on the LSD Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

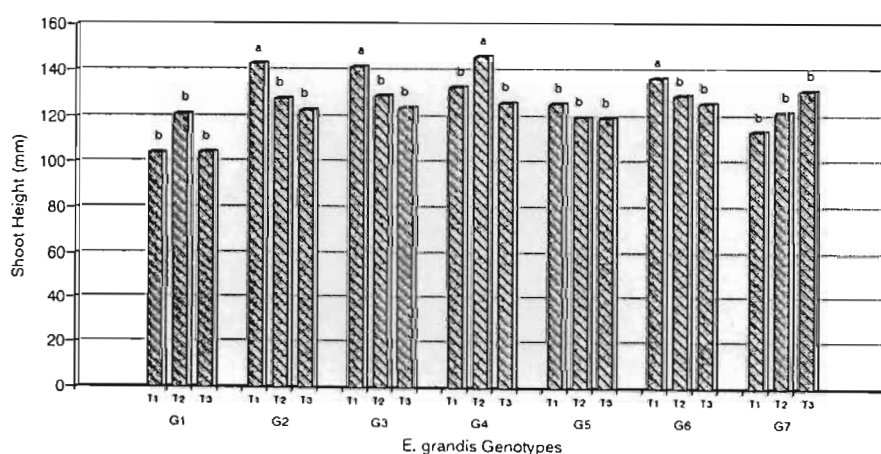
As was the case for average root number per rooted microcutting, the average root length varied with the genotype and with the treatment within a genotype (Figure 45). No significant difference in root length, irrespective of the treatment, was found for genotypes SGR042 (G3) and SGR482 (G7). Both bacterial cocktails (T1 and T2) significantly increased the root length (up to 2 fold) of genotypes SGR046 (G4) and SGR047 (G5) compared to the control treatment (T3). Bacterial cocktail T2 significantly improved root length (up to 4 fold) of rooted cuttings of genotypes

SGR038 (G1) and SGR394 (G6), compared to treatments T2 and T3. Although the average root length of genotype SGR038 (G1) achieved after treatment T1 was 2.5 fold longer than that obtained on auxin containing medium (T3), the difference was not significant. In the case of genotype G6 the root length achieved on auxin medium (T3) was significantly longer (1.6 fold) than that obtained after treatment with bacterial cocktail T1. For genotype SGR041 (G2), the average root length per rooted microcutting resulting after treatment T2 was significantly lower than that achieved with treatments T1 and T3, which were not significantly different from each other.



**Figure 45.** Average root length of roots developed on each microcutting of the seven *E. grandis* genotypes (see Table 2) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS media (see section 4.2.3.5). Based on the LSD Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

The average shoot heights of microcuttings of *E. grandis* genotypes SGR038 (G1), SGR047 (G5) and SGR481 (G7) rooted with bacterial cocktails T1 and T2, were not significantly better than those of control microcuttings rooted on auxin-containing medium (T3) (Figure 46). The shoot heights of genotypes SGR041 (G2), SGR042 (G3) and SGR394 (G6) rooted with bacterial cocktail T1, were significantly higher than those obtained with the other treatments, which did not significantly differ from one another. Similarly, the average shoot height of microcuttings from genotype SGR046 (G4) rooted with bacterial cocktail T2 was significantly higher than that obtained with the other treatments, which again were not significantly different from each other. The improved shoot heights found for certain clones, after bacterial treatments, can probably be attributed to the improved root quality and hence nutrient uptake.

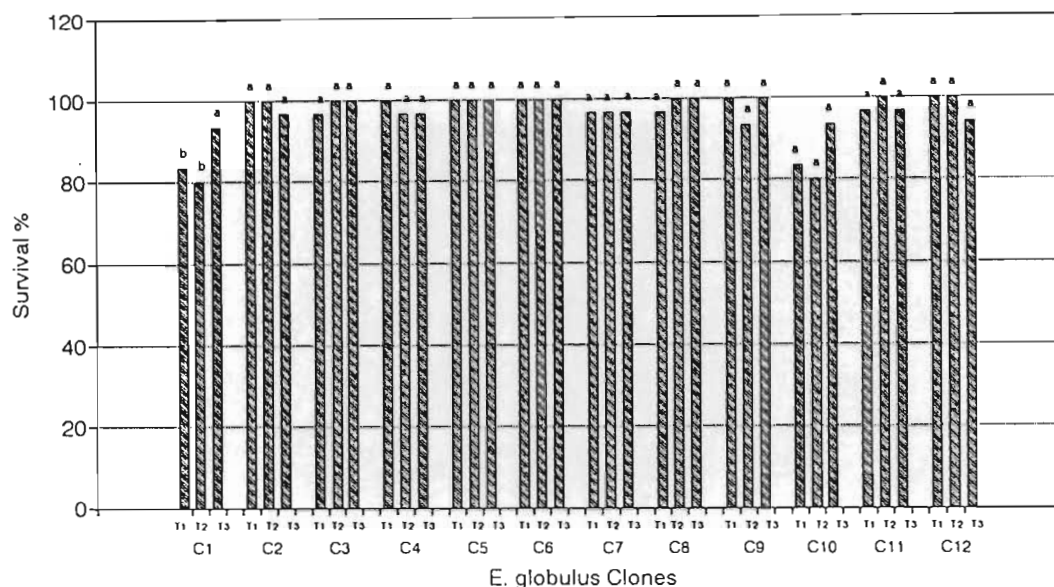


**Figure 46.** Average shoot height of the microcuttings from the seven *E. grandis* genotypes (see Table 2) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS media (see section 4.2.3.5). Based on the LSD Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

#### 4.3.9 *In Vitro* Rooting of Twelve *In Vitro* Developed Clones of *E. globulus* Using *A. rhizogenes* Cocktails

The auxin-induced rooting abilities of 12 *E. globulus* clones (Table 15) were compared to those induced by two bacterial cocktails (see section 4.2.3.4). Survival of these clones *in vitro*, was good ranging from 80 to 100 % (Figure 47). With the exception of *E. globulus* clone C1 (C33xC29/17), no significant effect of treatments on survival was evident. Survival of clone C1 was significantly lower after treatment with both bacterial cocktails (T1 and T2) than after the control treatment (T3). The difference in survival between the bacterial cocktail treatments was however, not significant. This decrease in survival suggests that the bacteria could have a detrimental effect on this *E. globulus* clone.

It must be borne in mind that in this trial the *A. rhizogenes* bacterial cocktail method was modified in that, after bacterial and auxin treatments, all microcuttings were re-inverted onto auxin-containing medium to further stimulate rooting. Under these conditions the rooting ability of *E. globulus* clone C33xC29/17 (C1) was significantly increased by 32 and 50 % after treatments with bacterial cocktail T1 and T2 respectively, compared to rooting achieved after the control treatment (T3 - auxin alone) (Figure 48). Rooting induced by bacterial cocktail T2 was significantly better (by 18 %) than that achieved with bacterial cocktail T1 for this clone.

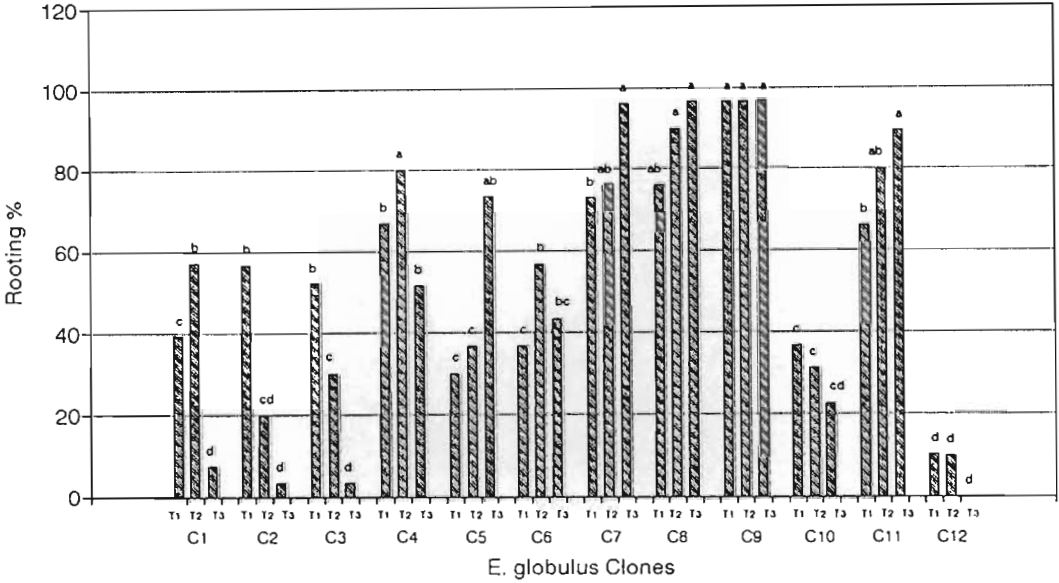


**Figure 47.** *In vitro* survival (%) of microcuttings of the 12 *E. globulus* clones (see Table 6) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS medium (RM<sub>g</sub>, see section 4.2.3.6). Based on the LSD Test any two means having a common letter are not significantly different at the P=0.05 level.

Interestingly, survival of clone C1 (Figure 47) appeared to be adversely affected by the bacterial treatments, but rooting was stimulated compared to the control treatment (Figure 48). Bacterial cocktail T2 also significantly enhanced rooting of clone C4 (C29xC37/34) compared to the other treatments. There was no significant difference between treatments T1 and T3 for this clone. Although treatment T2 gave the highest rooting percentage for clone C6 (C33xC25/69) it was not significantly different from that achieved after treatment T3. It was however, significantly better

(20 %) than that obtained with treatment T1, which was not significantly different from treatment T3. The rooting abilities of clones C2 (C33xC29/84) and C3 (C29xC37/8) showed similar trends (Figure 48). Rooting induced by bacterial cocktail T1 was notably the highest (up to 52 %) compared to that achieved with treatments T2 and T3. Treatment T2 yielded the next best rooting percentages for both these clones, however, differences in rooting between treatments T2 and T3 was only significant for clone C3. In the case of clone C5 (C33xC25/61) however, the bacterial treatments T1 and T2 appeared to significantly reduce (by up to 45 %) the rooting ability of this clone compared to treatment T3. This suggests that the bacterial cocktails could have an inhibitory effect on root formation of this clone. The survival of this clone was however, not affected by either bacterial treatment. In the case of clone C7 (C37xC11/40) treatment T3 also produced the highest rooting percentage (98 %), however, it was not significantly different from that achieved with bacterial cocktail treatment T2 (78 %). Rooting induced by bacterial cocktail T1 (75 %) was significantly lower than that induced by treatment T3 but was not significantly different from that induced by treatment T2. The same trends in rooting were found for clone C11 (Mu10xC37/78). Although the rooting ability of clone C8 (C37xC11/24) increased sequentially across treatments T1 to T3 inclusive, the differences were not significant. In the case of clone C10 (C18xC29/7), a progressive decrease in rooting ability was observed across treatments T1 to T3. Here again, the differences were not significant. The rooting

ability of clones C9 (C18xC29/28) was excellent, with 98 % rooting being achieved with all three treatments. Clone C12 (Mu10xC37/63) did not root in the presence of auxin (T3), suggesting that auxin may inhibit rooting. However, rooting could be increased to 10 % after treatment of this clone with either of the bacterial cocktails (Figure 48).

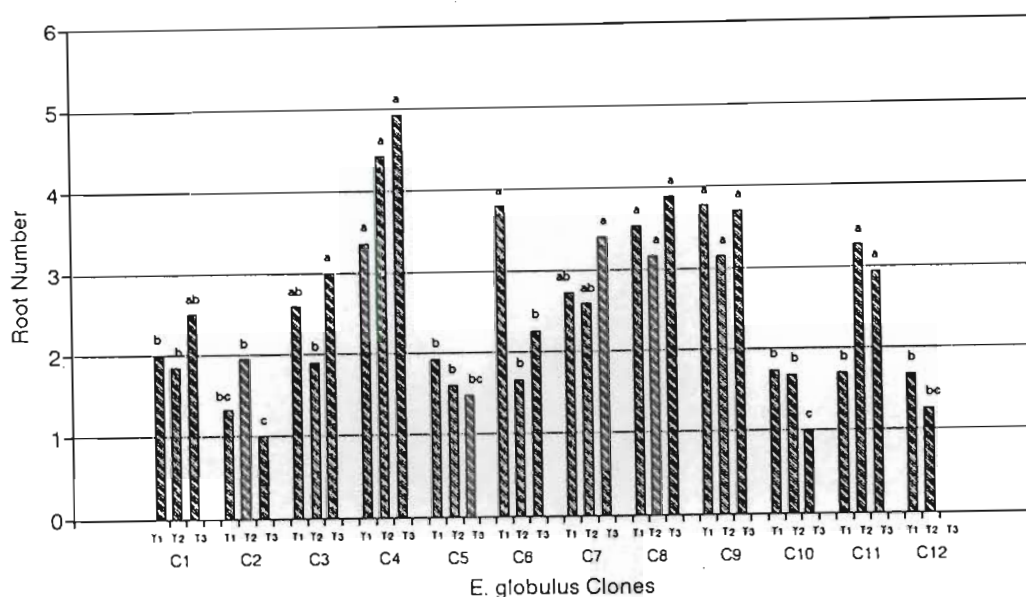


**Figure 48.** *In vitro* rooting (%) of microcuttings of the 12 *E. globulus* clones (see Table 6) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS medium (RM<sub>g</sub>, see section 4.2.3.6). Based on the LSD Test any two means having a common letter are not significantly different at the P=0.05 level.

The average number of roots that developed on each microcutting varied from clone to clone for *E. globulus* and was influenced by treatment in certain clones (Figure

49). The average root number per rooted microcutting of clones C1 (C33xC29/17), C4 (C29xC37/34), C5 (C33xC25/61), C7 (C37xC11/40), C8 (C37xC11/24) and C9 (C18xC29/28) were not significantly affected by treatment. Bacterial treatment T2 significantly increased root numbers of clone C2 (C33xC29/84) compared to that obtained after treatment T3. Although the root number achieved with treatment T1 was also higher than that achieved with treatment T3, the difference was not significant. Treatment T3 produced the greatest root number for clone C3 (C29xC37/8), but this was not significantly different from that achieved with bacterial treatment T1. It was however, significantly better than the root number obtained after treatment T2. Treatment T2 was not significantly different from treatment T1 for this clone. Bacterial cocktail T1 produced a 2.4 and 1.7 fold increase in root number for clone C6 (C33xC25/69), compared to treatments T2 and T3 respectively. These results were significant. The differences in root number between treatments T2 and T3 were however, not significant for this clone (Figure 49). The average root number per rooted cutting of clone C10 (C18xC29/7) after bacterial cocktail treatments T1 and T2 was not significantly different but they were significantly higher than that achieved with treatment T3. In the case of clone C11 (Mu10xC36/78), treatment T2 gave a slightly better root number (3.4 roots per cutting) than treatment T3 (2.9 roots per cutting) but the difference was not significant. These results were however, significantly higher than that achieved after treatment T1 (up to 1.9 fold). Finally there was no significant difference in root

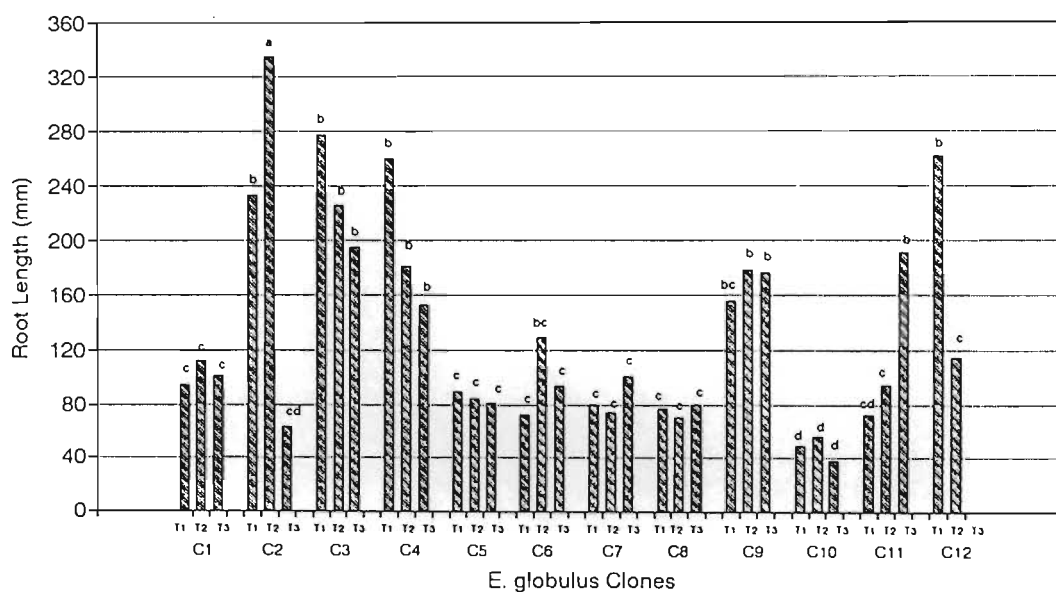
number obtained for clone C12 (Mu10xC36/78) after treatments T1 and T2. These results were significantly better than that obtained after treatment T3, for which no rooting occurred.



**Figure 49.** Average number of roots per rooted microcutting of the 12 *E. globulus* clones (see Table 6) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS medium (RM<sub>g</sub>, see section 4.2.3.6). Based on the LSD Test any two means having a common letter are not significantly different at the P=0.05 level.

As was found for root number, the root length varied from clone to clone for *E. globulus* and treatment-wise within a clone (Figure 50). Although some variation in root length was found for clones C1, C3, C4, C5, C6, C7, C8, C9 and C10 after treatments T1, T2 and T3, the differences were not significant. The root length of clone C2 was however significantly improved with bacterial cocktail treatments T1 and T2, by 5.2 and 3.6 fold, compared to that achieved with treatment T3. Bacterial

cocktail treatment T2 also yielded significantly better root lengths (1.4 fold increase) compared to bacterial cocktail treatment T1 for this clone. The root lengths of clone C11, achieved after bacterial cocktail treatments T1 and T2 were not significantly different. They were however, significantly shorter (up to 2.5 fold) than root lengths achieved after treating this clone with auxin alone (T3). As already mentioned, auxin treatment (T3) did not induce rooting of clone C12, however, the bacterial cocktail treatments (T1 and T2) did. The longest roots (265 mm) were achieved after treating this clone with bacterial cocktail T1, these were significantly longer (2.1 fold) than the roots induced using treatment T2.

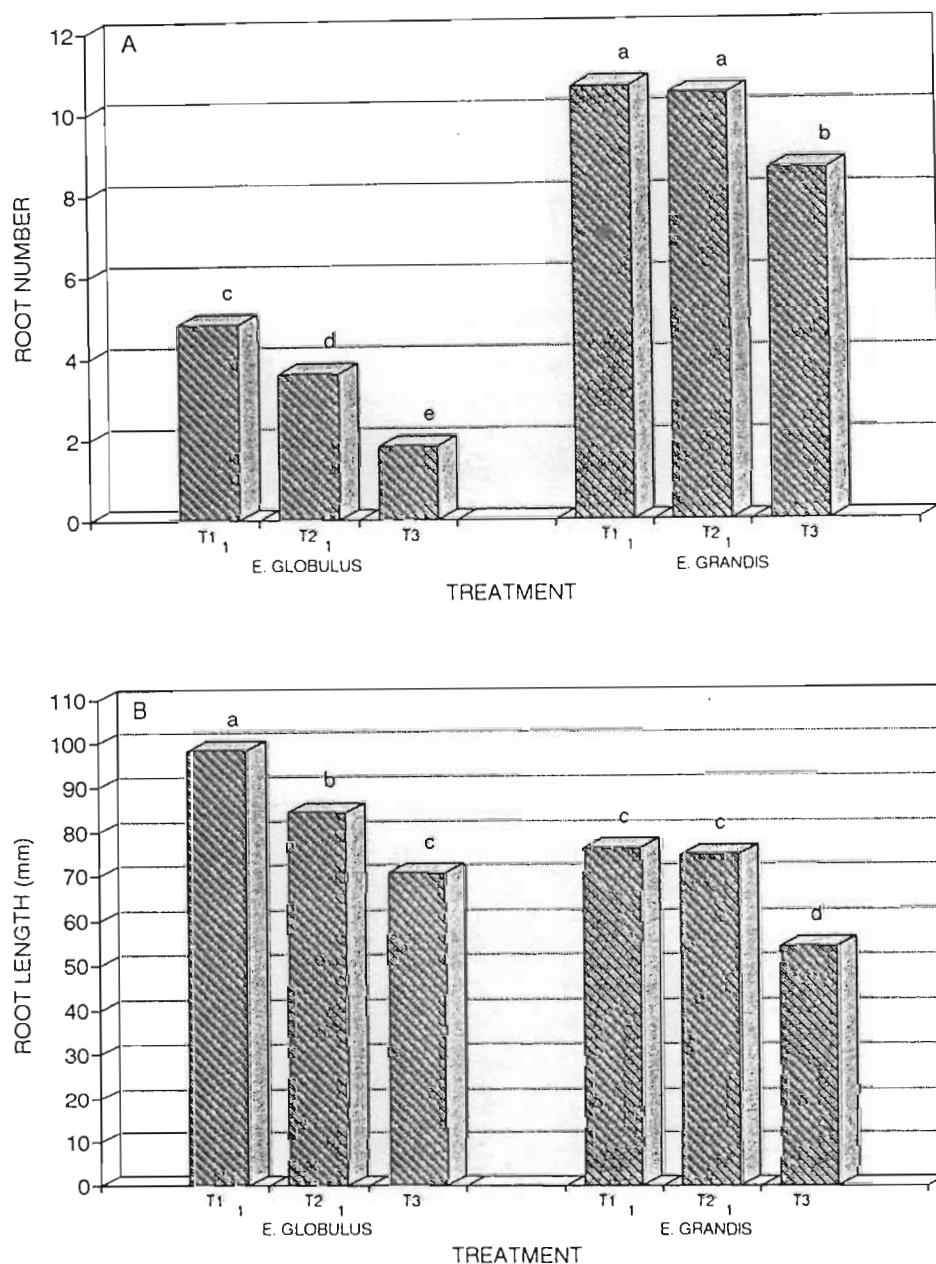


**Figure 50.** Average root length of roots developed on each microcutting of the 12 *E. globulus* clones (see Table 6) inoculated with two *Agrobacterium* cocktails, T1 and T2 (see section 4.2.2) and uninoculated control - T3, six weeks after inversion onto half-strength MS medium (RM<sub>g</sub>, see section 4.2.3.6). Based on the LSD Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

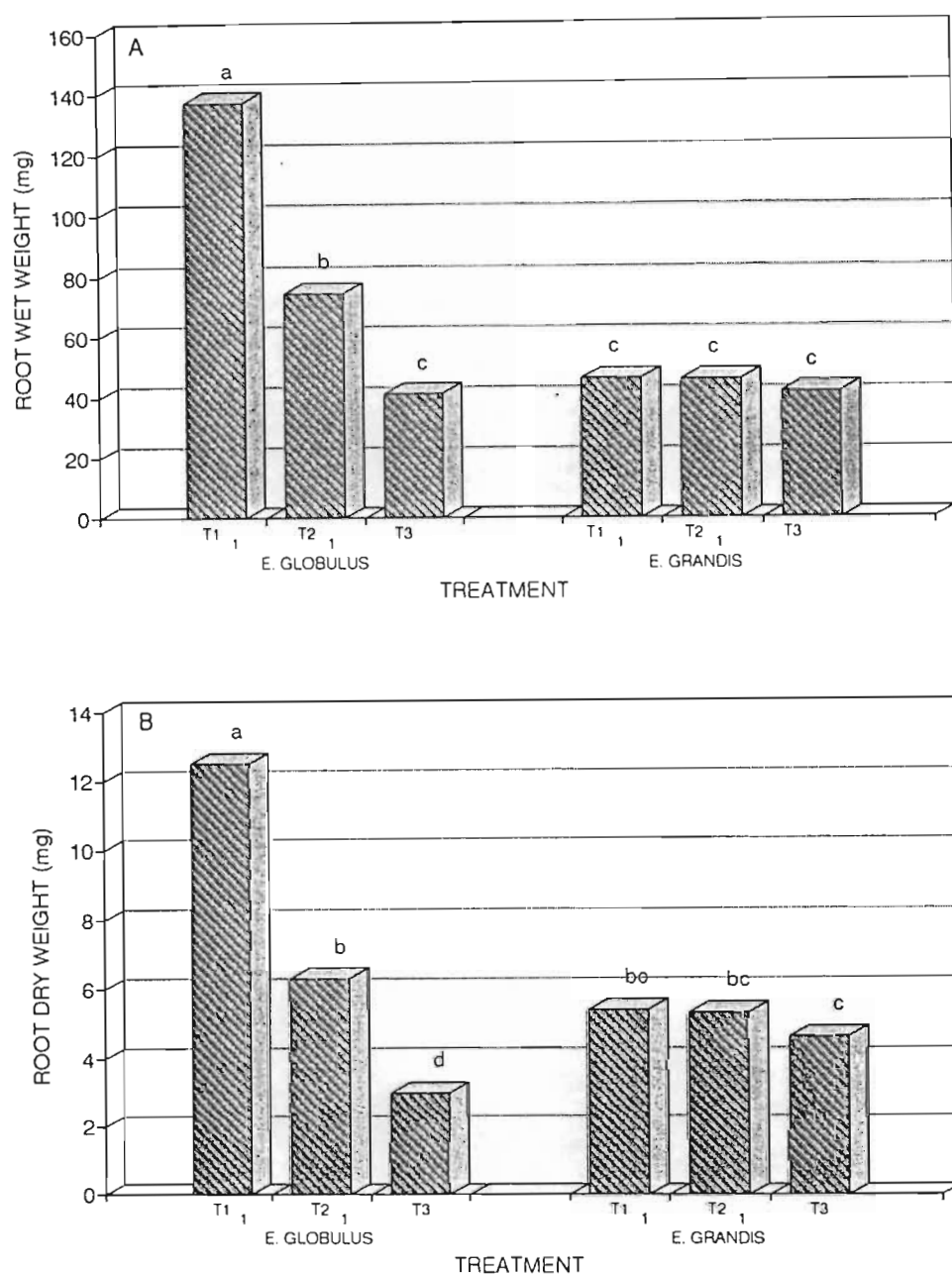
#### 4.3.10 *In Vitro* Rooting of *E. globulus* and *E. grandis* Using *A. rhizogenes* Cocktails

The effectiveness of rooting *E. grandis* and *E. globulus* C59 microcuttings by two *Agrobacterium* cocktails comprising strains LBA9402, TR8,3 and R1601 plus 1 mg l<sup>-1</sup> IBA (T1<sub>1</sub>) and these strains alone (T2<sub>1</sub>) was compared to that of 1 mg l<sup>-1</sup> IBA (T3). One hundred percent rooting was achieved with all the treatments for both *Eucalyptus* species. However, variation in the number of roots (Figure 51A), root length (Figure 51B), root fresh and dry mass (Figure 52A and B), shoot height (Figure 53) and shoot fresh and dry mass (Figure 54A and B) was observed for the different treatments with both *Eucalyptus* species tested.

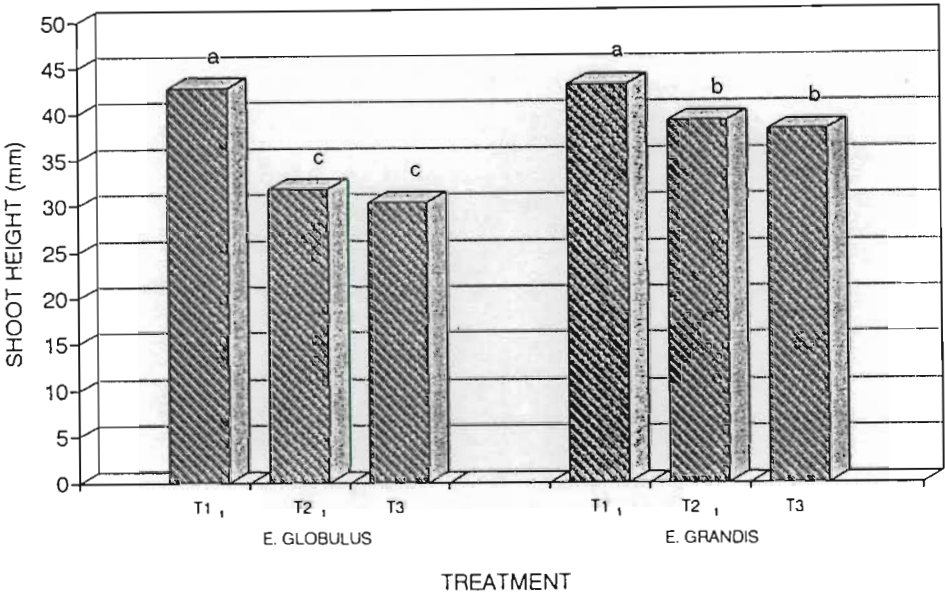
For all measurements made on *E. globulus* plantlets there was a significant difference between treatments, with the *A. rhizogenes* cocktail containing auxin (T1<sub>1</sub>) producing the best results and the auxin (T3) treated plantlets producing the poorest results (Figures 51 to 54). This trend was not apparent with *E. grandis*. No significant difference was found in root number and root length measurement for either of the *A. rhizogenes* cocktails (T1<sub>1</sub> and T2<sub>1</sub>) but they were significantly better than those for the IBA treated plantlets (51A and B). No significant difference was found between root fresh and dry mass for the three treatments (Figure 52A and B). The shoot height and wet mass measurements of *E. grandis* treated with *A.*



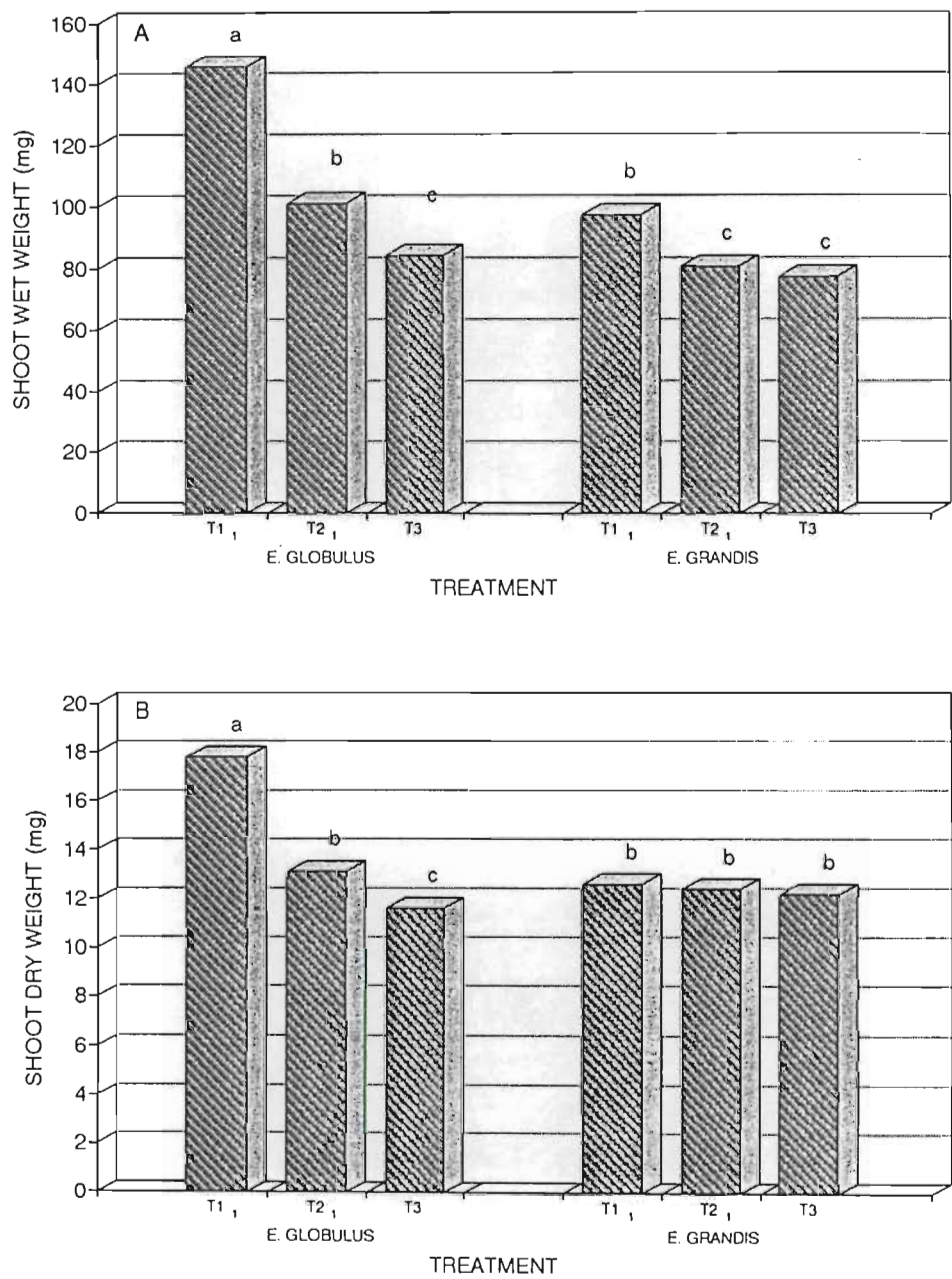
**Figure 51.** Root number (A) and length (B) of *in vitro* *E. grandis* and *E. globulus* chimeric plants produced after inoculation with two *A. rhizogenes* cocktails, T1<sub>1</sub> and T2<sub>1</sub> (see section 4.2.2), and 1mg l<sup>-1</sup> IBA - T3, eight weeks after inversion onto half-strength MS medium (see section 4.2.3.7). Based on the Scheffé test any two means having a common letter are not significantly different at the P=0.1 level.



**Figure 52.** Root wet (A) and dry (B) weights of *in vitro* *E. grandis* and *E. globulus* chimeric plants produced after inoculation with two *A. rhizogenes* cocktails, T1<sub>1</sub> and T2<sub>1</sub> (see section 4.2.2), and 1 mg l<sup>-1</sup> IBA - T3, eight weeks after inversion onto half-strength MS medium (see section 4.2.3.7). Based on the Scheffé test any two means having a common letter are not significantly different at the P=0.1 level.



**Figure 53.** Shoot height of *in vitro* *E. grandis* and *E. globulus* chimeric plants produced after inoculation with two *A. rhizogenes* cocktails, T1<sub>1</sub> and T2<sub>1</sub> (see section 4.2.2), and 1 mg l<sup>-1</sup> IBA - T3, eight weeks after inversion onto half-strength MS medium (see section 4.2.3.7). Based on the Scheffé test any two means having a common letter are not significantly different at the P=0.1 level.



**Figure 54.** Shoot wet (A) and dry (B) weights of *in vitro* *E. grandis* and *E. globulus* chimeric plants produced after inoculation with two *A. rhizogenes* cocktails, T1<sub>1</sub> and T2<sub>1</sub> (see section 4.2.2), and 1 mg l<sup>-1</sup> IBA - T3, eight weeks after inversion onto MS media. Based on the Scheffé test any two means having a common letter are not significantly different at the P=0.1 level.

*rhizogenes* cocktail T2<sub>1</sub> and IBA (T3) alone were not significantly different from one another but were significantly lower than those obtained with the *A. rhizogenes* cocktail containing IBA (T1<sub>1</sub>) (Figure 53 and 54A). No significant difference was found in shoot dry mass between the three treatments (Figure 54B).

#### 4.4 DISCUSSION

The aim of this research was to overcome rooting deficiencies and improve root quality in micropropagated *Eucalyptus* species and clones. Inoculation of *Eucalyptus grandis* with *A. rhizogenes* LBA9402 resulted in root development at the wound site. This demonstrated the susceptibility of this species to *A. rhizogenes*.

Transformation of the bases of *in vitro* propagated *Eucalyptus* shoot explants was achieved by inoculating the cut surface with the bacteria. This resulted in the production of chimeric plants with normal shoots and transformed roots. Root initiation occurred within the first two to four days after inoculation of *Eucalyptus* species with *A. rhizogenes* strains. All three *A. rhizogenes* strains were able to induce rooting in the three *Eucalyptus* species tested. However, the percentage rooting was dependent on the genotype of both the species and the bacterium. This

was also reported by LAMBERT and TEPFER (1991) for apples. No root development occurred on uninoculated control explants before inversion whereas rooting occurred on all inoculated explants. This root development could have resulted from the transfer of the auxin genes from the Ri plasmid into the plant genome. *A. rhizogenes* LBA9402 consistently gave better rooting on all three *Eucalyptus* species. In addition it efficiently induced rooting on *in vitro* propagated *E. globulus* microcuttings. This strain showed the greatest potential for transformation of clonal material.

Four weeks after re-inverting the explants, spontaneous rooting of uninoculated control explants was detected in hormone-free medium. Rooting of control plants was increased to approximately 50 percent in all species by the addition of  $1\text{ mg l}^{-1}$  IBA to the medium. *A. rhizogenes* LBA9402 induced 80 % rooting in all three *Eucalyptus* species and strain TR 8,3 produced 76 % rooting in *E. grandis* only. Root induction by R1601 was of the same order as that achieved by the addition of IBA for the three *Eucalyptus* species.

The growth of transformed roots which developed on the *Eucalyptus* explants was extensive when compared to the control. A visual comparison of root quality of control plants and chimeric plants six weeks after inoculation clearly demonstrated the improved root quality in chimeric plantlets. *In vitro* propagated *Eucalyptus*

plantlets were hardened off and extensive lateral root development occurred. No morphological abnormalities were observed in plantlets with transformed roots, suggesting that this procedure could be used to improve rooting in difficult-to-root *Eucalyptus* species.

Genetic transformation was assessed using both opine detection and molecular hybridisation with varying degrees of success. Normal, non-transformed roots could not be grown in liquid culture, however transformed roots could. This ability of the transformed roots to grow in liquid culture can therefore be taken as an indication that genes on Ri plasmid have been inserted into the plant genome and are being expressed.

The potential use of transformed root cultures as a fibre source for the Pulp and Paper Industry was investigated. The fibre yield from eucalypts is usually in the region of 52 % (Personal Communication Dr. J. S. M. Venter). The fibre yield from the root cultures was found to be extremely low, 12.6 % and therefore of little value to the Industry.

The plant genotype and physiological state are major determinants in rooting cuttings both *in vivo* and *in vitro*. These determinants are also crucial to the success of the molecular approach to improving rooting. A third determinant, the genotype of the

bacterium, plays an equally important role in this approach. The rooting data presented supported this.

Wild-type *A. rhizogenes* strain A4 and genetically modified *A. tumefaciens* strains LBA4404(pGA643) and LBA4404 (pGA643-12) were able to effectively induce rooting in both *E. grandis* and *E. nitens*. There was no significant difference in root induction by these strains compared to that induced by wild-type *A. rhizogenes* strains LBA9402, TR8,3 and R1601 on *E. grandis*. However, root enhancement induced by all the strains was significantly better than that induced by auxin treatment, which suggests that bacterial inoculation has the potential to alter the inherent ability of *Eucalyptus* genotypes to produce roots. These findings differ from those of LAMBERT and TEPFER (1991) who found that although *A. tumefaciens* LBA4404(pGA643-12), carrying the ORF12 gene from the A4 Ri TL-DNA in a binary vector system under the control of a strong CaMV 35S promoter, was significantly better at rooting apple cuttings than wild-type *A. rhizogenes* strains, it was not significantly better than auxin treatment. OFR12 is a member of a highly divergent gene family present in Ri- and Ti-DNA and is thought to facilitate organogenesis (LEVESQUE *et al.*, 1988; LAMBERT and TEPFER, 1991). Thus the *Agrobacterium* strains tested were not able to improve the inherent ability of apples to root. It was also found that *A. tumefaciens* LBA440(pGA643-12) induced apple roots which had a curly, corkscrew phenotype; this was not the case for

eucalypts. The fact that rooting of *Eucalyptus* by wild-type *A. rhizogenes* strains and genetically modified *A. tumefaciens* strains carrying the ORF12 gene in a binary vector was not significantly different, suggests that chromosomal background does not influence the infection process and hence the successful induction of roots.

Early results suggested that host specificity is one of the critical determinants in successful root induction by *A. rhizogenes* strains. With the aim of overcoming the host range limitation of a bacterium in successful transformation and rooting the concept of developing and using bacterial cocktails to improve rooting of a wide range of *Eucalyptus* genotypes/clones was investigated. Two *Agrobacterium* cocktails, T1 comprising three *A. rhizogenes* strains, LBA9402, TR8,3 and R1601 and T2 comprising the latter three strains and two (genetically modified) *A. tumefaciens* strains, LBA4404(pGA643) and LBA4404(pGA642-12) were screened on a range of *E. grandis* clones and genotypes, and *E. globulus* clones.

The genetic variation in auxin-induced rooting ability of the *E. grandis* SGR047 and SGR481 clones is emphasised by the data presented. Similarly a tremendous variation in response of these clones to the two bacterial treatments was also observed. Although these results still have to be statistically proven, they do suggest that *Agrobacterium* cocktails have potential to induce and improve rooting on certain clones.

The survival rates of microcuttings of the seven *in vitro* propagated *E. grandis* genotypes in an *Agrobacterium* cocktail-mediated rooting trial showed that the bacterial cocktails did not generally have an adverse effect on the *E. grandis* genotypes tested. The one exception was genotype SGR041 (G2) where survival after bacterial cocktail T1 treatment was significantly lower than that achieved after the other treatments. The reason for this is not known, however, the relatively poor condition of the microcuttings may have contributed to this decrease. Bacterial cocktails T1 and T2 significantly improved the rooting abilities of *E. grandis* genotypes SGR042 (G3) and SGR481 (G7), respectively. Although the results showed that one or both of the bacterial cocktails increased the rooting abilities of the seven *E. grandis* genotypes compared to auxin alone the differences were not always significant. In 25 % of the *E. grandis* genotypes tested, root number and root length were significantly improved by either one or both of the bacterial cocktails, compared to the auxin treatment. However, the results also show that the root number and length of certain clones appeared to be decreased owing to treatment by the bacterial cocktails, suggesting that they may have an adverse effect on root quality of certain *E. grandis* genotypes. The reason for this is unclear at present. The results from this trial did however, also show that the *Agrobacterium* cocktails have potential to induce and improve rooting on certain *E. grandis* genotypes.

The high survival rates of microcuttings in the *in vitro* *E. globulus* transformation

rooting trial showed that *Agrobacterium* cocktails did not generally have an adverse effect on the *E. globulus* clones tested. The one exception was clone C1 (C33xC29/17) where a slight (15 %) but significant decrease in survival was detected. The bacterial cocktails did however, significantly improve rooting of this clone compared to the auxin treatment. How the bacterial cocktail treatments can adversely affect survival and yet enhance rooting is difficult to explain. Other factors such as the condition of the microcuttings of this clone may however, have contributed to this decrease in survival. These results support the findings from the *E. grandis* genotype and clone trials. The rooting ability of 7 of the 12 clones tested was improved by one or both of the bacterial cocktails compared to auxin alone. These improvements were significant in 5 of the 7 clones. A significant decrease in rooting after bacterial cocktail treatment T1 was observed in clones C37xC11/40 (C7) and Mu10xC37/78 (C11) only. The root number developed per rooted cutting was generally unaffected by treatments for the *E. globulus* clones tested, however, for 33 % of the clones one or both of the bacterial cocktails significantly enhanced root number when compared to auxin treatment. In 25 % of the clones one of the two bacterial cocktails appeared to significantly reduce the root number when compared to auxin treatment. The results from this trial also suggested that the bacterial cocktail treatments generally did not adversely affect root length and were as efficient as auxin alone at enhancing root lengths of the *E. globulus* clones tested. However, for some clones, C33xC29/84 (C2) and Mu10xC37/63 (C12), the bacterial

cocktails significantly improved the root length compared to auxin treatment, while for one of the clones (C18xC29/7 (C10)) they appeared to have an adverse effect on root length.

The results from the *Agrobacterium* cocktail rooting trials showed that the two bacterial cocktails were able to improve rooting and root quality (as determined by root number and length) of certain *Eucalyptus* clones, compared to auxin treatment. However, these cocktails could not completely overcome the host range limitations of the individual *Agrobacterium* strains.

The concept of using *A. rhizogenes* cocktails was therefore further developed by testing *A. rhizogenes* cocktails comprising wild-type strains LBA9402, TR8,3 and R1601 and these strains plus 1mg l<sup>-1</sup> IBA. These cocktails were found to be as effective as auxin alone, in inducing rooting on *in vitro* propagated *E. grandis* and *E. globulus* C59, with 100 % rooting being achieved. This suggested that these *Eucalyptus* genotypes are easy-to-root genotypes. However, when the quality of the roots produced on *E. globulus*, as determined by root number and fresh and dry mass, was examined, the auxin-containing bacterial cocktail significantly improved the root quality over the other treatments and showed that the transformed roots were sensitive to auxin. The improved root quality is reflected in the improved shoot quality. In the case of *E. grandis*, there was no significant difference in root quality

obtained with the two *Agrobacterium* cocktails. The root quality resulting from the auxin treatment was however, significantly poorer. These findings are important as root quality is an important factor in field establishment and plant vigour. The improved root quality observed in this study supports the finding of ERICKSON *et al.* (1990) who found that inoculation of tomato transplants with *A. rhizogenes* improved root mass and vigour. Based on these results the potential use of an *A. rhizogenes* cocktail supplemented with auxin to improve rooting in *in vitro* propagated eucalypts is proposed.

In conclusion, the results from this study firstly show that *A. rhizogenes* strains have the potential to improve rooting and root quality in certain *Eucalyptus* species and clones. The resultant chimeric plants have the Ri-TL DNA in all or some of the roots but have morphologically normal aerial parts. A potential drawback to the use this approach would be the dissemination of *A. rhizogenes* into the soil. The use of antibiotics *in vitro* however, greatly reduces this possibility.

Secondly, the results show that the implementation of the molecular approach to improving rooting in eucalypts could be achieved in one of two ways. If *Agrobacterium* strains are used individually, a bank of strains would have to be established and it would be necessary to screen these strains on a range of plant genotypes for a strain/genotype combination that results in successful root induction.

Alternatively, if *Agrobacterium* cocktails are selected for this purpose, further research is essential to establish their host range.

## CHAPTER 5

### *IN VIVO AGROBACTERIUM RHIZOGENES* TRANSFORMATION SYSTEMS FOR IMPROVING ROOTING OF TREE SPECIES

#### 5.1 INTRODUCTION

Fifteen years of research have culminated in clonal propagation of *Eucalyptus* by stem cuttings becoming an integral part of forestry in South Africa. Factors determining the success of clonal propagation are numerous and diverse and include the genetic material, silvicultural practice (BOWDEN, 1984), knowledge of site and genotype interactions (BARNES, 1984) and nursery practice (ADENDORFF and SCHÖN, 1991; DENNISON and KIETZKA, 1993).

The limiting factor in commercial propagation of *Eucalyptus* clones by stem cuttings is the ability of the genotype to root. Besides the genetic control of rooting, root formation on cuttings is influenced by a number of factors (see section 1.5.2.1) which affect the micro environment around the base of the cuttings. These include the availability of air and moisture which are determined by the type / physical structure of propagation medium used (HARTMANN and KESTER, 1983; ANON, 1988). A range of media including peat, perlite, sand, vermiculite, rockwool and

loam have been used to root *Eucalyptus* (CHAPERON, 1983; GEARY and LUTZ, 1985; LOACH, 1985; CUNNINGHAM and GEARY; 1989; REUVENI *et al.*, 1990; ADENDORFF and SCHÖN, 1991; CARTER and SLEE, 1991). The physical characteristics of these media that result in their success or failure as propagation media have not been well documented. Research on the relationship between successful rooting and water content of the medium has however, shown that for optimum rooting, stem tissue must not lose water (GRANGE and LOACH, 1983; CARTER and SLEE, 1991). Water is lost from stem tissue by transpiration through leaves and can be replaced through the stem base provided water is present and freely available in the medium around the base of the stem (LEAKEY, 1985). CARTER and SLEE (1991) recently examined the physical characteristics of four media, peat, perlite, sand and peat:perlite:sand, and their results suggest that a relatively high moisture content may be more important than high air content for maximum root formation. The choice of medium for rooting cuttings commercially seems to vary from company to company and from country to country. For example, a vermiculite based medium is used in South Africa while a peat:perlite medium has been selected in Portugal.

As previously mentioned, optimisation of physiological and environmental conditions in the nursery have resulted in a marked increase in the rooting capacity of some *Eucalyptus* clones (ADENDORFF and SCHÖN, 1991; DENNISON and KIETZKA,

1993). However, rooting of other clones exhibiting desirable wood properties cannot be further improved by using these conditions. The poor rooting capacity of such *Eucalyptus* clones under commercial nursery conditions, led to this study on the potential use of *Agrobacterium rhizogenes*-mediated transformation for improving rooting. The use of six *A. rhizogenes* strains on rooting of cuttings of *E. grandis* and *E. globulus* clones under nursery conditions and the effect of two commercially used propagation media on *A. rhizogenes*-induced rooting was investigated.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Bacteria and Culture Conditions

The *A. rhizogenes* strains used in this study are listed in Table 13. Bacterial cultures were prepared as described in section 4.2.2. Bacterial suspensions of  $10^8$  cfu ml<sup>-1</sup> were prepared in sterile tap water.

#### **Bacterial Cocktails:**

T1 comprised *A. rhizogenes* strains LBA9402, R1601 and TR8,3 supplemented with 1mg l<sup>-1</sup> IBA.

T2 comprised *A. rhizogenes* strains LBA9402, R1601 and TR8,3 and *A. tumefaciens* strains LBA4404(pGA643) and LBA 4404(pGA643-12) supplemented 1 mg l<sup>-1</sup> IBA.

Bacterial cocktails were prepared as described in section 4.2.2.

### 5.2.2 Plant Material

The *Eucalyptus* species and clones used in this study are listed in Table 16. Where the rooting abilities under nursery conditions have already been reported they have been detailed. Two *E. globulus* clones, developed at CELBI, Quinta do Furadouro, Portugal were selected for this trial. The sixteen *E. grandis* clones with a range of rooting abilities were selected from the clone bank at CSIR, Forestry Research Centre, Sabie.

Table 16. *Eucalyptus* species and clones used in this study.

<i>Eucalyptus</i> species/clone	Rooting ability %	Source
<i>E. globulus</i> (Trial 1)  HM15 AC17		Dr. P. J. Wilson, CELBI, PORTUGAL
<i>E. grandis</i> (Trial 2)  SGR052                    (C1)                    80 SGR132                    (C2)                    45 SGR096                    (C3)                    15 SGR050                    (C4)                    90 SGR171                    (C5)                    65 SGR124                    (C6)                    30		CSIR, FORESTEK / DWAF*, South Africa, Trial No. 1/01/08/02/EA62/07 Reported rooting % see Nel (1991)
<i>E. grandis</i> (Trial 3, 4 & 5)  SGR030                    (C1)                    85 SGR034                    (C2)                    95 SGR071                    (C3)                    90 SGR003                    (C4)                    50 SGR028                    (C5)                    70 SGR125                    (C6)                    60 SGR063                    (C7)                    35 SGR096                    (C8)                    15 SGR103                    (C9)                    25 SGR109                    (C10)                    45		CSIR, FORESTEK / DWAF*, South Africa, Trial No. 1/01/08/02/EA62/07 Reported rooting % see Nel (1991)

continued

Table 16 continued

<i>Eucalyptus</i> species/clone	Rooting ability %	Source
<i>Eucalyptus</i> Hybrid Clones (Trails 6 & 7)		Dr P. Schön H L & H, White River
GxC 005/015 (C1)	88.5	
GxC 016/008 (C2)	98.0	
GxN 018/CTG (C3)	55.8	
GxN 019/CTG (C4)	37.2	
GxN 055/CTG (C5)	44.9	
GxN 065/CTG (C6)	62.8	
GxN 227/CTG (C7)	37.7	
NxG 017/ICF (C8)	36.4	
NxG 049/ICF (C9)	50.5	
NxG 070/ICF (C10)	71.7	
NxG 071/ICF (C11)	57.1	

\* DWAF, Department of Water Affairs and Forestry.

### 5.2.3 Trial 1: *In Vivo* *A. rhizogenes*-mediated Rooting of Cuttings of Two *E. globulus* Clones.

#### Experimental Design

The trial was set up in a randomised block design with 5 replications as follows :

- 2 *E. globulus* clones (Table 16, Trial 1)

- 4 Treatments (T1 - *A. rhizogenes* LBA9402; T2 - *A. rhizogenes* R1601; T3 - *A. rhizogenes* TR8,3; T4 - Control (no hormones, no inoculum))
- 20 cuttings per plot
- 2 plots (treated and control) of 20 cuttings within each strain
- 1200 cuttings

Apical stem cuttings, generally two leaf pairs plus apex and between 8 and 12 cm in length, of two *E. globulus* clones, HM15 and AC17, were harvested from 10 litre potted plants growing outdoors, at the CELBI nursery facility, Quinta do Furadouro, Portugal. The larger leaves of the cuttings were severed across the lamina to reduce overlap when the cuttings were set at 4 x 4 cm in the propagation environment. The cuttings were immersed for 30 seconds in 2 g l<sup>-1</sup> of the systemic fungicide Benlate (active ingredient benomyl (benzimidazole) 50 g kg<sup>-1</sup>). The cut surface at the base of the cutting was dried and then dabbed for five seconds onto the surface of 48 hour bacterial cultures grown on MYA plates (in the treated cuttings). The cuttings were set in peat:perlite (1:1) to a depth of two to three centimetres in the glasshouse at CELBI, Quinta do Furadouro, Portugal.

Cuttings were dug up at the end of the 39 day propagation period and the following parameters recorded for each plot of 20 original cuttings: percentage survival, percentage rooting and root number. Percentage rooting was expressed as the percentage of survivors that rooted.

#### 5.2.4 Trial 2: *In Vivo A. rhizogenes*-mediated Rooting of Cuttings of Six *E. grandis* Clones on Vermiculite and Peat:Perlite Media.

##### Experimental Design

The trial was set up in a randomised block design with five replications as follows:

- 6 *E. grandis* clones (Table 16, Trial 2)
- 5 Treatments (T1 - Control (no hormones, no inoculum); T2 - *A. rhizogenes* LBA9402; T3 - *A. rhizogenes* R1601; T4 - *A. rhizogenes* TR8,3; T5 - Seradix No. 2)
- 8 Cuttings per row plot
- 2 Media (vermiculite and peat : perlite (1:1)).
- 12400 Cuttings

Sterile Unigro Speedling trays were filled with vermiculite (8 kg medium grain vermiculite was mixed with 20 litres water and 100 ml Kelpak) or peat:perlite (1:1), shaken until just firm and placed in the greenhouse. Coppice material of the six *E. grandis* clones (Table 1) were cut from the clone bank at CSIR, Forestry Research Station, Sabie, in the morning to avoid excessive moisture loss. They were placed directly into 20 litre buckets containing 3 % sucrose solution. The material was also sprayed with water to prevent stress due to drying out. Cuttings, 2 to 5 mm thick, and between 6 and 10 cm in length were then prepared. Two leaves, which were cut in half, were left on the cuttings. The cuttings were then dipped into 0.1 % Benlate

for three seconds and shaken to remove the excess. The bases of the cuttings were then treated either by dabbing the cut surface for five seconds onto the surface of 48 hour bacterial cultures grown on MYA plates or by dipping into a hormone powder (Seradix No. 2). They were then firmly set in the prepared media to a depth of 2 to 3 cm, placed in the greenhouse and sprayed with Multifeed (30 g 15 l<sup>-1</sup> water). The cuttings remained in the greenhouse for a period of 28 days and were then moved to a shade net area under mist irrigation. The leaf temperature in the greenhouse was maintained between 18 and 20°C by misters which were set to spray as soon as 80% of leaf area was dry. Media temperature was maintained between 23.8 and 29.4 °C by placing the trays on heated sand beds. After 40 days the trial was assessed and the percentage rooting, expressed as the percentage survivors that rooted, was determined.

#### 5.2.5 Trial 3: *In Vivo A. rhizogenes*-mediated Rooting of Cuttings of Ten *E. grandis* Clones on Vermiculite Medium.

##### Experimental Design

The trial was set up in a randomised block design with 10 replications as follows :

10 *E. grandis* clones (Table 16, Trial 3)

- 8 Treatments (T1 - Control (no hormones, no inoculum); T2 - *A. rhizogenes* LBA9402; T3 - *A. rhizogenes* R1601; T4 - *A. rhizogenes* TR8,3; T5 - *A. tumefaciens* LBA4404(pGA643); T6 - *A. tumefaciens* LBA4404(pGA643-12); T7 - *A. rhizogenes* A4; T8 - Seradix No. 2)
- 8 Cuttings per row plot
- 6400 Cuttings

Vermiculite medium and cuttings were prepared as described in 5.2.4. Cuttings were inoculated either by dipping into the bacterial suspensions ( $10^8$  cfu ml<sup>-1</sup>) for at least 5 to 10 seconds or by dipping into a hormone powder (Seradix No. 2). They were then set and grown as described above. After 60 days the trial was assessed and the following data recorded: percentage survival, percentage rooting, average number of roots per cutting and root dry mass. Rooting ability was expressed as the percentage of survivors that rooted.

#### 5.2.6 Trials 4 and 5: *In Vivo* Rooting of Cuttings of Ten *E. grandis* Clones by Two *Agrobacterium* Cocktails

##### Experimental Design

The trial was set up in a randomised block design with 10 replications as follows :

- 10 *E. grandis* clones (Table 16, Trial 4 & 5)
- 3 Treatments (T1 and T2 (see section 5.2.1), and T3 - Seradix no. 2)
- 8 Cuttings per row plot
- 2400 Cuttings

Vermiculite medium and cuttings were prepared as described in section 5.2.4. Cuttings were inoculated by either dipping into the bacterial cocktail suspensions ( $10^8$  cfu ml<sup>-1</sup>) for at least 5 to 10 seconds or a hormone powder (Seradix No.2). They were then set and grown as described in section 5.2.4, in the SAFCOL nursery at Frankfort. After 60 days the trial was assessed and the following records made: percentage survival, percentage rooting, average number of roots per cutting and root dry mass. Rooting ability was expressed as the percentage of survivors that rooted.

#### 5.2.7 Trials 6 and 7: *In Vivo* Rooting of Cuttings of Eleven *Eucalyptus* Hybrid Clones by Two *Agrobacterium* Cocktails

##### Experimental Design - Trial 6

The trial was set up in a randomised block design with three replications as follows:

- 11 *Eucalyptus* hybrid clones (Table 13, Trial 6)

- 3 Treatments (T1 and T2 (see section 5.2.1), and T3 - Seradix no. 2)
- 8 Cuttings per row plot
- 1584 Cuttings

### Experimental Design - Trial 7

The trial was set up in a randomised block but unbalanced design (as the availability of material was limited) as follows :

- 11 *Eucalyptus* hybrid clones (Table 16, Trial 7)
  - 3 Treatments (T1 and T2 (see section 5.2.1), and T3 - Seradix no. 2)
- Replication (reps) and number of cuttings per row plot (C) were as follows:  
 C1 - 2 reps of 8 C + 1 rep of 3 C; C2 - 1 rep of 8 C + 1 rep of 7 C; C3 - 1 rep of 8 C + 1 rep of 6 C; C4 - 1 rep of 7 C; C5 - 1 rep of 8 C + 1 rep of 6 C; C6 - 1 rep of 8 C + 1 rep of 3 C; C7 - 1 rep of 8 C + 1 rep of 6 C; C8 - 2 reps of 8 C; C9 - 1 rep of 8 C + 1 rep of 3 C; C10 - 3 reps of 8 C; C11 - 2 reps of 8 C + 1 rep of 4 C.

These trials were set up and assessed in Durban as described in section 5.2.4, with the exception that the cuttings were inoculated by either dipping in the bacterial cocktail suspensions (T1 and T2) for at least 5 to 10 seconds or by dipping into a hormone powder (Seradix No. 2 (T3)). The cuttings were maintained in a shade tunnel at ambient temperature under mist irrigation (10 seconds on / 15 minutes off during the day and 10 seconds on / 30 minutes off during the night) for the duration

of the propagation period (60 days).

#### 5.2.8 Statistical Analysis of Data

For all trials, survival and rooting percentages were based on plot means and the number of roots and dry mass were based on the number of cuttings that had survived and formed roots. Survival percentage and rooted percentage data were angular-transformed before analysis. The SAS, two-way (clone x treatment) Analysis of Variance (ANOVA) was conducted for each of the transformed parameters and multiple comparisons across treatment and clone combinations achieved by Duncan's Multiple Range Test (DUNCAN, 1975; MILLER, 1981) unless otherwise stated. In Trial 3 the two-way ANOVA from the SAS General Linear Model Procedure (GLM) were used to analyse the data. The survival and rooting percentages were also based on plot means. The number of roots and dry mass were based on the number of cuttings that had survived and formed roots. Comparisons across treatment and clone were achieved by REGWQ Multiple Range Test (RYAN, 1959; RYAN, 1960; GABRIEL, 1978; WELSCH, 1977). Comparisons across treatment and clone was achieved by the Tukey Test (ANON., 1987) for *E. grandis* clone trials 4 and 5 and by the Least Significant Difference (LSD) Test (ANON., 1987) for *Eucalyptus* hybrid clone trials 6 and 7.

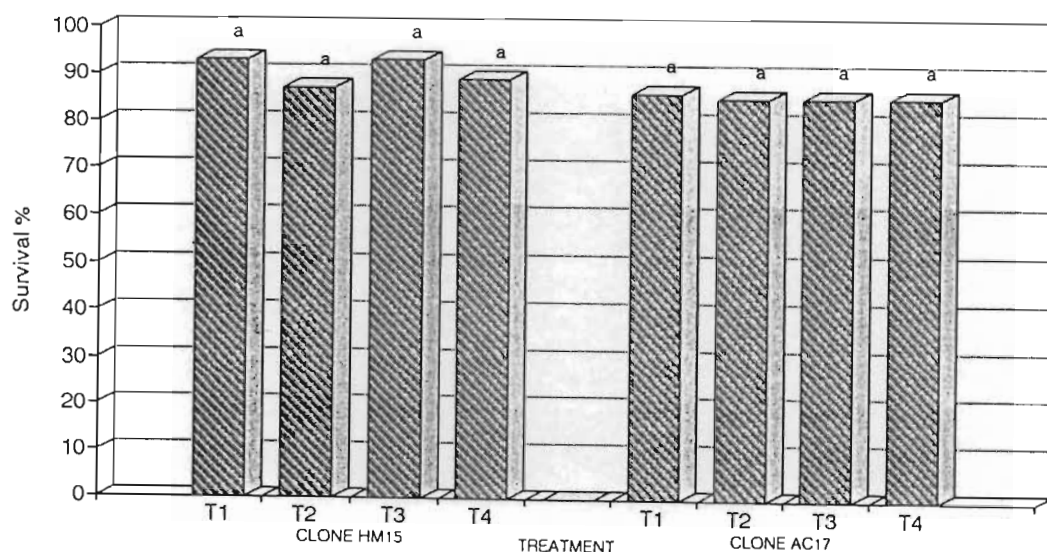
Significant differences between clones / treatments are indicated with an alphabetic letter. Groups of means with the same letter or which are overlapped with the same letter are not significantly different at the  $P = 0.05$  level based on either the Duncan's or the REGWQ Multiple Range Tests or the Tukey test, or at the  $P = 0.1$  level based on the LSD test. Non-overlapping groups with differing letters are significantly different at these levels.

### 5.3 RESULTS

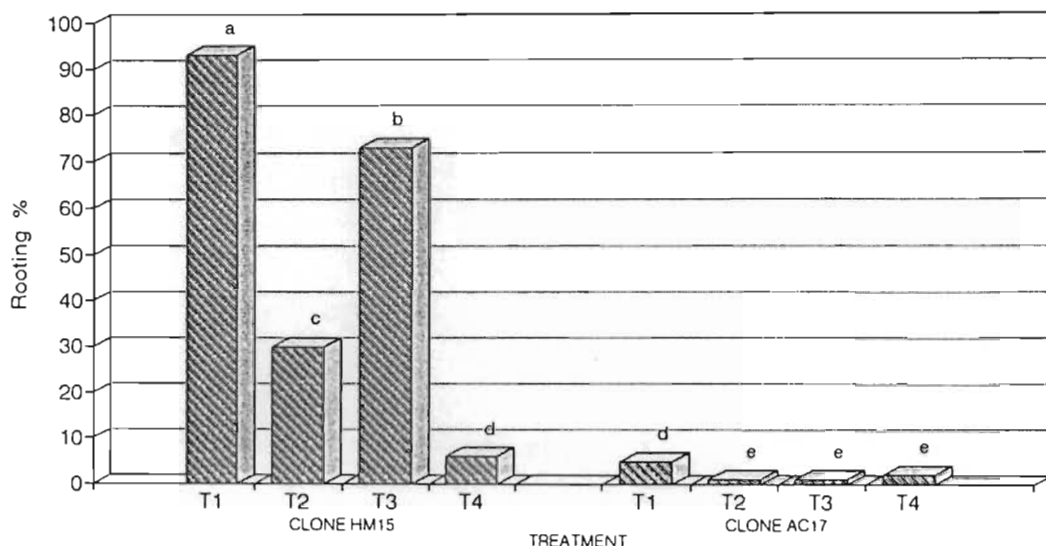
#### 5.3.1 Trial 1: *In Vivo A. rhizogenes*-Mediated Rooting of Cutting of Two *E. globulus* Clones.

Auxin treatment is not used for the commercial propagation of *E. globulus* as it was found not to improve rooting of this species. In some instances it reduced the rooting ability of certain *E. globulus* clones (Dr Philip J. Wilson, Personal communication). Hence auxin treatment was not used in this trial. The inherent rooting abilities of two *E. globulus* clones, HM15 and AC17 were compared to those induced by three *A. rhizogenes* strains. Survival of both clones under nursery conditions was good for all treatments, ranging from 85 to 93 %. The *A. rhizogenes* treatments did not affect

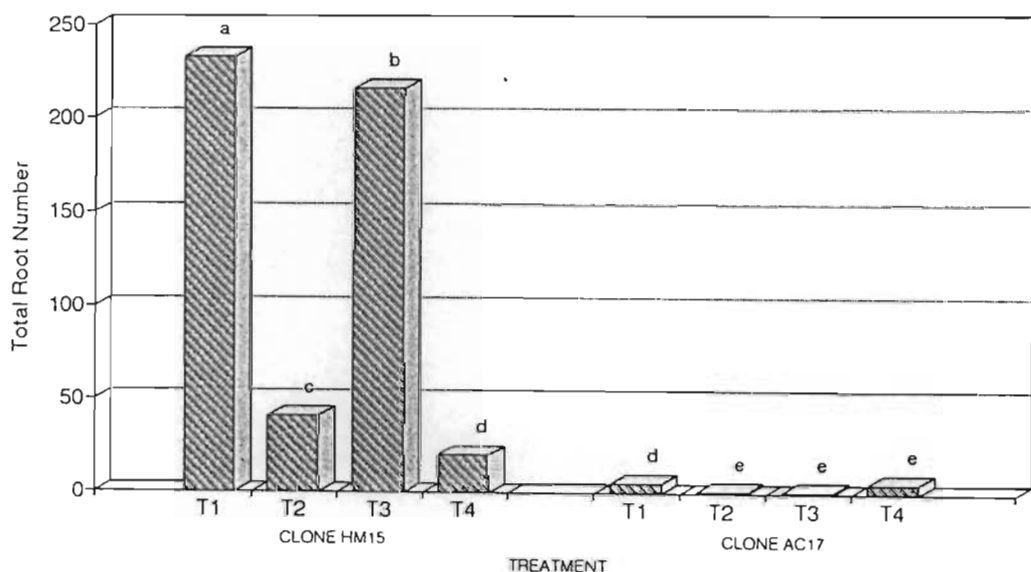
survival in either clone (Figure 55). The rooting ability of *E. globulus* AC17 was low and no strain of the bacterium had any effect. After treatment with *A. rhizogenes* strains R1601 and LBA9402, the rooting ability of clone HM15 was however, markedly increased, by 65 % and 86 % respectively . The total root number induced by these strains was at least 10 fold higher than that of the control. *A. rhizogenes* TR8,3 was less effective, but compared to the control (inherent rooting ability) still significantly increased the rooting percentage by 25 % (Figure 56). Root number was increased 2 fold (Figure 57).



**Figure 55.** Survival (%) of stem cuttings of two *E. globulus* clones, HM15 and AC17, 39 days after no treatment (T4) or treatment with one of three strains of *A. rhizogenes* LBA9402 (T1), R1601 (T2) and TR8,3 (T3). Based on the Duncan's Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.



**Figure 56.** Rooting ability (%) of stem cuttings of two *E. globulus* clones, HM15 and AC17, 39 days after no treatment (T4) or treatment with one of three strains of *A. rhizogenes* LBA9402 (T1), R1601 (T2) and TR8,3 (T3). Based on the Duncan's Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.



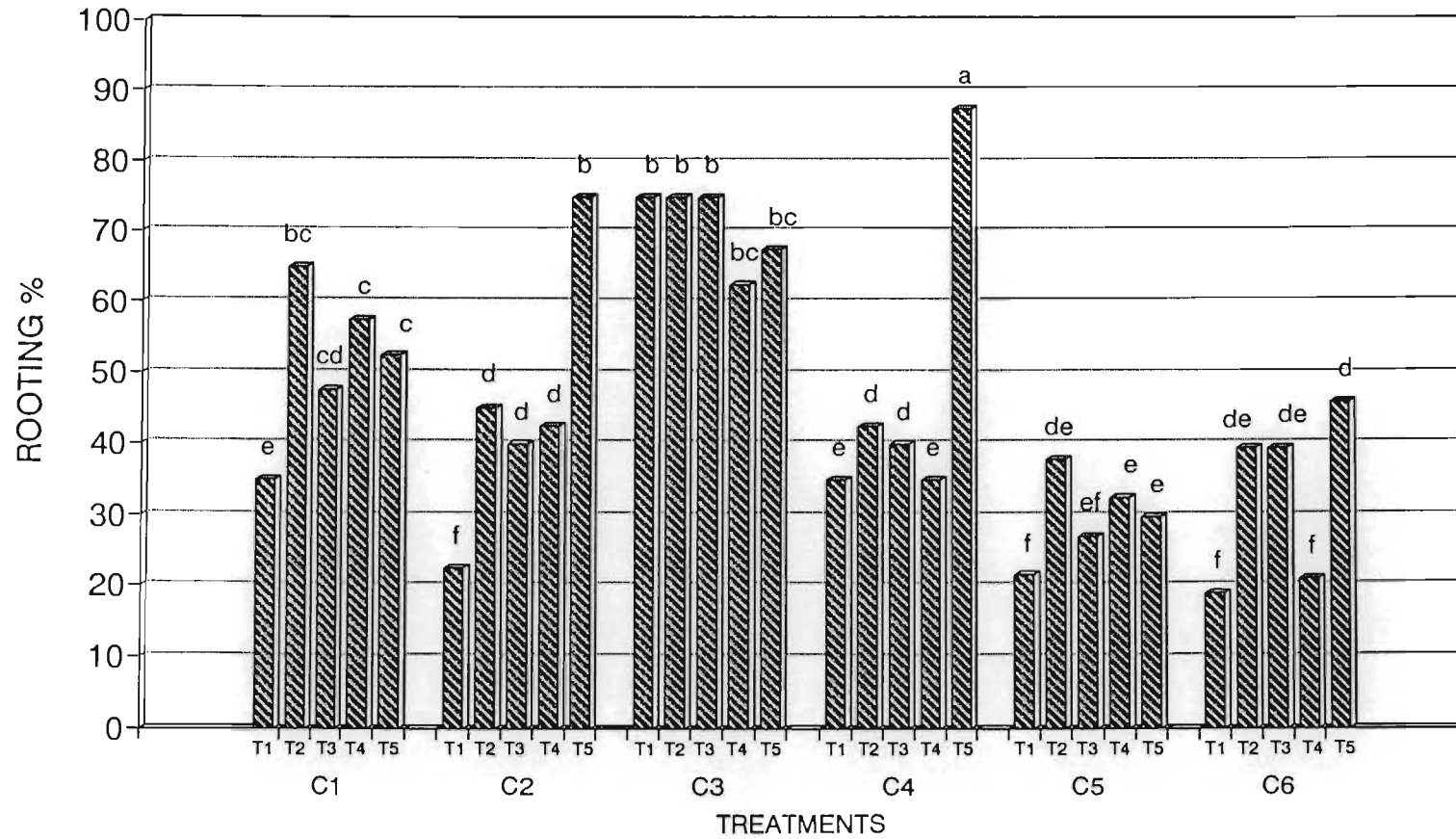
**Figure 57.** Total number of roots on stem cuttings of two *E. globulus* clones, HM15 and AC17, 39 days after no treatment (T4) or treatment with one of three strains of *A. rhizogenes* LBA9402 (T1), R1601 (T2) and TR8,3 (T3). Based on the Duncan's Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

### 5.3.2 Trial 2: *In Vivo A. rhizogenes*-Mediated Rooting of Cuttings of Six *E. grandis* Clones on Vermiculite and Peat:Perlite Media

Seradix (No. 2) is used extensively for the commercial propagation of *Eucalyptus* clones in South Africa and was therefore used as the positive control treatment in all cutting transformation trials.

The inherent rooting abilities of six *E. grandis* clones (see Table 16, Trial 2) were compared to rooting induced by auxin and by three *A. rhizogenes* strains (see section 5.2.4) on two different media: vermiculite (used for propagating cuttings in commercial nurseries in South Africa) and peat:perlite (1:1) (used in commercial nurseries in Portugal).

The inherent rooting abilities (T1) of the six *E. grandis* clones on vermiculite medium was low, ranging from 18 % for SGR124 (C6) to 35 % for SGR052 (C1). Seradix (T5) significantly enhanced rooting of all the clones except SGR096 (C3) (Figure 58). Although the Seradix (T5)-induced rooting ability of this clone (68 %), was slightly lower than its inherent rooting ability (T1) (75 %), the difference was not significant. The *A. rhizogenes* LBA9402 treatment (T2) significantly enhanced rooting of all clones, except clone C3, compared to their inherent rooting abilities (T1) (Figure 58). In the case of *E. grandis* SGR052 (C1) this bacterial strain

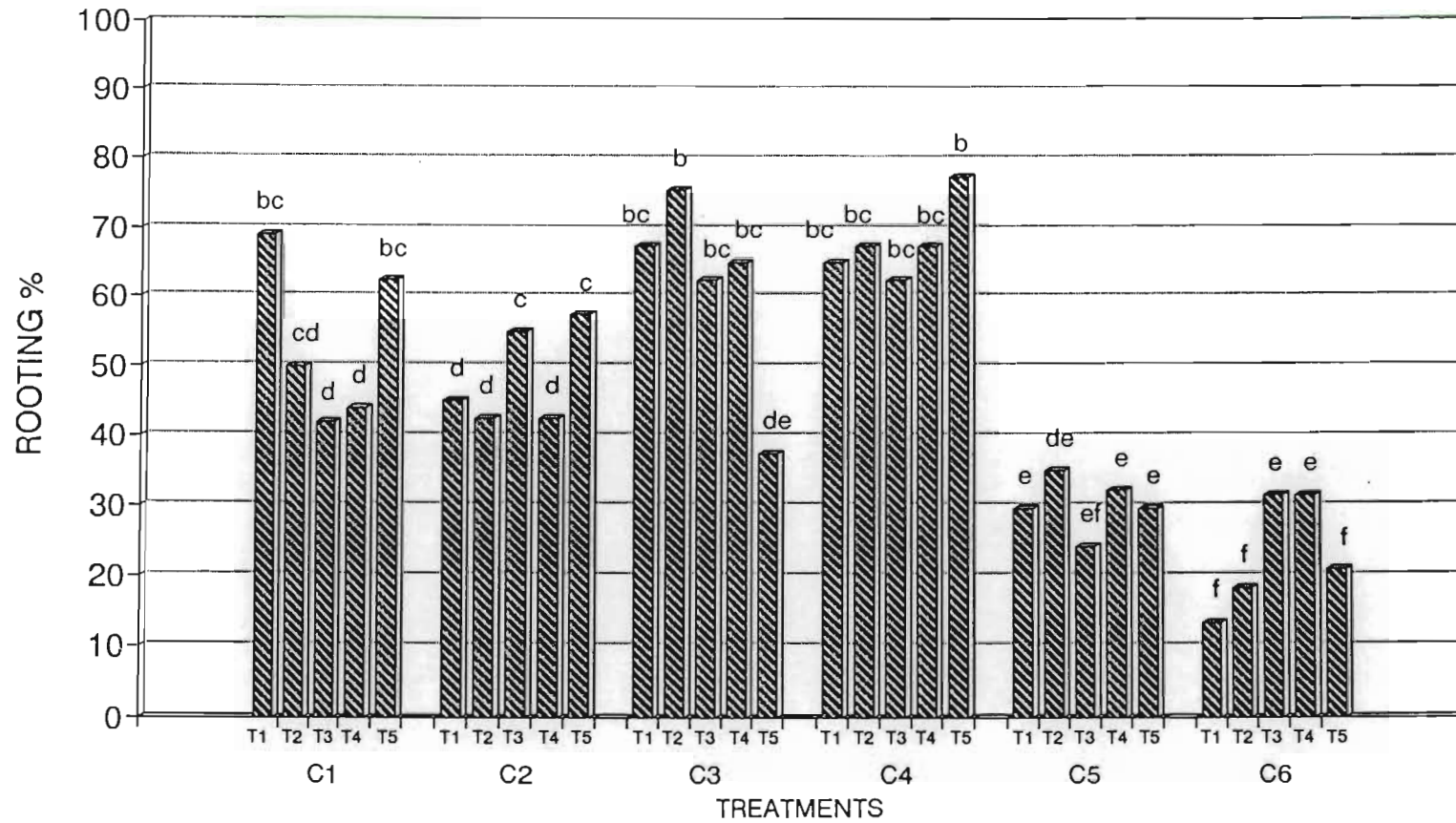


**Figure 58.** Rooting ability (%) of stem cuttings of six *E. grandis* clones (see Table 16, Trial 2) in vermiculite, after no treatment, treatment with one of three strains of *A. rhizogenes* or treatment with Seradix (see section 5.2.4). Based on the Duncan's Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

produced the best rooting (65 %) although it was not significantly different from that achieved with the other two bacterial strains (T3 and T4) (48 and 58 %, respectively) or Seradix (T5) (52 %). The same trend was observed for clone SGR171 (C5). There was no significant difference in the rooting percentages obtained with the three bacterial strains (T2 to T4) on SGR132 (C2), however, they were significantly lower (up to 35 %) than that obtained with Seradix (T5). For clone SGR096 (C3) none of the treatments was able to enhance rooting. The rooting ability of clone SGR050 (C4) was not improved by *A. rhizogenes* TR8,3 (T4) treatment compared to its inherent rooting ability. However, this clones rooting ability was significantly enhanced by the other bacterial treatments, T2 and T3. Seradix (T5) was however, the most effective treatment, resulting in 87.5 % rooting. Unlike *A. rhizogenes* LBA9402 (T2) and R1601 (T3), TR8,3 (T4) was ineffective in enhancing rooting of SGR124 (C6). The rooting abilities induced by the former two bacterial strains were however not significantly different from those obtained with Seradix (T5) (Figure 58).

The rooting abilities of the six *E. grandis* clones in peat:perlite medium yielded very different results (Figure 59) from those obtained when vermiculite was used as the rooting substrate. The inherent rooting ability (T1) of the clones ranged from 14 % for SGR124 (C6) to 78 % for SGR052 (C1). No significant difference was found in rooting of clone SGR052 (C1) with *A. rhizogenes* strains TR8,3 (T4) and R1601 (T3). However these were 25 % and 28 % lower than the inherent rooting ability

(T1) of this clone. In addition they were significantly lower than that achieved with Seradix (T5). Rooting by *A. rhizogenes* LBA9402 (T2) was 12 and 19 % lower than the Seradix-induced rooting (T5) and the inherent rooting abilities (T1) of clone SGR052 (C1), but the differences were not significant. Rooting induced by Seradix (T5) was also lower (7 %) than this clone's inherent rooting ability (T1) but here again the difference was not significant (Figure 59). Seradix (T5) and *A. rhizogenes* R1601 (T3) treatment produced significantly better rooting than the other treatments for SGR132 (C2). The other two bacterial strains (T2 and T4) were however, ineffective as rooting percentages achieved were similar to the inherent rooting ability (T1). *A. rhizogenes* LBA9402 (T2) induced the highest rooting percentage (75 %) in *E. grandis* clone SGR096 (C3), although it was not significantly better than that achieved with the other two bacterial strains (T3 and T4) or the inherent rooting ability (T1) of this clone. Seradix (T5) however, appears to significantly reduce rooting of this clone on peat:perlite medium by up to 47 %. Rooting of SGR050 (C4) was best with Seradix treatment (T5) although this was not significantly different from any of the other treatments. Although treatment of SGR175 (C5) with *A. rhizogenes* LBA9402 (T2) resulted in a slightly higher mean rooting percentage, no significant improvement in rooting of this clone was achieved with any of the treatments (Figure 59). *A. rhizogenes* R1601 (T3) and TR8,3 (T4) significantly enhanced rooting ability of clone SGR124 (C6) compared to the other treatments (T2 and T5) and to this clone's inherent rooting ability (T1).



**Figure 59.** Rooting ability (%) of stem cuttings of six *E. grandis* clones (see Table 16, Trial 2) in peat : perlite (1:1), after no treatment, treatment with one of three strains of *A. rhizogenes* or treatment with Seradix (see section 5.2.4). Based on the Duncan's Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

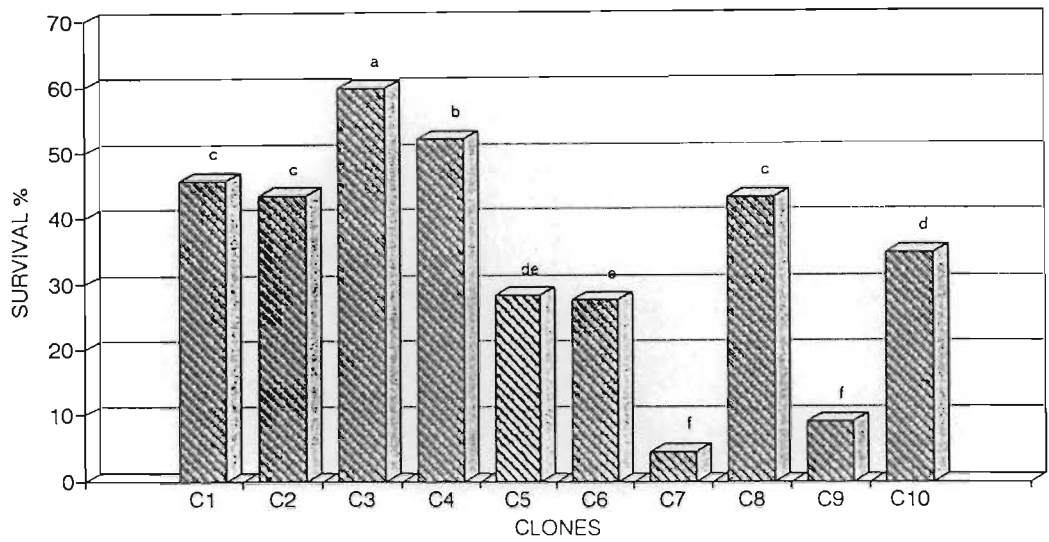
The inherent rooting abilities (T1) of clones SGR052 (C1), SGR132 (C2), SGR050 (C4) and SGR171 (C5) were significantly higher by 34, 23, 30 and 9 %, respectively on peat:perlite medium than vermiculite medium (Figures 56 and 57). Seradix-induced rooting (T5) was however, generally poorer on the peat:perlite medium, with rooting percentages of clones C2 (SGR132), C3 (SGR096), C4 (SGR050) and C6 (SGR124) significantly decreased, by 18, 31, 10 and 15 % compared to rooting percentages achieved with Seradix (T5) on vermiculite medium.

However, rooting of clones C1 (SGR052) and C5 (SGR171) in these media was not significantly different. Seradix-induced rooting (T5) of clone C3 (SGR096) in peat:perlite medium was significantly lower than the inherent rooting ability (T1) of this clone. *A. rhizogenes* strains had either little or no effect on improving rooting of all the clones propagated on peat:perlite medium, with the exception of clone C6 (SGR124). The rooting of this clone was significantly improved by up to 19 %, after inoculation with *A. rhizogenes* strains R1601 (T3) and TR8,3 (T4), compared to the other treatments. Strains R1601 (T3) and TR8,3 (T4) significantly reduced the rooting ability of clone C1 (SGR052) compared to this clone's inherent rooting ability on peat:perlite medium.

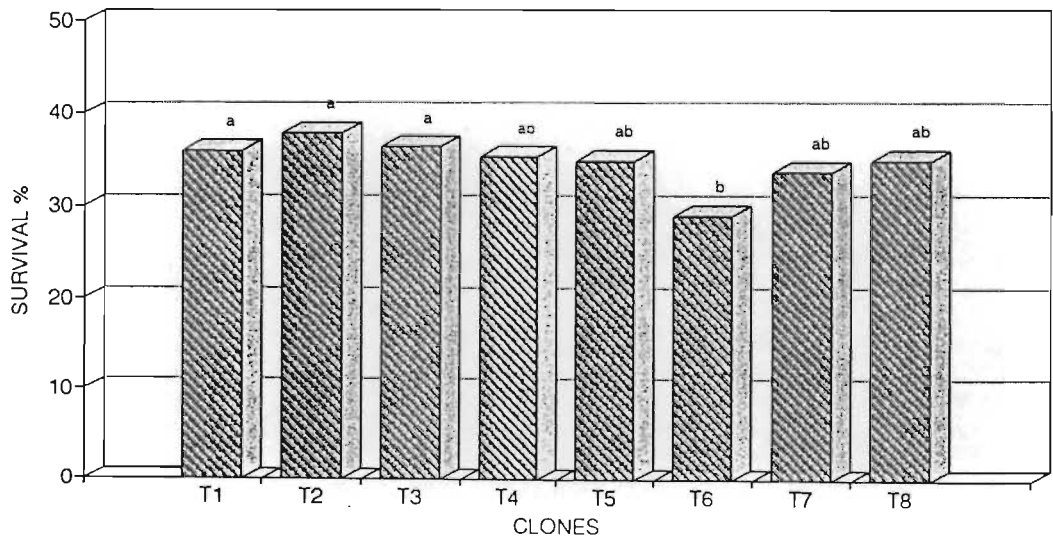
### 5.3.3 Trial 3: *In Vivo A. rhizogenes*-Mediated Rooting of Cuttings of Ten *E. grandis* Clones on Vermiculite Medium

The inherent rooting abilities of ten *E. grandis* clones (see Table 16) were compared to those induced by auxin and six *A. rhizogenes* strains (see section 5.2.5). The survival of cuttings in this trial was poor with only 34.8 % of the cuttings surviving and of these only 12.3 % rooted. As a result of the low survival rates it was not possible to make a comparison of treatment/clone interactions as was envisaged at the start of this trial. However, a comparison of survival (%), rooting ability (%), average root number and root dry mass of the clones and the treatments was made. There were significant differences between the ten clones and between the eight treatments for these measurements. Rankings of clone means and treatment means are detailed in Figures 60 to 67.

*E. grandis* clone SGR071 (C3) had the best survival rate (59.7%) and it was significantly different from the other clones. SGR003 (C4) was second best with 52.0%, also significantly different. Treatments *A. rhizogenes* LBA9402 (T2), *A. rhizogenes* R1601 (T3) and untreated control (T1) had the best survival rates namely 37.9%, 36.4% and 36.0%, respectively. They were not significantly different from each other, but were significantly better than *A. tumefaciens* LBA4404(pGA643-12) (T6) which had the worst survival rate of 29% (Figures 60 and 61).

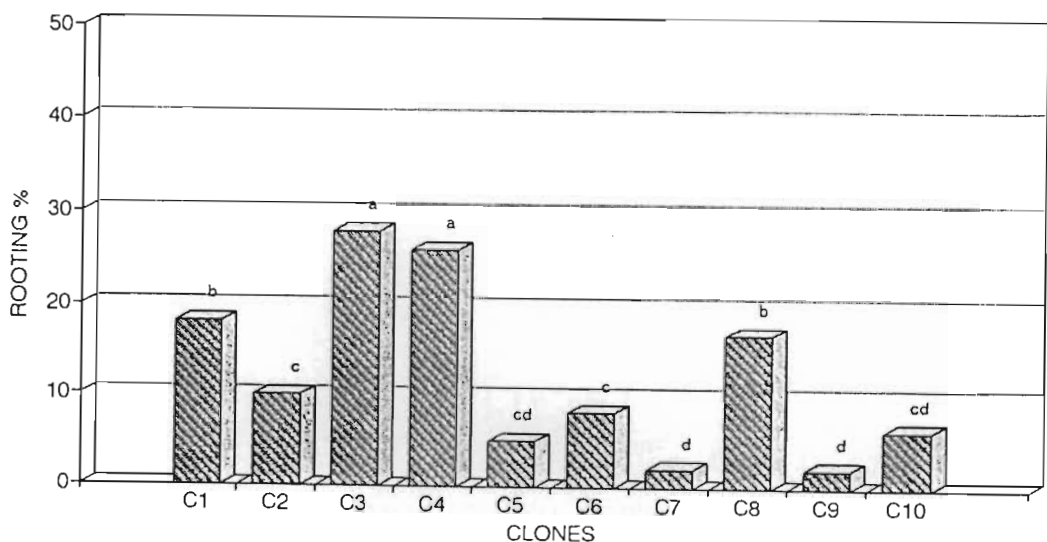


**Figure 60.** Survival rates (%) of the ten *E. grandis* clones C1 to C10 (see Table 16, Trial 3). Based on the REGWQ Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

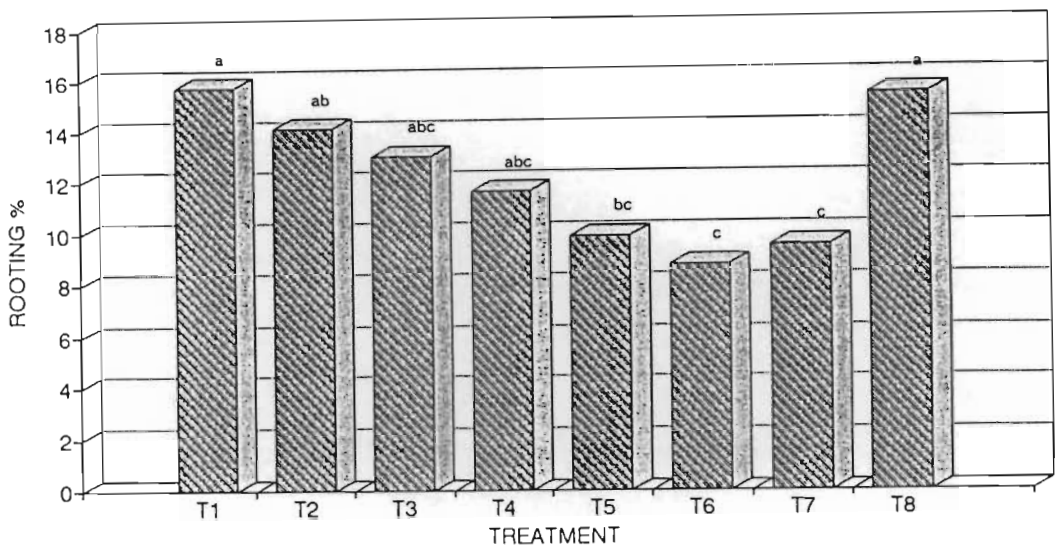


**Figure 61.** Survival rates (%) of the eight treatments T1 to T8 (see section 5.2.5). Based on the REGWQ Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

Based on rooting ability, *E. grandis* clones SGR071 (C3) (27.9 %) and SGR003 (C4) (25.9 %) again gave the best results. The results for these clones were not significantly different from each other, but they significantly out-performed SGR030 (C1) (18.3 %) and SGR096 (C8) (16.9 %). The control treatments T1 (untreated) and T8 (Seradix) gave the best rooting percentages (15.8 % and 15.4 % respectively) but did not differ significantly from T2 (*A. rhizogenes* LBA9402), T3 (*A. rhizogenes* R1601), and T4 (*A. rhizogenes* TR8,3). They gave significantly better rooting than treatments T7 (*A. rhizogenes* A4) (9.5 %) and T6 (*A. tumefaciens* LBA4404(pGA643-12) (8.8 %) (Figures 62 and 63).

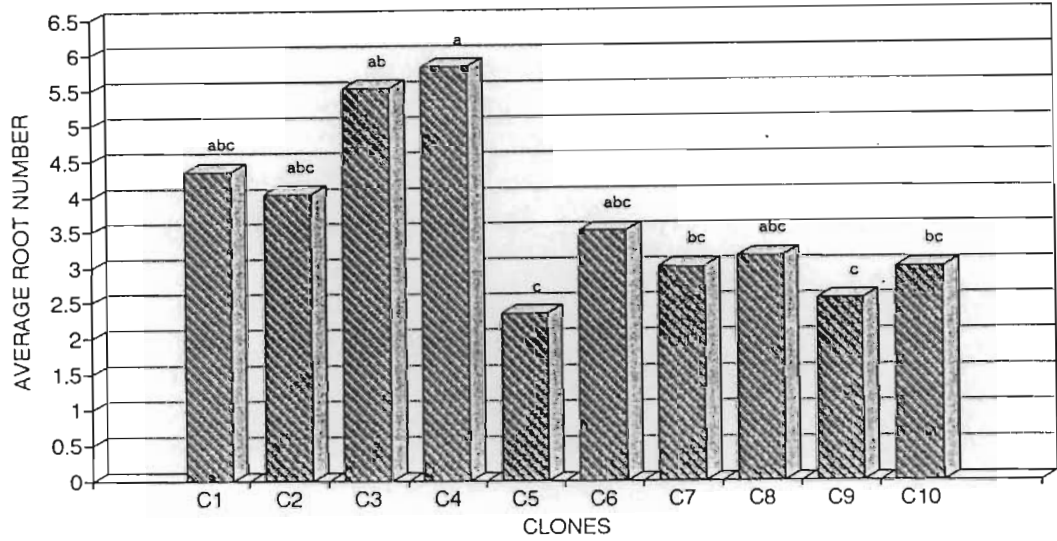


**Figure 62.** Rooting abilities (%) of the ten *E. grandis* clones C1 to C10 (see Table 16, Trial 3). Based on the REGWQ Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

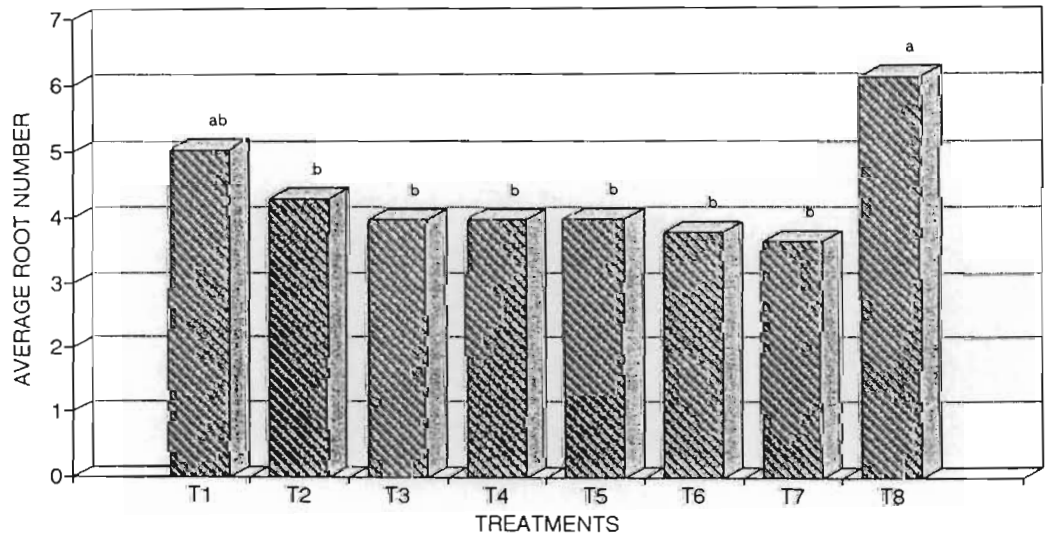


**Figure 63.** Rooting abilities (%) of the eight treatments T1 to T8 (see section 5.2.5). Based on the REGWQ Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

*E. grandis* SGR003 (C4) and SGR071 (C3) gave the highest average number of roots per clone (5.8 and 5.5 respectively), but results did not significantly differ from SGR030 (C1), SGR034 (C2), SGR125 (C6) and SGR096 (C8). Clones SGR028 (C5) and SGR103 (C9) produced significantly lower numbers of roots than the other clones. Control treatments T1 (untreated) and T8 (Seradix) again yielded the highest number of roots (6.1 and 5.0 respectively). Seradix treatment (T8) was significantly better than the rest of the treatments with the exception of T1 (untreated control) (Figures 64 and 65).

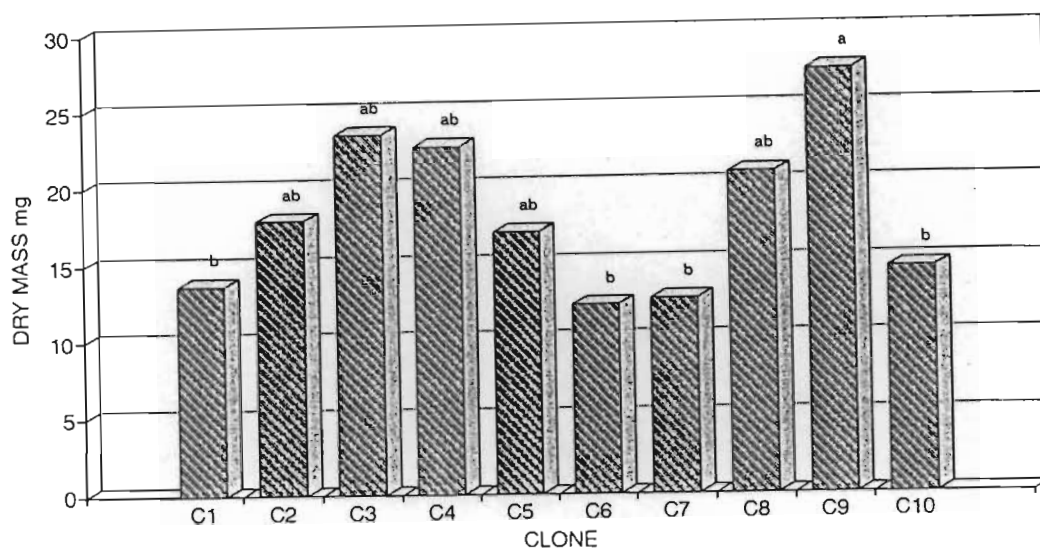


**Figure 64.** Average number of roots per surviving rooted cutting for each of the ten *E. grandis* clones C1 to C10 (see Table 16, Trail 3). Based on the REGWQ Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

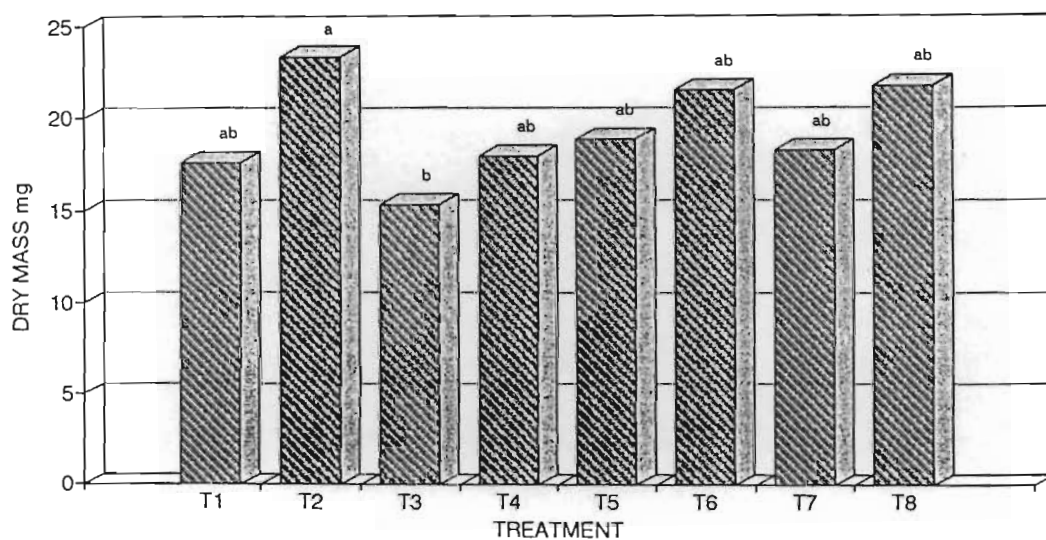


**Figure 65.** Average number of roots per surviving rooted cutting for each of the eight treatments T1 to T8 (see section 5.2.5). Based on the REGWQ Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

*E. grandis* clone SGR103 (C9) had the highest root dry mass (27.4 mg) but this did not significantly differ from SGR071 (C3) (23.4 mg), SGR003 (C4) (22.6 mg), SGR096 (C8) (20.6 mg), SGR034 (C2) (17.8 mg) or SGR028 (C5) (16.9 mg). One should not be misled by clone SGR103 (C9); it had fewer but bigger roots, but most importantly it had the worst rooting percentage and the second worst survival rate. *E. grandis* clones SGR071 (C3) and SGR003 (C4) therefore had the best rooting abilities compared to the other clones tested. Treatment with *A. rhizogenes* LBA9402 (T2) yielded the highest root dry mass of 23.3 mg, which was significantly higher than that achieved by treatment with *A. rhizogenes* R1601 (T3) (15.4 mg). However, the mass of roots produced by the *A. rhizogenes* LBA9402 (T2) treatment was not significantly better than for the other treatments. The Seradix treatment (T8) resulted in the second highest root dry mass while the untreated control (T1) gave the second lowest value (Figures 66 and 67).



**Figure 66.** Average dry mass of roots from cuttings of each of the ten *E. grandis* clones C1 to C10 (see Table 16, Trial 3). Based on the REGWQ Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.



**Figure 67.** Average dry mass of roots from cuttings of each of the eight treatments T1 to T8 (see section 5.2.5). Based on the REGWQ Multiple Range Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

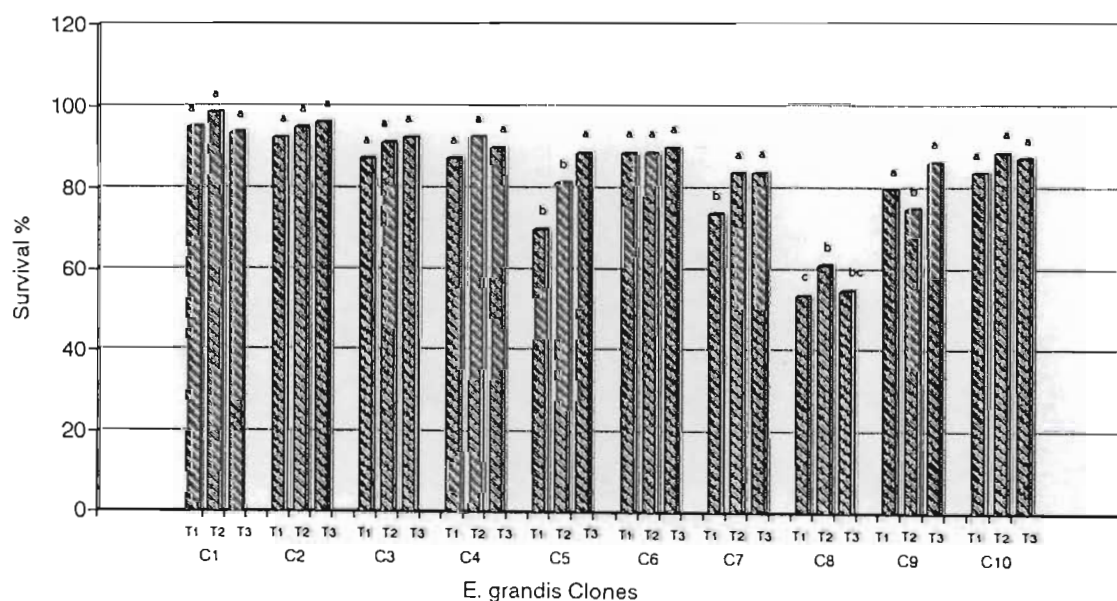
#### 5.3.4 Trials 4 and 5: *In Vivo* Rooting of Cuttings of Ten *E. grandis* Clones by Two *Agrobacterium* Cocktails

The Seradix-induced rooting abilities of ten *E. grandis* clones (see Table 16) were compared to rooting induced by two bacterial cocktails (see section 5.2.1) under commercial nursery conditions at the SAFCOL nursery at Frankfort. Two trials were set up at a two month interval from October 1993.

##### **Trial 4 :** October 1993 to December 1993

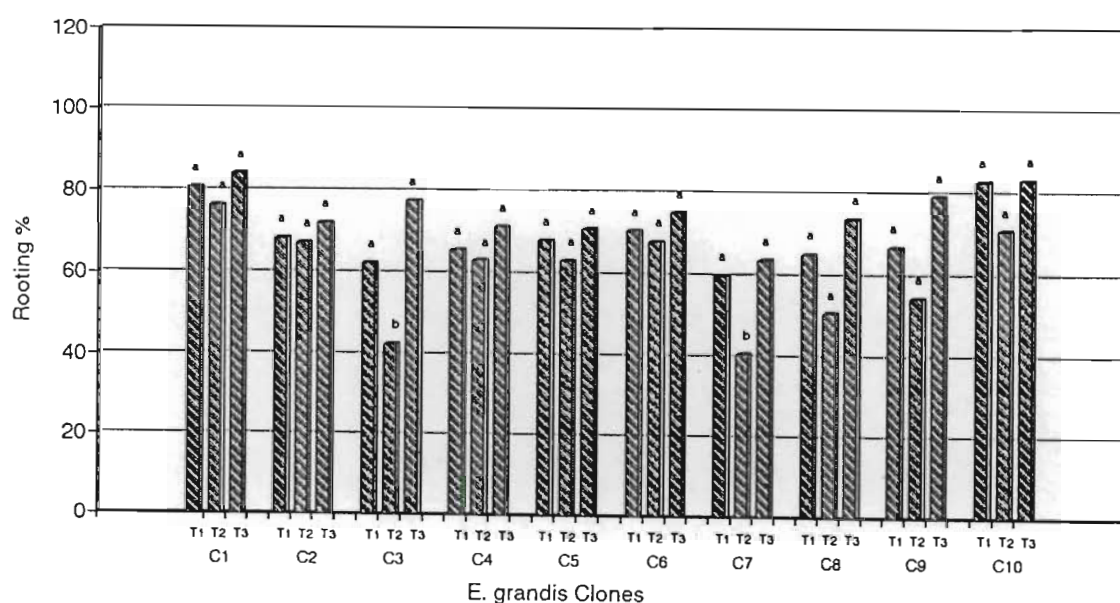
With the exception of clone SGR096 (C8) survival of all the clones was good ranging from 70 to 100 %. No significant differences in survival, irrespective of treatment, were found for clones SGR030 (C1), SGR034 (C2), SGR071 (C3), SGR003 (C4), SGR125 (C6) and SGR109 (C10) (Figure 68). In the case of clone C5 (SGR028) both bacterial cocktails (T1 and T2) significantly reduced the survival rate of this clone by 20 and 9 % respectively, compared to that achieved after Seradix treatment (T3). No significant difference in survival was found in clone C7 after treatment with bacterial cocktail T2 and Seradix (T3), however, a significant decrease (10 %) in survival was observed after treatment of this clone with bacterial cocktail T1. In the case of clone SGR096 (C8) survival rate ranged from 54 to 61 % depending on the treatment, however, there was no significant difference between treatments T1 and T3 and T2 and T3 for this clone. The difference in survival rates

of clone C8 after treatments T2 and T1 were however, significant, with a 7 % increase in survival being achieved with treatment T2. Finally, survival of clone C9 (SGR103) after bacterial cocktail treatment T1 was not significantly different from that achieved after Seradix treatment (T3). Bacterial cocktail T2 however, significantly reduced survival by up to 10 % compared to the other treatments. These results suggest that the bacterial cocktails could have a slight adverse affect on certain *E. grandis* clones under the SAFCOL nursery conditions used at Frankfort.



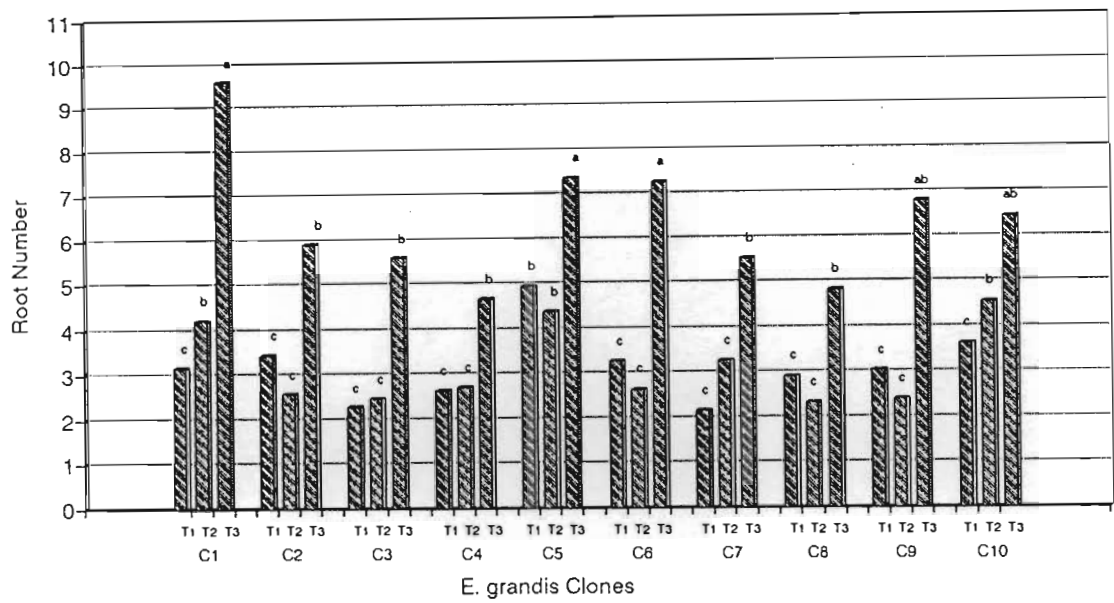
**Figure 68.** Survival (%) of stem cuttings of ten *E. grandis* clones (see Table 16, Trial 4), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1) Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

No significant differences in the rooting abilities as a result of the treatments was obtained for clones SGR030 (C1), SGR034 (C2), SGR003 (C4), SGR028 (C5), SGR125 (C6), SGR096 (C8), SGR063 (C9) and SGR109 (C10) (Figure 69). In the case of clone SGR071 (C3), Seradix induced rooting (T3) was 16 % better than that achieved after treatment with bacterial cocktail T1, however, this difference was not significant. The rooting ability of clone SGR071 (C3) was significantly reduced by up to 36 % after treatment with bacterial cocktail T2 compared to treatments T1 and T3. A similar trend was observed for clone SGR063 (C7). These results suggest that bacterial cocktail T2, comprising 5 bacterial strains and auxin could have an adverse effect on rooting of *E. grandis* clones C3 and C7. Why this should be is not known.



**Figure 69.** Rooting ability (%) of stem cuttings of ten *E. grandis* clones (see Table 16, Trial 4), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

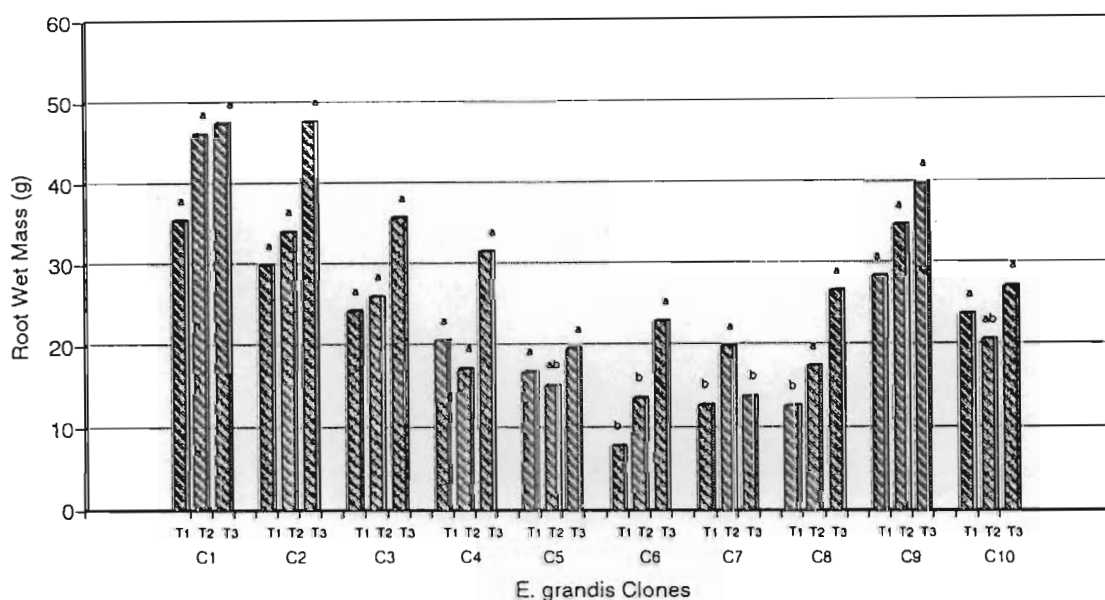
The Seradix treatment (T3) resulted in the greatest root number per cutting for all the clones tested in this trial (Figure 70). No significant differences were observed in average root number per rooted cutting of clones SGR034 (C2), SGR071 (C3), SGR003 (C4), SGR028 (C5), SGR125 (C6), SGR063 (C7), SGR096 (C8) and SGR103 (C9) obtained after treatment of these clones with bacterial cocktails T1 and T2. However, for each clone, they were significantly lower than that obtained with Seradix, suggesting that the bacterial cocktails were not able to stimulate an increase in root number for these clones to the same extent as Seradix. Bacterial cocktail T2 however, yielded a significantly higher number of roots (1.3 fold) than bacterial cocktail T1 for clones SGR030 (C1) and SGR109 (C10). A significant sequential increase in root number was observed across treatments T1 to T3 inclusive for clone C1. Although a similar trend was observed for clone C10 the increase in root number after treatments T2 and T3 was not significant.



**Figure 70.** Average number of roots on rooted stem cuttings of ten *E. grandis* clones (see Table 16, Trial 4), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

The average root wet masses of clones C1, C2, C3, C4, C5, C9 and C10 were unaffected by the three treatments, i.e., no significant differences were found between treatments within a clone (Figure 71). The root wet mass of clone C6 after Seradix treatment was significantly greater by up to 2.8 fold compared to that obtained after treatment with bacterial cocktails T1 and T2. In the case of clone C7, there was no significant difference in root wet masses after treatments T1 and T3. However, treatment T2 significantly increased wet mass of this clone by up to 1.5

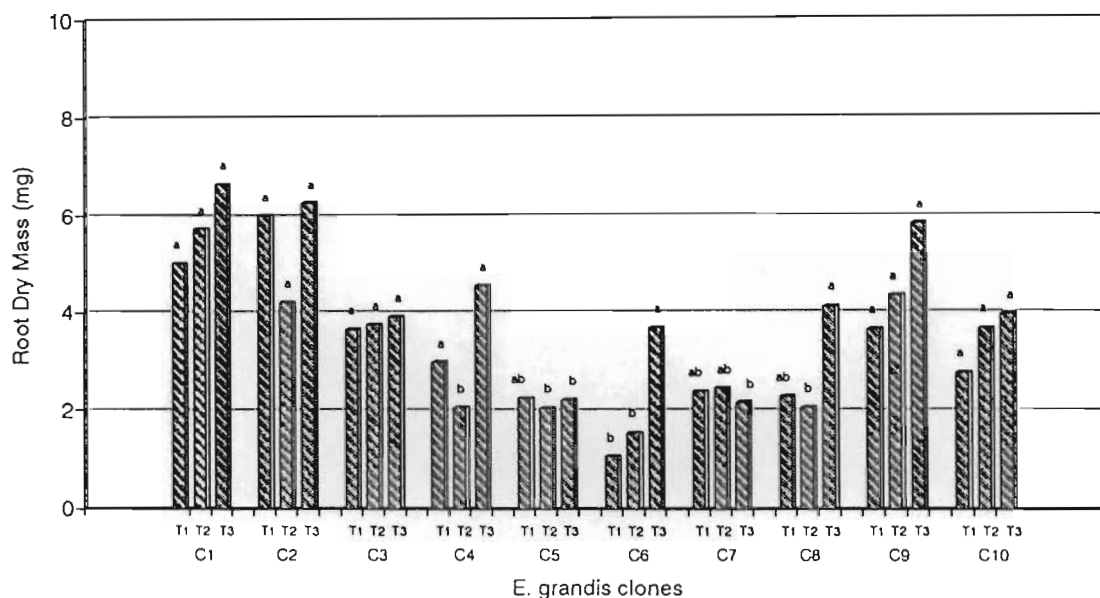
fold. Finally, no significant difference was found in the wet masses of roots arising after treatment of clone C8 with the bacterial cocktail T2 and Seradix T3. The wet mass of roots arising from this clone after treatment with bacterial cocktail T1 was however, up to 2.2 fold lower than that achieved with the other treatments.



**Figure 71.** Average wet mass of roots from cuttings of ten *E. grandis* clones (see Table 16, Trial 4), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

When the root dry mass data were considered (Figure 72), no significant differences in this parameter, irrespective of treatment, were detected for clones C1, C2, C3, C5, C7, C9 or C10. These results suggest that the bacterial treatments were able to

induce roots with similar dry masses to that achieved with Seradix. The dry masses of roots obtained after treatment of clone C4 with bacterial cocktail T1 and Seradix T3 were not significantly different from each other, however, they were significantly better, by up to 2.3 fold, than that obtained after treatment of this clone with bacterial cocktail T2. No significant difference was found in the average dry masses of roots of clone C6 after bacterial cocktail treatments T1 and T2. Treatment of this clone with Seradix (T3) however, significantly improved root dry mass compared to the other treatments. After treatment of clone C8 with bacterial cocktails T1 and T2, no significant difference was found in the resultant root dry masses. However, these results were significantly different (lower by up to 2 fold) from those achieved after Seradix treatment (T3). Seradix treatment yielded the greatest root mass for this clone.

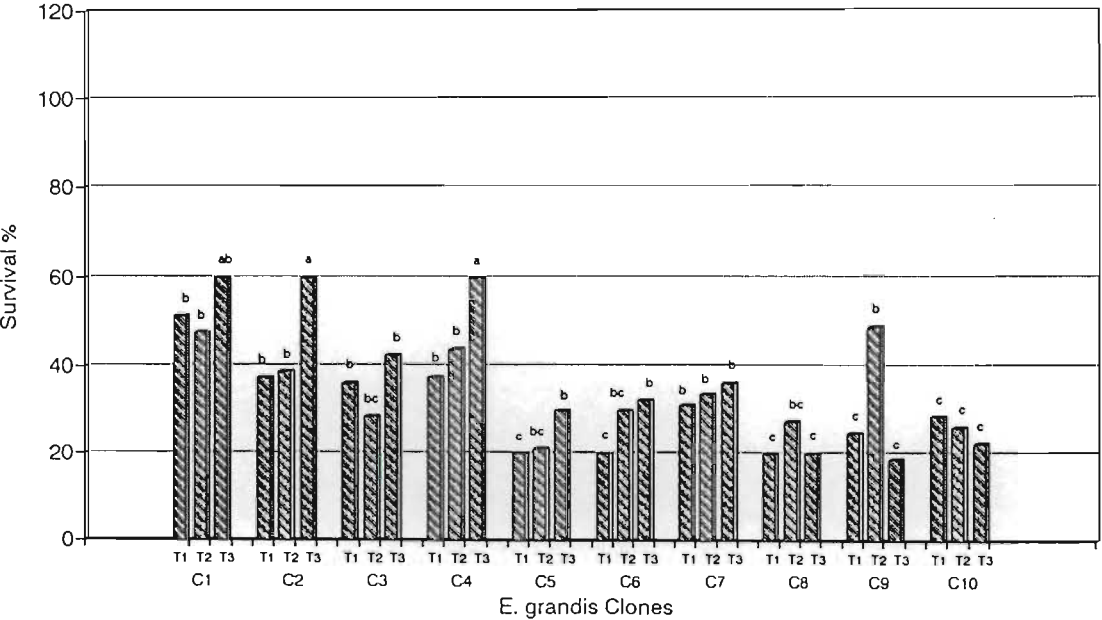


**Figure 72.** Average dry mass of roots from cuttings of ten *E. grandis* clones (see Table 16, Trial 4), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

#### Trial 5 : December 1993 to February 1994

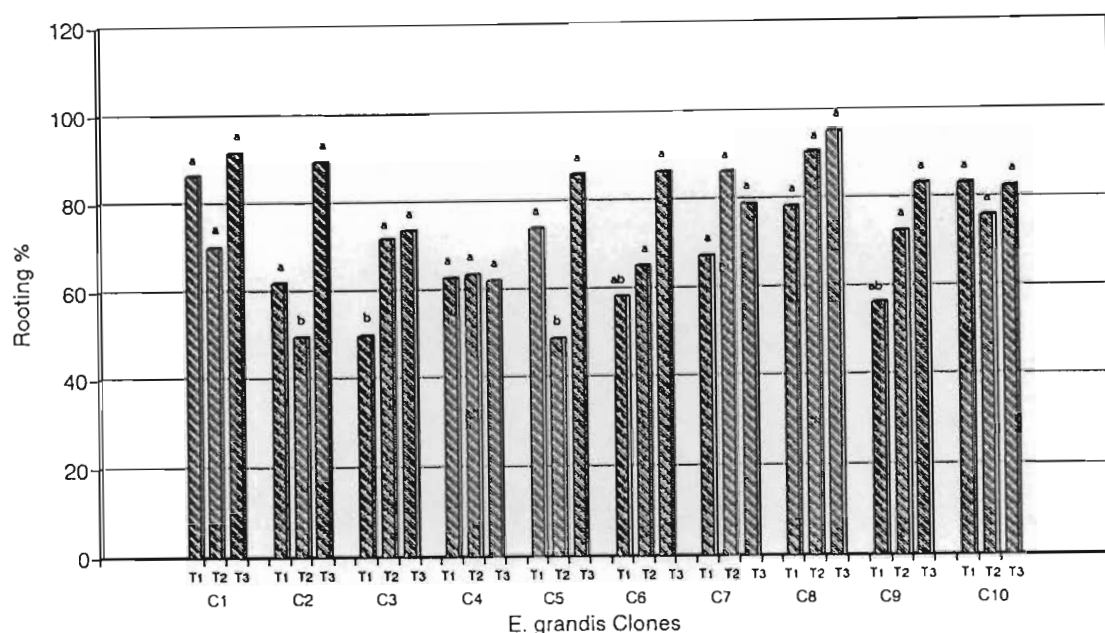
Survival rates of the ten *E. grandis* clones used in the trial established in December 1993 were extremely poor ranging from as low as 19 to 60 % (Figure 73). The main reason for this was a disease problem which was experienced at the SAFCOL nursery at Frankfort. The survival rates of clones SGR030 (C1), SGR071 (C3), SGR063 (C7), SGR096 (C8) and SGR109 (C10) were unaffected by the bacterial treatments, as no significant differences were observed in survival percentage

between the three treatments for these clones. The survival of clone SGR034 (C2) was significantly lower (22 %), after treatment with bacterial cocktails T1 and T2, than after treatment with Seradix (T3). In the case of clones SGR028 (C5) and SGR125 (C6), no significant difference in survival was found between treatments T1 and T2 and T2 and T3. However, survival of these clones after treatment T3 was significantly better (by up to 14 %) than after treatment T1. No significant difference was found in survival of clone C9 (SGR103) after treatments T1 and T3, however, treatment T2 significantly increased survival of this clone by up to 32 %.



**Figure 73.** Survival (%) of stem cuttings of ten *E. grandis* clones (see Table 16, Trial 5), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

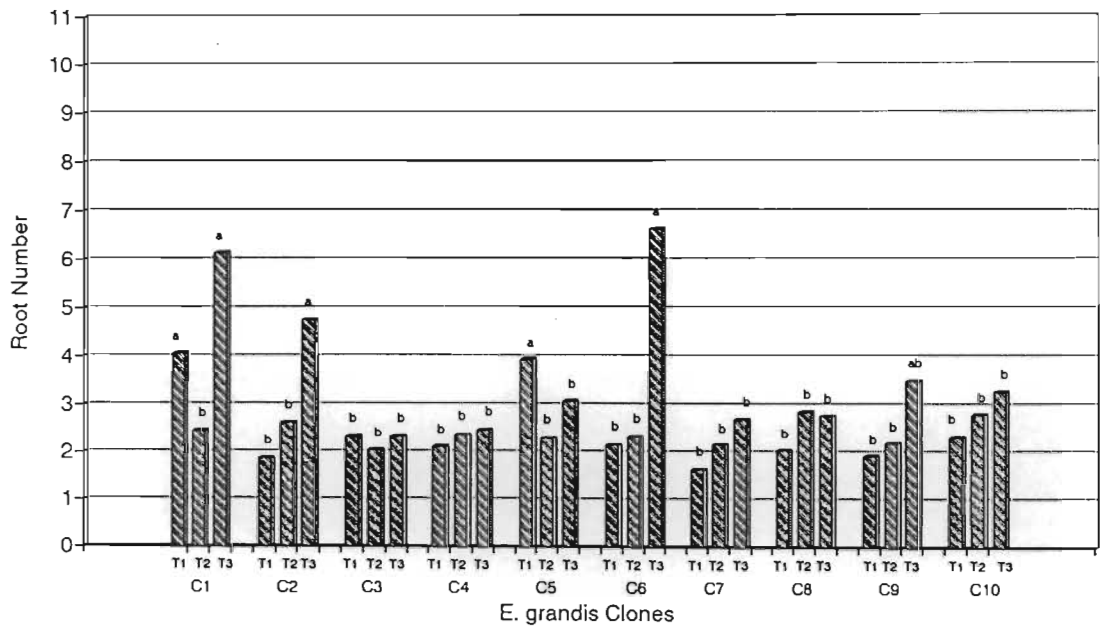
Rooting of the ten *E. grandis* clones ranged from 50 % to 98 % depending on the rooting treatment applied. No significant differences were observed in rooting ability of surviving cuttings of clones SGR030 (C1), SGR003 (C4), SGR125 (C6), SGR063 (C7), SGR096 (C8), SGR103 (C9) and SGR109 (C10), after treatment of these clones with the three rooting treatments (Figure 74). These results imply that all treatments could be effectively used to root these *E. grandis* clones. Although Seradix-induced rooting of clone SGR034 (C2) was 23 % higher than that achieved with bacterial cocktail T1, the difference was not significant. Both these treatments did however, yield significantly better rooting percentages (12 and 40 %, respectively) than bacterial cocktail T2. No significant difference was found in the rooting ability of clone SGR071 (C3) after treatment with bacterial cocktail T2 and Seradix (T3). Rooting of this clone after treatment with bacterial cocktail T1 was however, significantly lower by 25 % compared to the other two treatments. The rooting ability of clone SGR028 (C5) was also affected by the treatments, in that there was no significant difference between rooting achieved after treatments T1 and T3, however, rooting after treatment T2 was significantly lower (by up to 36 %) than that achieved with the other treatments.



**Figure 74.** Rooting ability (%) of stem cuttings of ten *E. grandis* clones (see Table 16, Trial 5), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

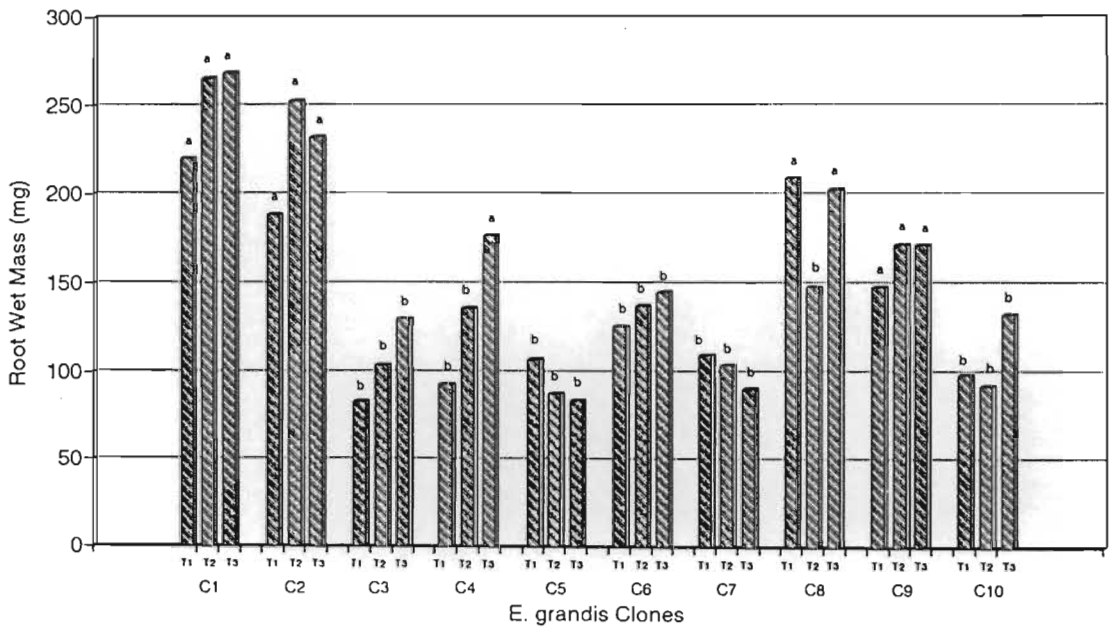
The average number of roots obtained on rooted cuttings of clone SGR030 (C1) after Seradix treatment (T3) was 1.5 fold greater than that obtained after bacterial cocktail treatment T1, however, this difference was not significant (Figure 75). The number of roots obtained after treatment T2 for this clone was significantly lower (by up to 2.5 fold) than that obtained after the other treatments. Seradix treatment (T3) of clone SGR034 (C2) yielded a significantly higher number of roots (up to 1.7 fold) per cutting than bacterial treatments T1 and T2, which were not significantly different from one another. None of the treatments significantly improved root

number on cuttings of clones SGR071 (C3), SGR003 (C4), SGR063 (C7), SGR096 (C8), SGR103 (C9) and SGR109 (C10). A similar trend was found for clone SGR125 (C6) with the exception that no significant difference in root number was found between treatments T1 and T2. The number of roots on cuttings of clone SGR028 (C5) after treatment with bacterial cocktail T1 were significantly greater (up to 1.8 fold) than that obtained after treatments T2 and T3, which were not significantly different from each other. The root number obtained after these treatments was however, significantly lower (approximately 3.1 fold) than that obtained after Seradix treatment (Figure 75).



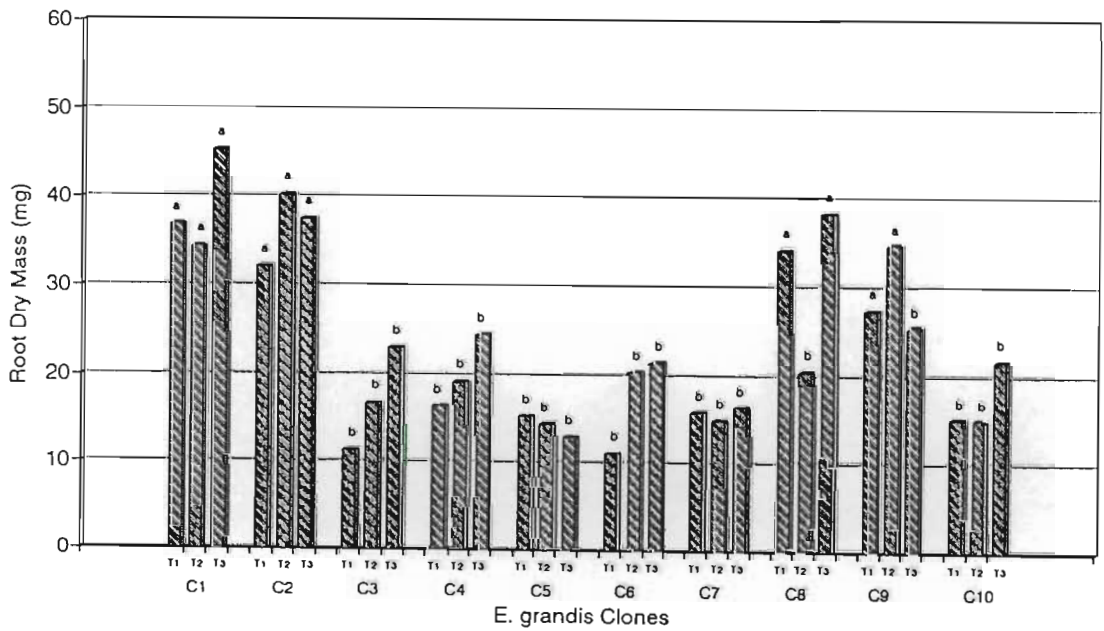
**Figure 75.** Average number of roots on rooted stem cuttings of ten *E. grandis* clones (see Table 16, Trial 5), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

No significant difference in root wet mass was found between the three treatments for *E. grandis* clones SGR030 (C1), SGR034 (C2), SGR071 (C3), SGR028 (C5), SGR125 (C6), SGR063 (C7), SGR103 (C9) and SGR109 (C10) (Figure 76). The root wet masses of cuttings of clone SGR003 (C4) after treatments T1 and T2 were not significantly different from one another, but were significantly lower (by as much as 1.8 fold) than treatment T3. In the case of clone SGR096 (C8) the root wet masses obtained after treatments T1 and T3 were not significantly different, whereas, a significant decrease in this parameter was found after treatment T2 (Figure 76).



**Figure 76.** Average wet mass of roots from cuttings of ten *E. grandis* clones (see Table 16, Trial 5), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the P=0.05 level.

The trends in average root dry mass differ slightly from those found in average root wet mass. No significant difference in root dry masses between treatments was observed for clones C1, C2, C3, C4, C5, C6, C7 and C10 (Figure 77). In the case of clone C8, however, the root dry masses obtained after treatments T1 and T3 were not significantly different, while bacterial cocktail treatment T2 significantly reduced the root dry mass of this clone. The best root dry mass (35 mg) for clone C9 was achieved after treatment T2. This was not significantly different from treatment T1 (28 mg). These root dry masses were, however, significantly better (up to 1.4 fold) than that obtained with treatment T3. These results imply that the bacterial treatments increased secondary root formation and hence the root mass of this clone.



**Figure 77.** Average dry mass of roots from cuttings of ten *E. grandis* clones (Table 16, Trial 5), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

A comparison of the rooting percentages (Seradix -induced) obtained for the ten *E. grandis* clones in trials 5 and 6 (Figures 69 and 74) to their reported rooting percentage (NEL, 1991) was made (Table 17). This was possible as rooting in these trials was carried out under the same propagation conditions used for establishing the reported rooting abilities of these clones.

**Table 17.** Comparison of the rooting abilities of ten *E. grandis* clones (see Table 16) obtained in trials 5 and 6 to their reported rooting ability (NEL, 1991).

CLONE	TRIAL 5 ROOTING %	TRIAL 6 ROOTING %	REPORTED* ROOTING %
C1	84	94	85
C2	73	90	95
C3	78	76	90
C4	72	62	50
C5	72	87	70
C6	76	88	60
C7	64	79	35
C8	74	97	15
C9	79	84	25
C10	84	83	45

\* The reported rooting abilities of these clones are based on a series of rooting trials set up over a propagation season.

The reason for selecting *E. grandis* clones with a range of rooting abilities for the *Agrobacterium*-mediated rooting trials was to establish whether agrobacteria could be used to effectively enhance rooting and root quality of not only "poor" but "average" and "good" rooting clones as well. However, when the actual (as

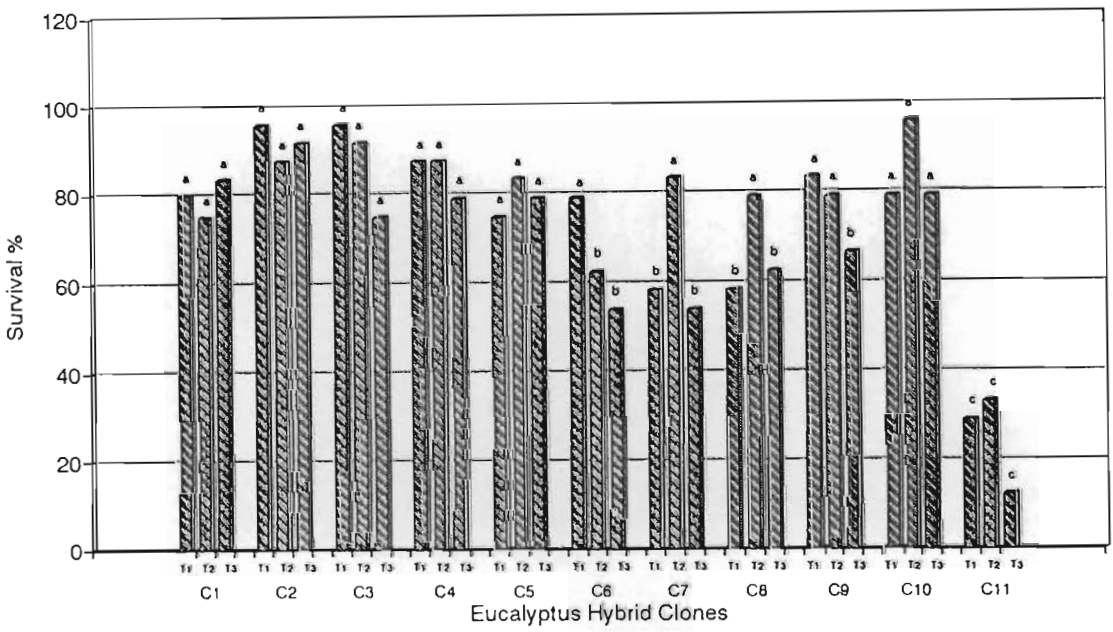
established with Seradix in these trials) and reported rooting abilities of the ten *E. grandis* clones were compared (Table 17) large differences were found. All the "poor" and "medium" rooters gave very good rooting percentages. In fact all the clones rooted at or above the commercially acceptable level of 70 %. The rooting abilities of these clones will have to be revised. From the results it can be concluded that, compared to the rooting and root quality obtained with Seradix, the *Agrobacterium* cocktails were generally not able to improve rooting or root quality (as determined by root number, length, and wet and dry masses) of the ten *E. grandis* clones tested in these trials.

### 5.3.5 Trials 6 and 7: *In Vivo* Rooting of Cuttings of Eleven *Eucalyptus* Hybrid Clones by Two *Agrobacterium* Cocktails

Only a small number of stock plants (3 to 5 ramets) of each of the *Eucalyptus* hybrid clones were available for these nursery trials. As a result the trial sizes were smaller than those set up for the *E. grandis* clones. Two small trials were established in September and November 1993, to compare Seradix-induced rooting of eleven *Eucalyptus* hybrid clones developed by HL&H, with rooting induced by two bacterial cocktails (see section 5.2.1). Propagation was carried out at ambient temperature, in a shade tunnel, under mist irrigation, in Durban.

## Trial 6

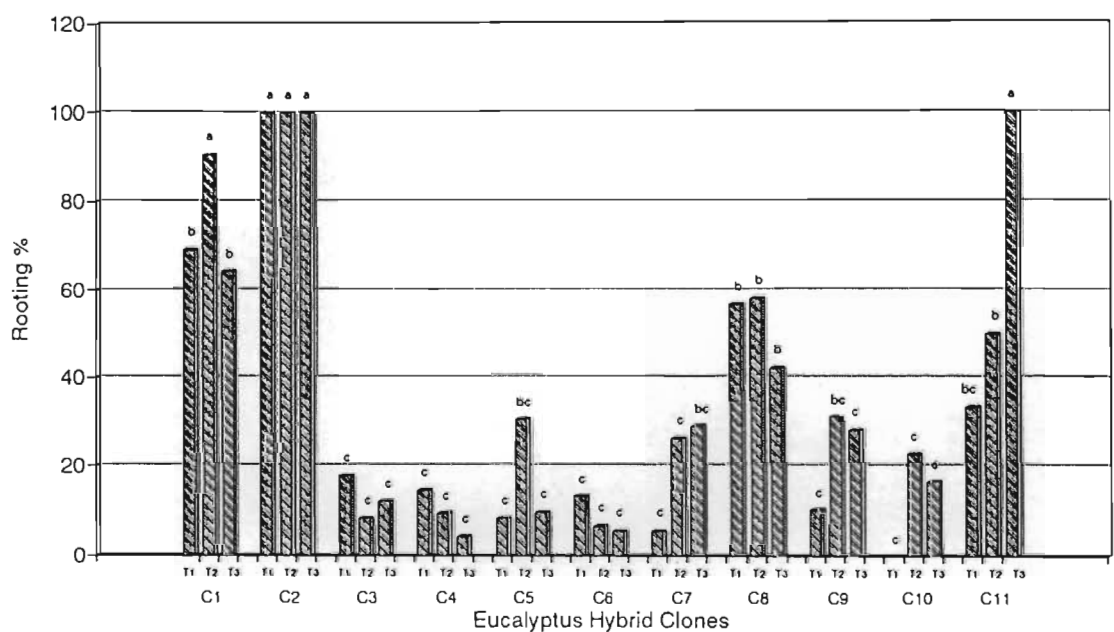
The survival rate of the eleven hybrid clones ranged from 14 to 95 % depending on the clone and treatment. No significant differences in survival rates due to treatments were observed between hybrid clones GxC 005/015 (C1), GxC 016/008 (C2), GxN 018/CTG (C3), GxN 019/CTG (C4), GxN 055/CTG (C5), NxG 070/ICF (C10) and NxG 071/ICF (C11) (Figure 78). Clone C11 had the poorest survival rate. No significant difference in survival of clone GxN 065/CTG (C6) was found after treatment with bacterial cocktail T2 and Seradix T3. Survival of this clone after treatment with bacterial cocktail T1 was however, significantly better (up to 23 %) than that achieved with the other treatments. In the case of clone GxN 227/CTG (C7), survival was significantly better after treatment T2, by 25 and 27 % compared to treatments T1 and T3, respectively. The difference between treatments T1 and T3 was however, not significant. The same trend was observed in clone NxG 017/ICF (C8). Although there was no significant difference between survival of clone NxG 049/ICF (C9) after treatment with bacterial cocktails T1 and T2, survival after these treatments was significantly better (up to 16 %) than that achieved with Seradix (T3). The implication here is that Seradix may have an adverse effect on the survival of this *Eucalyptus* hybrid clone.



**Figure 78.** Survival (%) of stem cuttings of eleven *Eucalyptus* hybrid clones (see Table 16, Trial 6), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

The genetic variation in rooting ability of the eleven *Eucalyptus* hybrid clones was evident from the data presented in Figure 79. Rooting ranged from as low as 0 % to 100 % depending on clone and treatment. Ninety percent rooting was achieved after treatment of *Eucalyptus* hybrid clone C1 with bacterial cocktail T2. This was

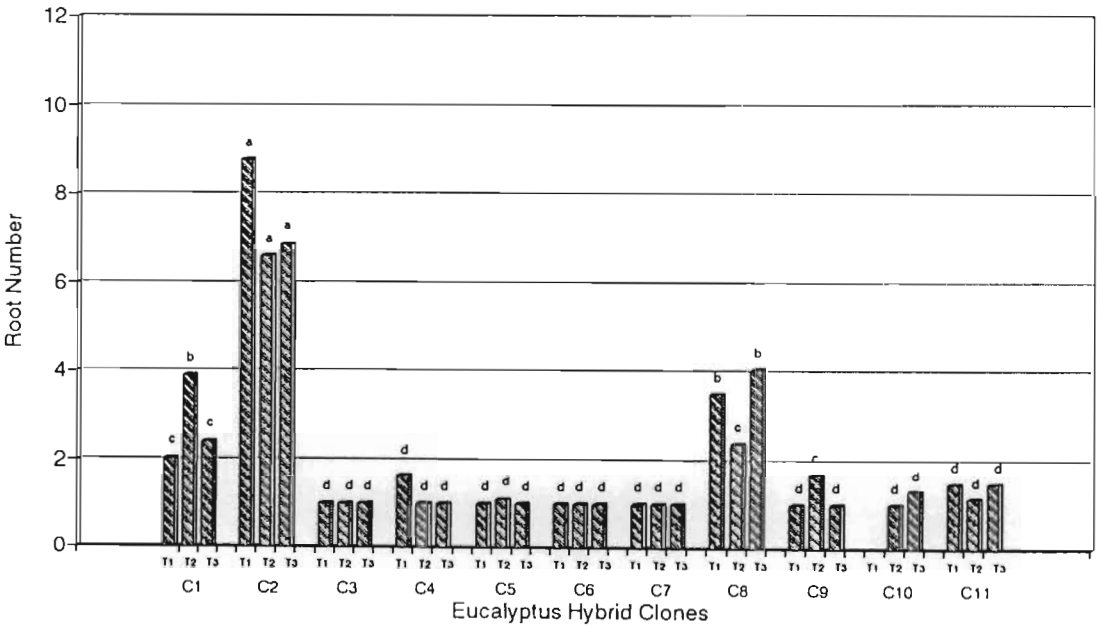
significantly higher, by 20 and 24 % respectively, compared to treatments T1 and T3. There was however, no significant difference in the rooting abilities of this clone after treatment with bacterial cocktail T1 and Seradix (T3). Although variation in rooting ability between clones was observed, the three treatments did not significantly affect rooting of clones C2, C3, C4, C5, C6, C7, C8, C9 and C10. Clone C2 was a good rooter with 100 % rooting being achieved irrespective of the treatment. In the case of clone C10, rooting due to treatments T2 and T3 were not significantly different. No rooting was achieved with treatment T1 for this clone. Although rooting of clone C11 with bacterial cocktail treatment T2 was 16 % higher than that achieved with bacterial cocktail treatment T1, this difference was not significant. Seradix (T3) induced rooting was however, 100 % for this clone and significantly higher than that achieved with the other two treatments (Figure 79). These results show that the bacterial cocktails can overcome the genetic control on rooting of certain *Eucalyptus* hybrid clones (eg. C1). In some instances however, the bacterial cocktails appear to be unable to improve rooting to the same extent as Seradix (eg. C11). This has also been found to be the case for certain *E. grandis* and *E. globulus* clones both *in vitro* and under nursery conditions (see Chapter 4 and sections 5.3.2 and 5.3.4).



**Figure 79.** Rooting ability (%) of stem cuttings of eleven *Eucalyptus* hybrid clones (see Table 16, Trial 6), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

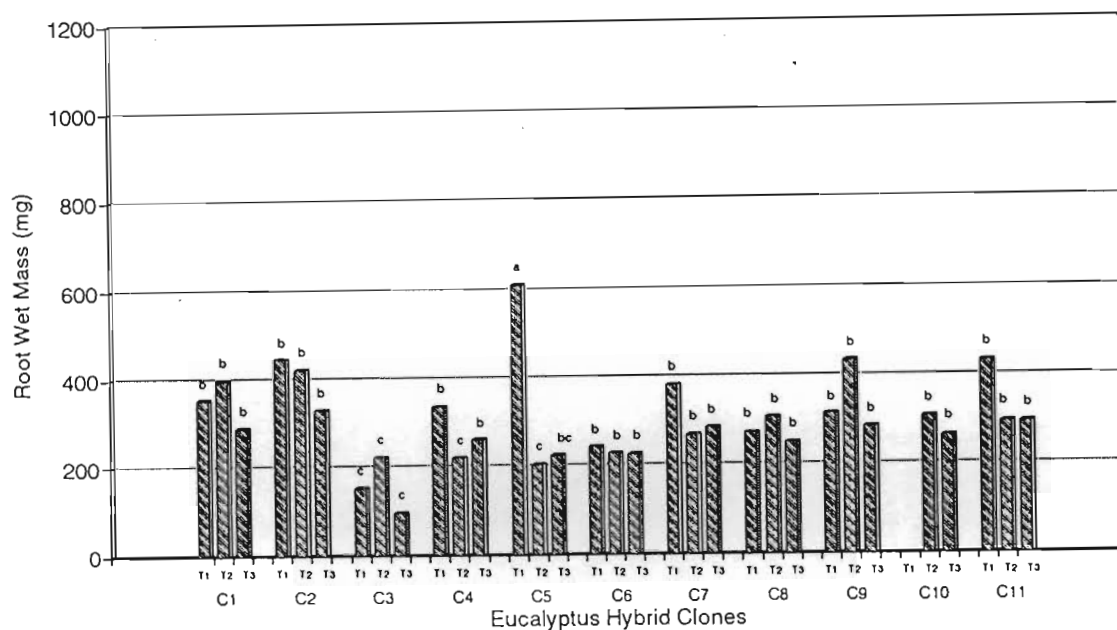
Although the average root number varied from clone to clone there was no significant difference in root number due to treatment for clones C2, C3, C4, C5, C6, C7, and C11 (Figure 80). Average root number on clone C2 after treatment T1 was up to 1.4 fold greater than that obtained with the other treatments, however, the

differences were not significant. Treatment T2 resulted in a significantly greater number of roots per rooted cutting of clone C1 compared to the other treatments, which were not significantly different from each other. The same trend was observed for clone C9. The root number on cuttings of clone C8 was significantly lower, by up to 1.6 fold, after treatment T2, compared to that achieved after treatments T1 and T3, which were not significantly different from each other. The number of roots on cuttings of clone C10, after treatments T2 and T3 were not significantly different (Figure 80). These results show that root number can be enhanced by the bacterial cocktails but this increase is dependent on the plant genotype.



**Figure 80.** Average number of roots on rooted stem cuttings of eleven *Eucalyptus* hybrid clones (see Table 16, Trial 6), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

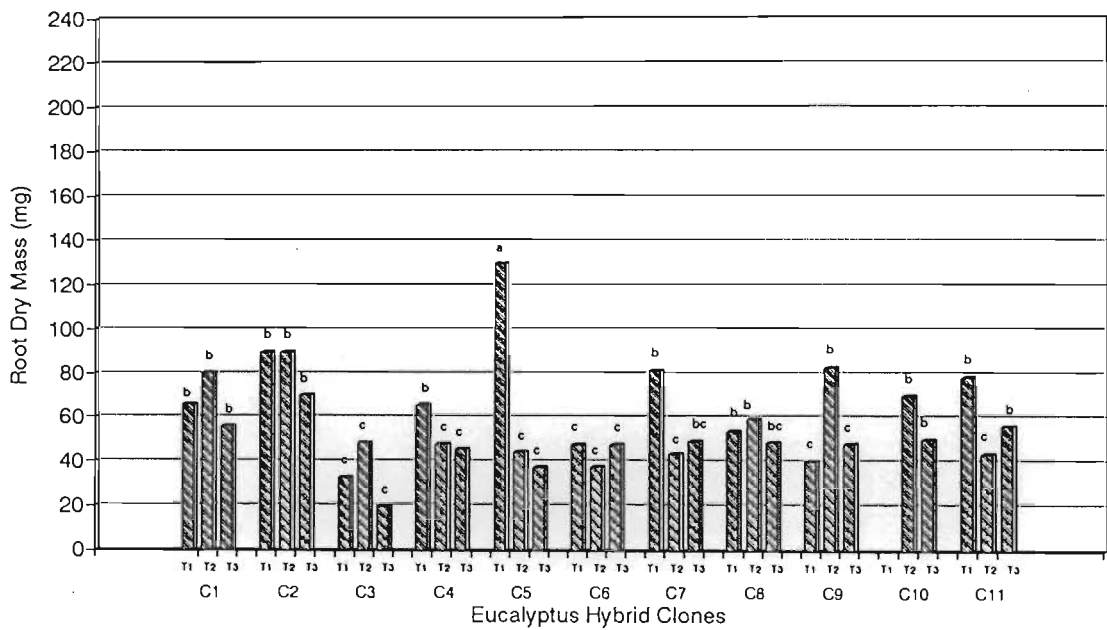
No significant differences were found in the average wet masses of roots from clones C1, C2, C3, C6, C7, C8, C9 and C11, irrespective of treatments (Figure 81). The root wet masses of cuttings of clone C4 after treatments T1 and T3 were not significantly different from one another, even though treatment T1 yielded the best root wet mass. These treatments did however, significantly increase root wet mass (up to 1.6 fold) compared to that achieved with treatment T2. In the case of clone C5, root wet masses obtained after treatments T2 and T3 were not significantly different. The root wet mass obtained after treatment T1 for this clone was however, significantly better, by up to 3 fold, than that achieved with the other treatments. Root wet mass results for clone C10, after treatments T2 and T3 were not significantly different. As already mentioned bacterial cocktail treatment T1 was ineffective on clone C10, with a complete inhibition of rooting occurring (Figure 81).



**Figure 81.** Average wet mass of roots from cuttings of eleven *Eucalyptus* hybrid clones (see Table 16, Trial 6), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

For the majority of the *Eucalyptus* hybrid clones the trends in average root dry mass data are slightly different from those found for the wet mass data. No significant differences in root dry masses between treatments were observed for clones C1, C2, C3, C6 and C8 (Figure 82). Bacterial cocktail T1 significantly increased the root dry masses of clones C4 and C5 by up to 1.5 and 3.4 fold respectively, compared to

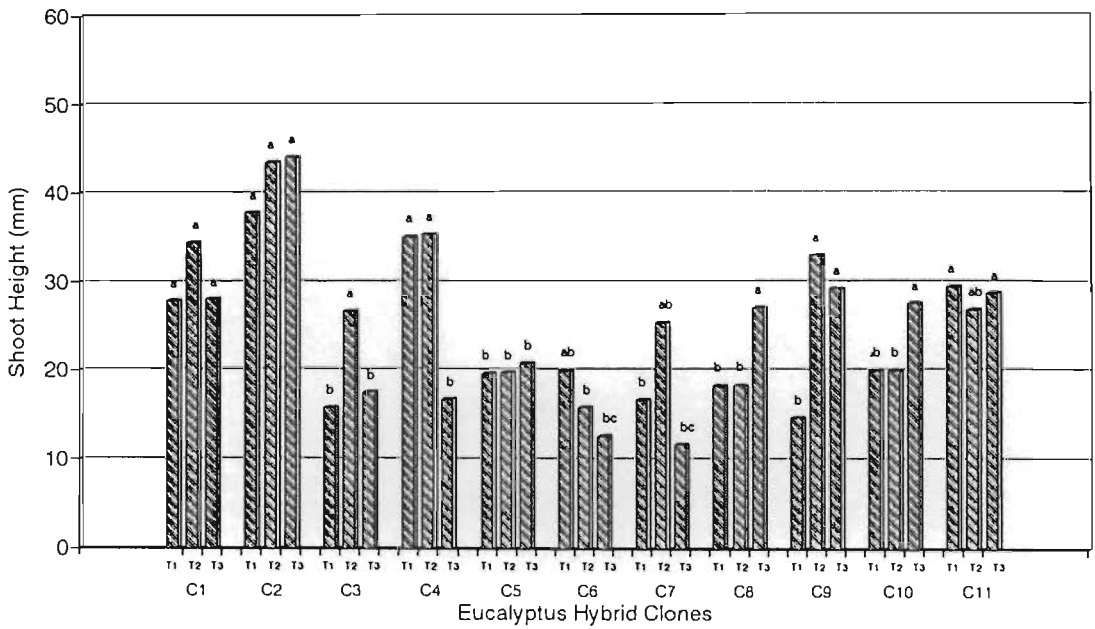
bacterial cocktail T2 and Seradix T3. The differences between treatments T2 and T3 were however, not significant for these clones. Bacterial cocktail treatment T1 produced the greatest root dry mass (82 mg) for clone C7. This was significantly higher than that achieved with bacterial cocktail treatment T2 (44 mg), but was not significantly better than that achieved with Seradix treatment T3 (50 mg). The differences between treatments T2 and T3 was also not significant. Bacterial cocktail T2 significantly increased the root dry mass of clone C9, by up to 2.1 fold, compared to the other two treatments. There was however, no significant difference in the root masses obtained after treatment of this clone with the bacterial cocktail T1 and Seradix T3. No significant difference was found in the average dry mass of roots from clone C10 after treatments T2 and T3 (Figure 82). The same trend was found in the root wet mass data for this clone (Figure 81). In the case of clone C11 bacterial cocktail treatment T1 gave the greatest root dry mass (79 mg) compared to the other two treatments. It was not significantly different from that obtained after treatment T3 (56 mg), but was significantly greater (1.9 fold) than that achieved after treatment T2 (Figure 82). The results from this trial show that the bacterial cocktails were able to increase root biomass of certain *Eucalyptus* hybrid clones, compared to Seradix.



**Figure 82.** Average dry mass of roots from cuttings of eleven *Eucalyptus* hybrid clones (see Table 16, Trial 6), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

A comparison was made of the average shoot heights of new shoots developing from axillary buds on the stem cuttings of the eleven *Eucalyptus* hybrid clones after treatment of the cuttings with two rooting bacterial cocktails (T1 and T2) and Seradix (T3), to establish whether the effects of these rooting compounds were reflected in shoot growth (Figure 83). No significant differences in shoot heights

were observed for clones C1, C2, C5, C6, C7, and C11, irrespective of treatment. The average shoot height of clone C3 after treatment T2 was significantly higher (up to 1.75 fold) than that obtained after treatments T1 and T3. The difference between bacterial cocktail treatments T1 and T2 was however, not significant for this clone. In the case of clone C4, shoot heights achieved after treatments T1 and T2 were not significantly different from each other, but were significantly higher than that obtained after treatment T3 (Seradix) (2.1 fold). The shoot heights obtained after treatment of clones C8 and C10 with bacterial cocktails T1 and T2 were not significantly different from each other, but were significantly lower than the shoot heights obtained after Seradix treatment T3. Treatment T1 seemed to significantly lower the average shoot height of clone C9 (up to 2.2 fold lower), compared to the other two treatments, which were not significantly different from each other (Figure 83).

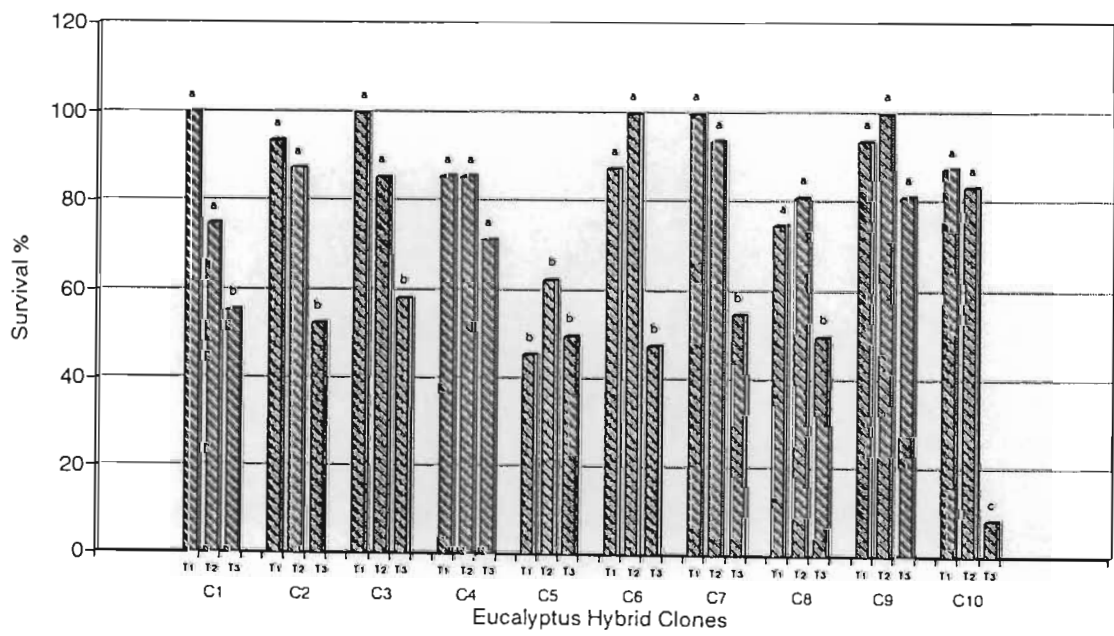


**Figure 83.** Average shoot height of new shoots developing from cuttings of eleven *Eucalyptus* hybrid clones (see Table 16, Trial 6), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the Tukey Test any two means having a common letter are not significantly different at the  $P=0.05$  level.

**Trial 7**

Only the survival rates and rooting abilities determined for the eleven *Eucalyptus* hybrid clones in this trial are presented (Figure 84 and 85), even though the other parameters were measured. The reasons for this are, the unbalanced data sets (see section 5.2.7) and the high mortality of cuttings of some of the clones after certain treatments (Seradix in particular) (Figure 84) which made interpretation of the data difficult and not very reliable.

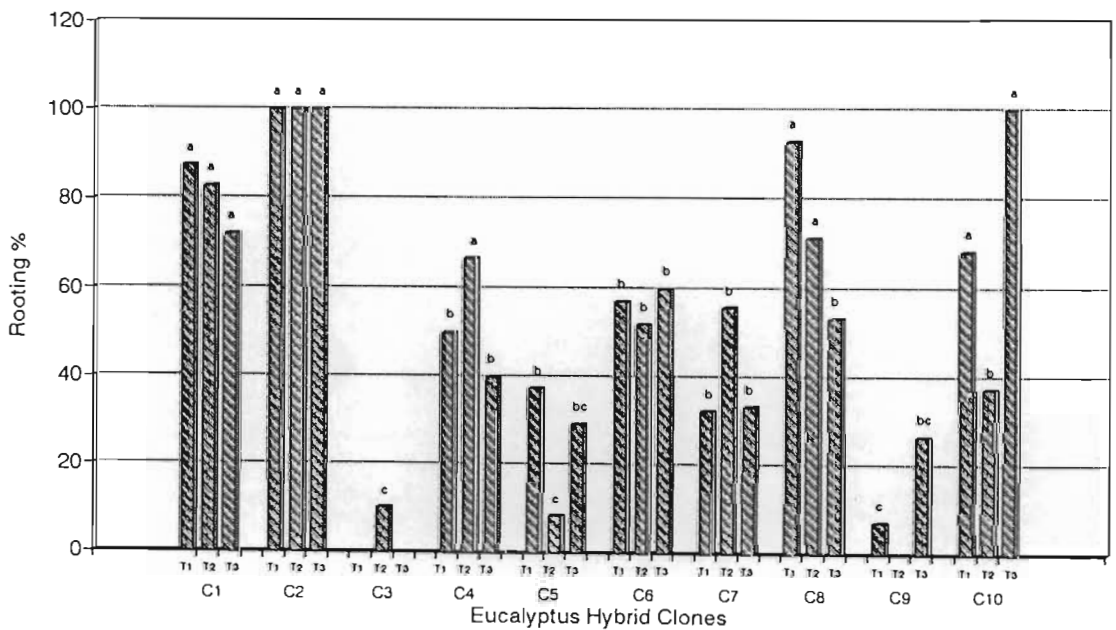
No significant treatment effect on the survival rates of clones GxC 019/CTG (C4), GxN 055/CTG (C5) and NxG 049/ICF (C9) were evident even though the survival rate after Seradix treatment (T3) tended to be lower than for the other treatments. Bacterial cocktail treatments T1 and T2 appeared to have a beneficial effect on some clones, significantly improving survival of clones GxC 005/015 (C1), GxC 016/008 (C2), GxN 018/CTG (C3), GxN 065/CTG (C6), GxN 227/CTG (C7), NxG 017/ICF (C8) and NxG 070/ICF (C10) (by up to 80 %) compared to treatment T3 (Seradix). All clone NxG 071/ICF (C11) cuttings died in this trial.



**Figure 84.** Survival (%) of stem cuttings of ten *Eucalyptus* hybrid clones (see Table 16, Trial 7), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the LSD Test any two means having a common letter are not significantly different at the P=0.1 level.

The rooting abilities of the eleven hybrid clones varied from clone to clone (from 0 to 100 %), irrespective of treatment. Although the bacterial cocktail treatments (T1 and T2) improved rooting ability of C1 compared to Seradix (T3), the differences were not significant (Figure 85). As was found in Trial 6, 100 % rooting was achieved for clone C2, irrespective of treatment. This clone is undoubtedly the best rooter of the hybrid clones. None of the surviving cuttings of clone C3 rooted after treatments T1 and T3, and only 10 % of the cuttings treated with bacterial cocktail treatment T2 rooted. Rooting of clone C4 was significantly better, by 16 and 26 %, after treatment T2, compared to treatments T1 and T3 respectively. There was however, no significant difference in rooting of this clone after treatments T1 and T3. The rooting abilities of clone C5 after treatments T1 and T3 were not significantly different from each other, however, they were significantly better, by up to 28 %, compared to treatment T2. There was no significant difference in the rooting ability of clone C6, irrespective of treatment. In the case of clone C7, rooting after treatment T2 was 24 % higher than that achieved with the other treatments, but the differences were not significant. Bacterial cocktail T1 induced the best rooting (94 %) for clone C8, however, although this was higher than that achieved with bacterial cocktail T2 (72 %), the difference was not significant. The rooting abilities induced by bacterial cocktails T1 and T2 were significantly better (up to 40 %) for this clone than that achieved with Seradix (T3). Seradix (T3) induced the best rooting for clone C9 (26 %), but this was not significantly different

from the rooting achieved with treatment T1 (8 %). No rooting was obtained after treatment of this clone with bacterial cocktail T2. Finally, rooting of clone C10 was best with Seradix (T3) (100 %). Although this was much higher than that achieved with treatment T1 (70 %) for this clone, the difference was not significant. Rooting of clone C10 after treatment T2 was significantly lower, by up to 62 %, than that achieved with the other treatments (Figure 85).



**Figure 85.** Rooting ability (%) of stem cuttings of ten *Eucalyptus* hybrid clones (see Table 16, Trial 7), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 comprised Seradix No 2. Based on the LSD Test any two means having a common letter are not significantly different at the  $P=0.1$  level.

## 5.4 DISCUSSION

The aim of this research was to overcome rooting deficiencies and improve root quality in nursery propagated *Eucalyptus* species and clones. The potential use of *A. rhizogenes* strains and bacterial cocktails for this purpose was investigated.

The high survival rates of cuttings in the *E. globulus* cutting transformation trial showed that *A. rhizogenes* treatments did not have a detrimental effect on the *E. globulus* clones tested. Rooting ability of one of the clones, HM15, was significantly improved after treatment with *A. rhizogenes* and the treated cuttings developed up to ten times as many roots as untreated cuttings. However, rooting of the second clone in this trial, AC17, which had a very poor rooting ability could not be improved using the bacterial treatments. This suggests that *A. rhizogenes* strains could be used to improve rooting of selected *E. globulus* genotypes.

The results from the rooting media trial (Trial 2) clearly showed the clonal variation not only in the inherent rooting abilities of the six *E. grandis* clones tested, but also in Seradix-induced and *A. rhizogenes*-induced rooting in both vermiculite and peat:perlite media. As was found for the inherent and Seradix-induced rooting, the success of *A. rhizogenes*-induced rooting of *E. grandis* clones is influenced by the rooting medium used. Seradix-induced rooting of clone C3 (SGR071) in peat:perlite medium was significantly lower than the inherent rooting ability of this clone,

suggesting that Seradix could have an inhibitory effect on rooting of certain *E. grandis* clones propagated in this medium. This supports the finding of P. J. Wilson (Personal communication), that Seradix can have an inhibitory effect on certain *E. globulus* clones propagated in peat:perlite. The results of the media trial also suggest that *A. rhizogenes* strains could under certain circumstances have an adverse effect on rooting of certain *E. grandis* clones. For example, rooting of *E. grandis* SGR052 (C1) induced by strains R1601 and TR8,3 was significantly lower than the inherent ability of this clone to root in a peat:perlite medium. A vermiculite based medium therefore appears to be more suited for propagation of *E. grandis* clones if Seradix or *A. rhizogenes* strains are used to induce rooting. However, if hormones are not used in the propagation process, a peat:perlite based medium would probably be more suitable.

A comparison of Seradix-induced rooting of cuttings of the six *E. grandis* clones in vermiculite (Trial 2), with the reported rooting percentages (which were also determined using Seradix and vermiculite) showed that rooting of clone SGR052 (C1) in this trial was 27.5 % lower than the reported rooting ability of this clone. Similarly rooting of clones SGR171 (C5) and SGR124 (C6) were 30 and 12.5 % lower than the reported values. However, rooting of SGR132 (C2) was 30 % higher. The rooting ability of SGR050 (C4) (87.5 %) was similar to the reported rooting percentage (90 %) for this clone. These deviances could be accounted for by a number of factors including health of the stock plants, seasonality, and

environmental conditions in the nursery at the time of this experiment. *E. grandis* clones SGR132(C2), SGR096 (C3) and SGR050 (C4) have potential for commercial propagation as their rooting abilities at least meet the 70 % rooting requirement in a vermiculite-based medium.

The interpretation of the results from Trial 3, which compared the ability of six *A. rhizogenes* strains to induce rooting on ten *E. grandis* clones, with the inherent rooting abilities and Seradix-induced rooting of these clones, was extremely difficult owing to the low survival rate. As already mentioned, it was not possible to make a comparison of treatment/clone interactions for this trial owing to the low survival rates. The results however, indicate that clones SGR071 (C3) and SGR003 (C4) would be the best choice if all variables are taken into consideration. On the whole, the control treatments gave a marginally better survival rate, rooting percentage and number of roots than the *Agrobacterium* treatments. This suggests that the bacterial inocula may possibly have some detrimental effect on survival rate and rooting of these clones. However, from the results of previous *E. grandis* trials using vermiculite as rooting medium this seems highly unlikely. In addition, when looking at the rankings of the treatments, there are no real significant differences between the controls (T1 and T8) and the bacterial treatments (T2, T3 and T4). The only observed difference of real significance was between the treatment at the top of the ranking and the one at the bottom. Further trials will however, have to be set up before definite conclusions can be made.

There was a significant interaction between clone and treatment for survival, rooting ability and number of roots, indicating some additional genotypic effects on treatment. The interaction causes a significant variance component which is not explained by clone and treatment. In practical terms this would mean that a clone would respond better (or worse) than expected to a certain treatment. To determine the exact nature of the interaction, a more detailed study would be necessary; however, the limited number of rooted survivors prevented such an analysis.

With the exception of dry mass, replication had a significant effect on all variables (survival, number rooted, number of roots) which indicates a significant amount of environmental noise. This environmental impact on replications should be taken into consideration in future experiments. Control of greenhouse conditions at Sabie were inadequate and this is felt to be one of the major reasons for the poor success of this trial. Other trials will have to be set up under more controlled greenhouse conditions, to eliminate environmental noise, before any conclusion can be drawn as to the effect of the various treatments on improving the rooting of these ten selected clones.

Nursery trials testing the root-inducing abilities of individual *Agrobacterium* strains do however, show that *A. rhizogenes* strains have potential to alter the inherent rooting abilities of certain *E. grandis* and *E. globulus* clones, enhance rooting and improve root quality. These findings differ from those of LAMBERT and TEPFER

(1991), who found that although the inoculation of apple cuttings altered the root system morphology it neither increased the frequency of rooting of the cuttings, nor their survival, nor did it alter the inherent ability of the apple lines tested to root.

Of the *A. rhizogenes* strains tested, LBA9402, has the broadest host range, improving rooting on a range of the *E. grandis* and *E. globulus* clones tested under nursery conditions. This confirmed the *in vitro* results and showed that the use of this molecular approach to rooting is dependent on the genotypes of both the bacterium and the plant.

With the aim of overcoming the host range limitation of individual bacterial strains in successful *Agrobacterium*-mediated rooting under nursery conditions, research was undertaken to explore the use of bacterial cocktails, developed in this study with *in vitro* material (Chapter 4), to improve rooting of a wide range of *Eucalyptus* clones (Trials 4 to 7). The other potential advantage of using bacterial cocktails would be that initial screening of *Agrobacterium* strains for host specificity may not be necessary. Two bacterial cocktails were tested, T1 comprising three *A. rhizogenes* strains, LBA9402, R1601 and TR8,3, supplemented with auxin and T2 comprising three *A. rhizogenes* strains, LBA9402, R1601 and TR8,3 and two genetically modified *A. tumefaciens* strains LBA4404(pGA643), and LBA4404(pGA643-12), supplemented with auxin, on a range of *E. grandis* and *Eucalyptus* hybrid clones. The results from these trials showed that the bacterial cocktails were able to

overcome the genetic control on rooting of certain but not all clones. The clonal variation in *Agrobacterium*-mediated rooting with bacterial cocktails under nursery conditions supported the *in vitro* *Agrobacterium* bacterial cocktail-mediated rooting results. The bacterial cocktails were however, generally more effective at improving rooting of the *Eucalyptus* hybrid clones than the *E. grandis* clones tested.

In Trial 6 the rooting ability of *Eucalyptus* hybrid clone GxC005/015 (C1) was significantly improved by the bacterial cocktail T2 compared to the bacterial cocktail T1 and Seradix. However, in the second trial set (Trial 7) no significant differences were found between the three treatments for this clone. In Trial 7 bacterial cocktail T2 significantly improved the rooting of *Eucalyptus* hybrid clone GxN019/CTG (C4) compared to the other two treatments, while both bacterial cocktails T1 and T2 significantly improved rooting of clone NxG017/ICF (C8). In Trial 6 however, no significant differences in rooting abilities were found between treatments for these clones. What this suggests is that other factors, such as the condition of the stock plants and the propagation conditions, influence the success of the *Agrobacterium*-mediated rooting. The variation in rooting abilities of a number of the clones tested in this study, from trial to trial, after either Seradix or bacterial cocktail treatments, emphasises the fact that many factors (eg. condition of stock plants and environmental and physiological conditions) influence the successful propagation of stem cuttings under nursery conditions. In conclusion although bacterial cocktails were able to improve rooting of certain clones, their use was also found to be

limited by host range.

Based on the findings of LAMBERT and TEPFER (1991), molecular hybridisation was not used to confirm transformation of *in vivo* inoculated cuttings as the risk of contaminating bacteria was too high. Opine detection, as discussed in Chapter 4, was used as confirmation.

The nursery problems experienced during these trials often made interpretation of results difficult. Further research to optimise nursery conditions and ensure good survival and successful rooting is advisable. This will ultimately be followed by field trials set up with the chimeric plants. However, before field trials can be considered, it would be necessary to ensure that *A. rhizogenes* is eliminated from the rhizosphere prior to field planting. Based on the suggestion by David Tepfer (personal communication) research into the effect of increasing the substrate temperature above 30 °C as a means of controlling *A. rhizogenes* populations in the rhizosphere of rooted cuttings has been initiated. Preliminary results suggest that increasing the substrate temperature to 37°C for a two day period, 30 days after the cuttings were inoculated with the *A. rhizogenes* strain reduced the rhizosphere population of the bacterium 100 fold.

## CHAPTER 6

### ANATOMICAL STRUCTURE AND FUNCTION OF *AGROBACTERIUM* *RHIZOGENES* TRANSFORMED ROOTS

#### 6.1 INTRODUCTION

The integration and expression of part of the DNA (T-DNA) of the root-inducing (Ri) plasmid present in *A. rhizogenes* in plant cells results in the production of transformed or hairy roots (Ri-roots) at the site of infection. The potential of this phenomenon to improve rooting and root quality of *Eucalyptus* was investigated in this study. A number of *A. rhizogenes* strains have been shown to induce root formation on both *in vitro* micro-cuttings (Chapter 4) and *in vivo* cuttings (Chapter 5) of a range of *Eucalyptus* species and clones. In order to establish whether the insertion and expression of the Ri T-DNA in plant cells affects the structure and function of the resultant roots, the anatomy of transformed and untransformed roots and their nutrient uptake abilities were examined using light and transmission electron microscopy and a radio-isotope nutrient uptake root bioassay.

The radio-isotope nutrient uptake bioassay which was developed by John Dighton and co-workers (DIGHTON and HARRISON, 1983; HARRISON and DIGHTON, 1990; HARRISON *et al.*, 1990; DIGHTON and JONES, 1991; JONES *et al.*,

1991a; JONES *et al.*, 1991b) provides an indication of the nutritional status of plantation trees by determining uptake of nitrogen (N), phosphorus (P) and potassium (K) by fine feeder roots at the soil surface. By examining the relationship between root uptake, the availability of these elements in the soil and the degree of deficiency of these nutrients in the plant, it is possible to determine precise nutrient/fertilization requirements. For instance, roots from nutrient deficient trees absorb these nutrients faster than their counterparts with adequate nutrient supplies. Indications are that the root bioassay technique is a valuable alternative or supplementary diagnostic tool to the more conventional foliar nutrient level procedure. Certainly, the root bioassay appears to offer greater sensitivity in detecting nutrient status, is less expensive to use in the long term and has particular application for evaluating clones from tree breeding studies. This assay was used to assess whether nutrient uptake by transformed roots was as effective as that of normal roots.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Plant Material

The plant material used in this study is detailed in Table 17.

**Table 18.** Chimeric and control *E. grandis* (g), *E. dunnii* (d) and *E. globulus* (gb) plants and root cultures used as a source of material for this study.

<i>Eucalyptus</i> Plants	Characteristics	Microscopy	Nutrient Uptake
<i>E. grandis</i>	gc <sub>1</sub> <sup>a</sup>	✓	✓
	gc <sub>2</sub> <sup>a</sup>	✓	✓
	gl <sup>a</sup>	✓	✓
	g2 <sub>1</sub> <sup>a</sup>	✓	✓
	g2 <sub>2</sub> <sup>a</sup>	-	✓
	g3 <sup>a</sup>	✓	✓
	gc <sup>i</sup>	✓	-
	gl <sup>i</sup>	✓	-
	gl <sup>r</sup>	✓	-
	g3 <sup>r</sup>	-	✓
<i>E. dunnii</i>	dc <sup>a</sup>	✓	✓
	d1 <sup>a</sup>	✓	✓
	d2 <sup>a</sup>	✓	✓
	d3 <sup>a</sup>	✓	✓
	d1 <sup>r</sup>	-	✓
<i>E. globulus</i>	gbc <sup>i</sup>	✓	✓
	gb1 <sup>i</sup>	✓	✓
	gb1 <sup>r</sup>	✓	✓
	gb2 <sup>r</sup>	-	✓
	gb3 <sup>r</sup>	-	✓
	gb4 <sup>r</sup>	-	✓

<sup>a</sup> acclimatized chimeric and control *Eucalyptus* plants; <sup>i</sup> *in vitro* control *Eucalyptus* plants; <sup>r</sup> transformed root cultures of *Eucalyptus*

✓ denotes material used for microscopy and/or nutrient uptake studies

- denotes material not used for these studies

## 6.2.2 Light Microscopy

### 6.2.2.1 Wax Embedding Technique

Roots from acclimatized, one-year-old chimeric and control *E. grandis* (g) and *E. dunnii* (d) (Table 18) plants produced *in vitro* (see Chapter 4) were selected for light microscopic examination. Three representative root samples were taken from each plant. From the middle zone of these roots, 5 x 5 mm segments were taken and fixed in approximately 2 to 3 ml of formalin acetic alcohol (FAA) [formaldehyde (27 - 40 %):acetic acid (glacial):ethanol (95 %):water (2:1:10:7)] for 24 hours at room temperature, dehydrated in a graded butanol:ethanol:water series and embedded in paraffin wax (melting point 52 °C) according to standard procedures (JOHANSEN, 1940; JENSEN, 1962) and poured into moulds. Butanol was used as the transition solvent for wax infiltration. Sections of 5 - 10  $\mu\text{m}$  were mounted on slides with Haupt's adhesive (JOHANSEN, 1940); stained in Safranine-Fast Green, viewed and photographed with an Olympus (Vanox AHBS3) photomicroscope. A minimum of 10 sections were examined for each root sample.

#### 6.2.2.2 Epoxy Resin Embedding Technique

*In vitro* root samples from *E. grandis* (gl<sup>1</sup>) root cultures (Table 18) and *in vitro* *E. grandis* seedlings were fixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2), and post-fixed in 0.5 % osmium tetroxide in 0.1 M phosphate buffer for 8 hours. Tissues were dehydrated in a graded acetone series and then infiltrated with Spurr's epoxy resin according to standard procedures (SPURR, 1969). Sections of 1 to 2  $\mu\text{m}$  were cut, mounted on slides and stained with 1 % toluidine blue.

#### 6.2.3 Transmission Electron Microscopy

Root specimens of *in vitro* *E. grandis* and *E. globulus* control and chimeric plants were selected from tip, middle and basal regions of roots and fixed for 8 hours in 3 % glutaraldehyde (buffered to pH 7.2 with 0.05 M sodium cacodylate). This was followed by two washes of 30 minutes in 0.05 M sodium cacodylate buffer. Post fixation was then carried out in 2 % osmium tetroxide followed by two washes in fresh buffer and dehydration in an ethanol series. The specimens were then embedded in Spurr's epoxy resin (SPURR, 1969).

Sections were cut on a LKB III ultramicrotome and picked up on a 200 mesh copper

grid and double stained with lead citrate and uranyl acetate. The sections were then viewed in a Jeol 100C Transmission Electron Microscope operating at an accelerating voltage of 80 kV.

#### 6.2.4. Nutrient Uptake Bioassays

Between 8 and 10 random samples of fine roots of chimeric and normal *Eucalyptus* plants growing in 10 litre containers (Table 18), were carefully teased from the top 5 cm of the soil horizon. Intact segments 10-20 cm in length and 0.5-2 mm in diameter were kept moist between wet tissue paper for transport to the laboratory. Roots were carefully washed to remove loose soil. Root samples from *in vitro* transformed root cultures were also used (Table 17). The bioassay technique was carried out within 48 hours of sampling the roots.

##### 6.2.4.1 Phosphorus Uptake Bioassay

Roots were "primed" by placing them in a  $5 \times 10^{-4}$  M  $\text{CaSO}_4$  solution, at  $18^\circ\text{C}$  for 30 minutes. They were then transferred for 15 minutes to a solution also at  $18^\circ\text{C}$ , containing  $\text{CaSO}_4$  at  $5 \times 10^{-4}$  M and  $\text{KH}_2\text{PO}_4$  at  $5 \times 10^{-6}$  M to which  $^{32}\text{P}$  was added

at about  $50 \mu\text{Ci l}^{-1}$ . Roots were removed from the solution and washed for 5 minutes under running water. A sub-sample ( $<200$  mg fresh weight) of roots was removed from each root sample and placed in 15 ml distilled water in a 20 ml standard, glass, counting vial and counted for  $^{32}\text{P}$  activity by Cerenkov light in a Tricarb 2000 liquid scintillation counter. Each root sub-sample was then removed from the vial, blotted and weighed and the vial recounted for  $^{32}\text{P}$ , which had diffused from the free space of the roots. In this way a measure was made of P which had not been metabolically absorbed by the roots. A subset of the roots was acid-digested and the digest counted, to allow for physical quenching of Cerenkov light. Both sets of  $^{32}\text{P}$  counts were adjusted for background, decay and colour quench of Cerenkov light (by the sample-channels ratio method). Recounts of vials without roots were then subtracted from counts of vials with roots, and the resulting counts further adjusted for the physical quench factor. Estimated  $^{32}\text{P}$  uptake by the roots was then converted to P uptake per unit root mass per 15 minute time period (expressed as  $\text{pg P mg}^{-1} \text{ root } 15 \text{ min}^{-1}$ ), by reference to the specific activity of the  $^{32}\text{P}$  solution into which the roots had been placed and the root mass previously measured.

Estimated  $^{32}\text{P}$  in roots was derived according to the equation (DIGHTON and HARRISON, 1983):  $Y_1 = 946 + 1.196X_1 - 48.3X_2 + 0.33(X_2)^2$  ( $R^2 = 0.96$ )

Where,  $Y_1 = \text{dpm } ^{32}\text{P}$  in root digest;  $X_1 = \text{dpm } ^{32}\text{P}$  in root samples estimated using previous equations; and  $X_2 = \text{root weight (mg)}$ .

#### 6.2.4.2 Potassium Uptake Bioassay

The same bioassay procedure as outlined above was followed using Rubidium ( $^{86}\text{Rb}$ ) as a tracer for  $\text{K}^+$  uptake.

#### 6.2.4.3 Nitrogen Uptake Bioassay

Roots were immersed in a presoak solution ( $5 \times 10^{-4}\text{M}$   $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ) for 30 minutes (50-100 roots  $\text{l}^{-1}$ ). They were then transferred to an uptake solution containing  $0.8 \text{ mg l}^{-1}$   $^{14}\text{NH}_4\text{Cl}$ ,  $0.2 \text{ mg l}^{-1}$   $^{15}\text{NH}_4\text{Cl}$  and  $5 \times 10^{-4}\text{M}$   $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , for 2 hours and agitated occasionally. Roots were then washed in running water for 15 minutes and blotted dry. Roots were placed into paper bags and oven dried at  $60 - 80^\circ\text{C}$ . Roots prepared using the N bioassay technique were analyzed using Nuclear Magnetic Resonance (NMR) spectroscopy at the Waikato Stable Isotope Unit, Waikato, New Zealand.

#### 6.2.4.4 Statistical Analysis of Data

The SAS, two-way (clone x treatment) Analysis of Variance (ANOVA) was conducted for each of the parameters and multiple comparisons across treatment and root sample combinations achieved by Duncan's Multiple Range Test (DUNCAN, 1975; MILLER, 1981). Significant differences between root sample / treatments are indicated by an alphabetic letter. Groups of means with the same letter or which are overlapped with the same letter are not significantly different at the  $P = 0.05$  level based on the Duncan's Multiple Range Tests, but non-overlapping groups with differing letters are significantly different at this level.

### 6.3 RESULTS

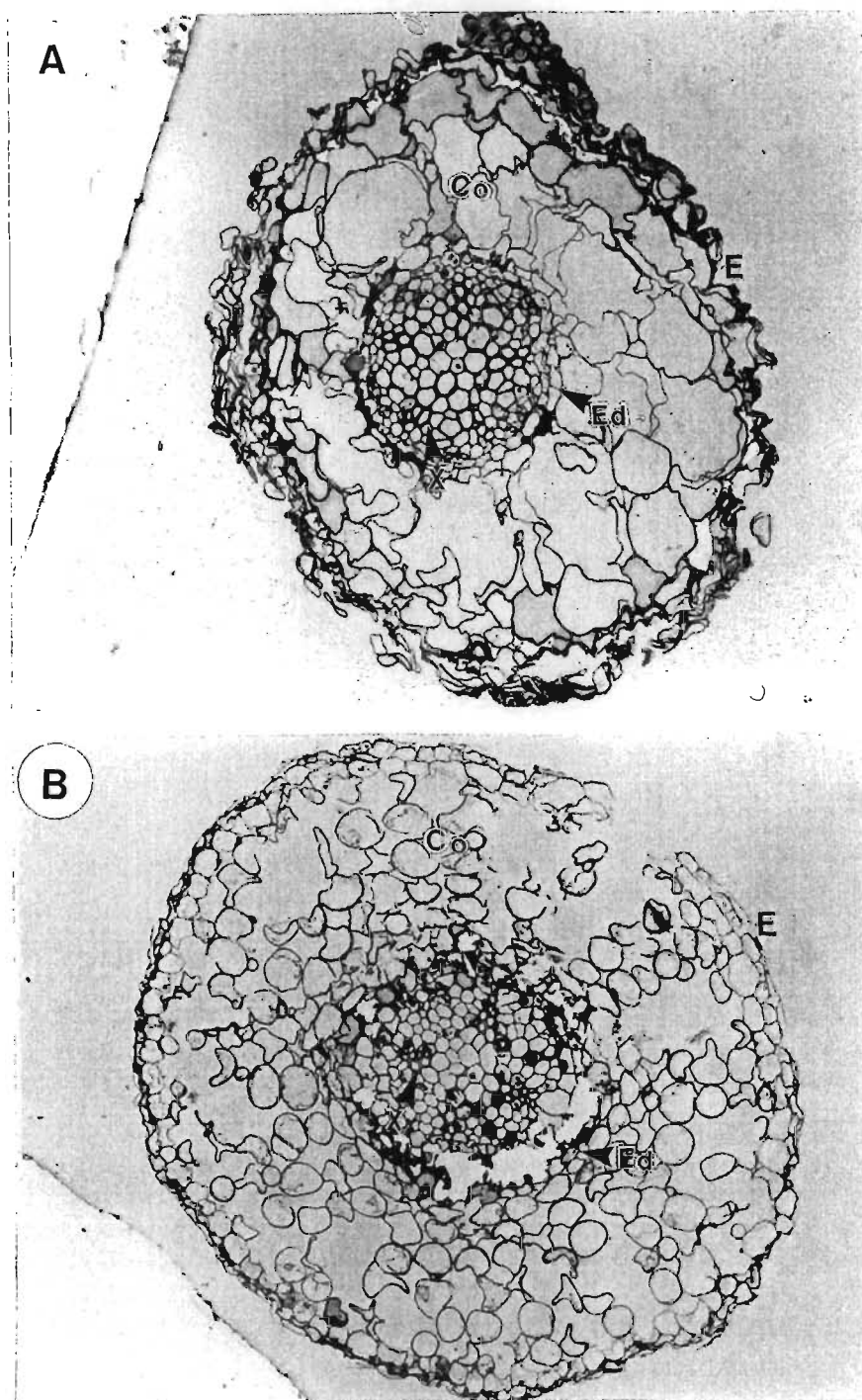
#### 6.3.1 Anatomy of Transformed and Non-transformed Roots

As non-transformed roots could not be cultured *in vitro*, the anatomical characteristics of roots from *in vitro* transformed root cultures (g1') developed from *E. grandis* after inoculation with *A. rhizogenes* LBA9402 were compared with roots taken from *in vitro* propagated *E. grandis* seedlings. It must, however, be borne in

mind that the roots were grown under different conditions and were of different ages, making direct comparisons difficult.

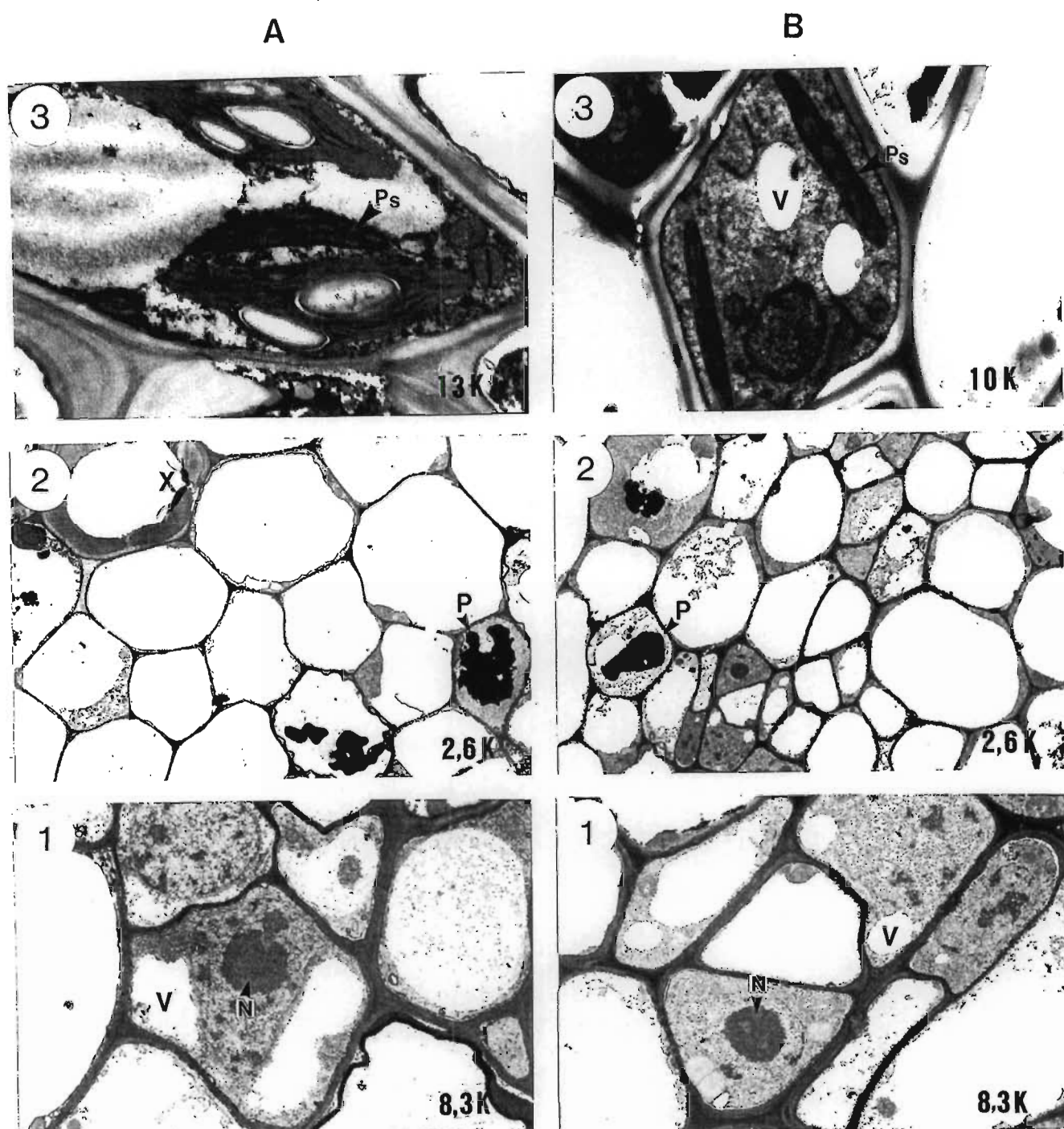
The roots of control *E. grandis* plants consisted of an irregular arrangement of epidermal cells (E) (Figure 86A). The cortex cells (Co) were large and irregularly shaped with many air spaces. Within a well-defined endodermis (Ed) and pericycle, the vascular tissue was of normal appearance. Protoxylem and meta xylem were observed and phloem fibres were present in the phloem.

In contrast, in tissue of transformed root cultures (gl'), the epidermis (E) (Figure 86B) was well defined with a regular arrangement. The cortical cells (Co) were small and mostly irregularly-shaped with many airspaces. The endodermis (Ed) and pericycle appeared indistinct. The vessel elements of the xylem (X) were present but were not regularly arranged as in the non-transformed roots. The phloem fibres of the phloem tissue were also visible but arranged differently because of the xylem (X) arrangement.

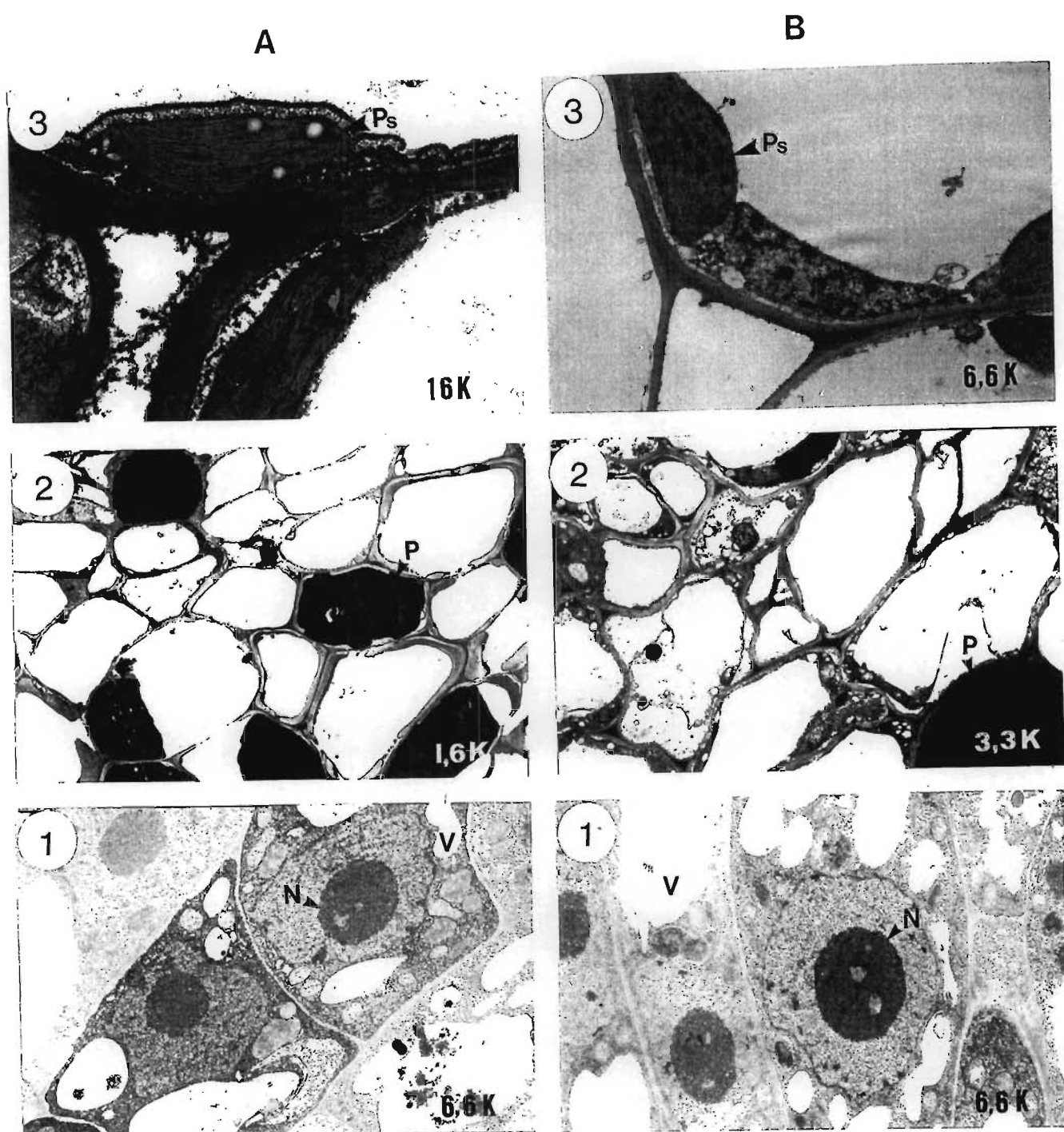


**Figure 86.** Light micrographs of transverse sections through non-transformed roots (A) taken from *in vitro* propagated *E. grandis* seedlings (gc') and transformed root (B) taken from root cultures of *E. grandis* transformed with *A. rhizogenes* LBA9402 (gl'). Abbreviations: cortex (Co), epidermis (E), endodermis (Ed) and xylem (xylem vessel elements) (X). Magnification X200.

Because of the difficulties associated with comparison between roots at the ultrastructure level, it was decided to compare cells from the tip, middle and basal regions of control (non-transformed) and transformed roots taken from *in vitro* propagated control and chimeric (*A. rhizogenes* LBA9402 transformed) *E. grandis* (gc<sup>i</sup> and gl<sup>i</sup>) and *E. globulus* (gbc<sup>i</sup> and glb<sup>i</sup>) seedlings using transmission electron microscopy. Cells from the root tip region of both *E. grandis* and *E. globulus* control and transformed roots showed no striking ultrastructural differences (Figure 87A1, 87B1, 88A1 and 88B1). Cells appeared metabolically active and were irregularly shaped. They contained cytoplasm, large nuclei (N) and vacuoles (V). Some cells from the middle region of roots from both *Eucalyptus* species examined were seen to contain electron-dense material, thought to be phenolics (P). Transformed and non-transformed cells of *E. grandis* from this region appeared to have more phenolics than cells from a comparable region in *E. globulus* (Figures 87A2, 87B2, 88A2 and 88B2). At the basal region (root stem interface region) of transformed and non-transformed *E. grandis* and *E. globulus* roots, cells also appeared normal. Plastids (Ps) were detected in some of the cells (Figures 87A3, 87B3, 88A3 and 88B3).



**Figure 87.** Transmission electron micrographs of transverse sections through root tip (1), mid-root (2) and root/stem interface (3) regions of control (*gc¹*) (A) and transformed roots (*gl¹*) (B) taken from *in vitro* propagated *E. grandis* seedlings. Eucalypts were transformed with *A. rhizogenes* LBA9402. Abbreviations: nucleus (N); xylem (X); phenolics (P); plastid (Ps); vacuole (V).

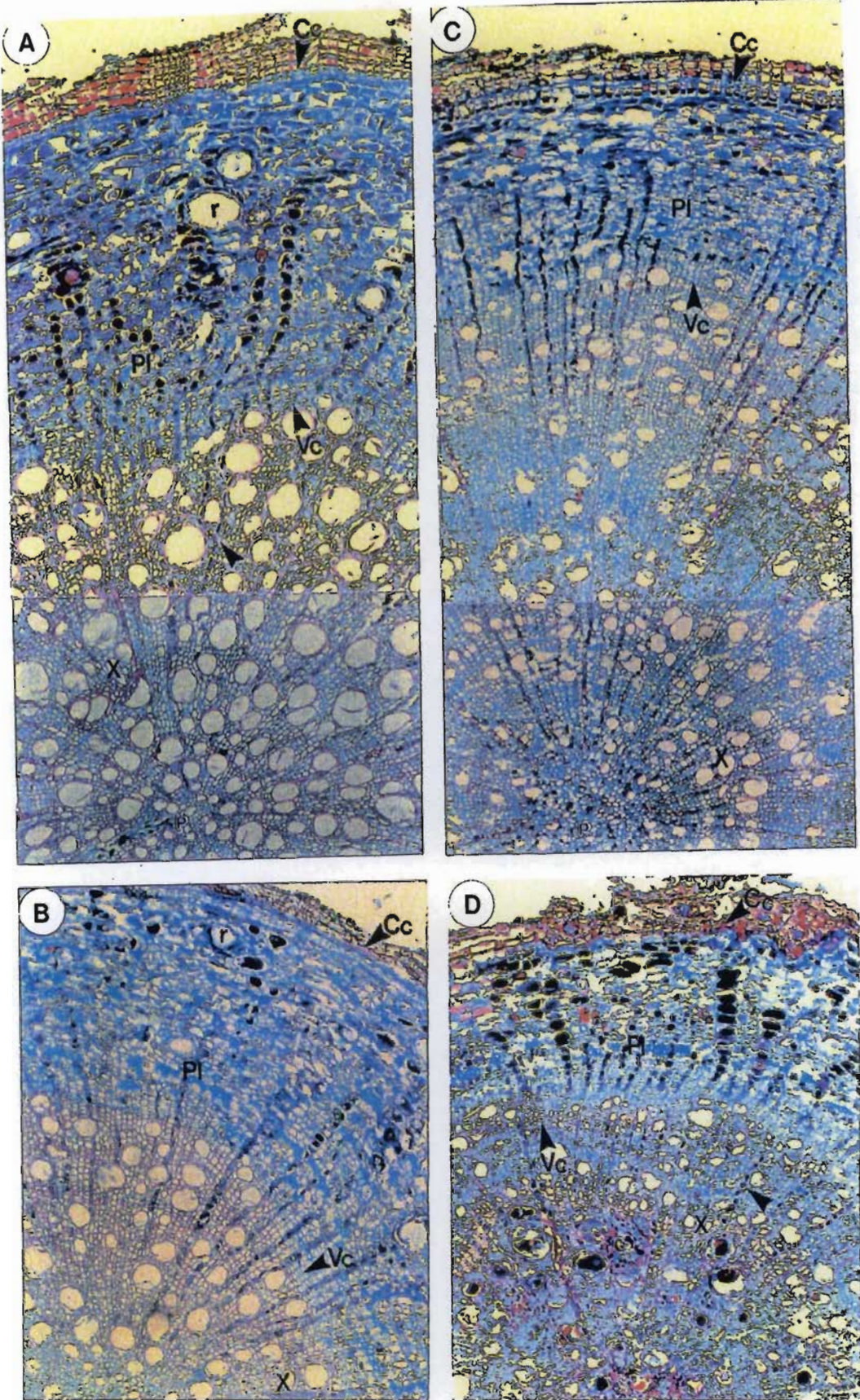


**Figure 88.** Transmission electron micrographs of transverse sections through root tip (1), mid-root (2) and root/stem interface (3) regions of control (*gbc*<sup>1</sup>) (A) and transformed roots (*gb1*<sup>1</sup>) (B) taken from *in vitro* propagated *E. globulus* seedlings. Eucalypts were transformed with *A. rhizogenes* LBA9402. Abbreviations: nucleus (N); xylem (X); phenolics (P); plastid (Ps).

The anatomy of transformed roots taken from one-year-old acclimatised *in vitro* propagated chimeric *E. grandis* and *E. dunnii* plants produced after *in vitro* inoculation with *A. rhizogenes* LBA9402, R1601 and TR8,3 were compared to roots of control plants of the same age and propagated in a similar manner (Figures 89 and 90).

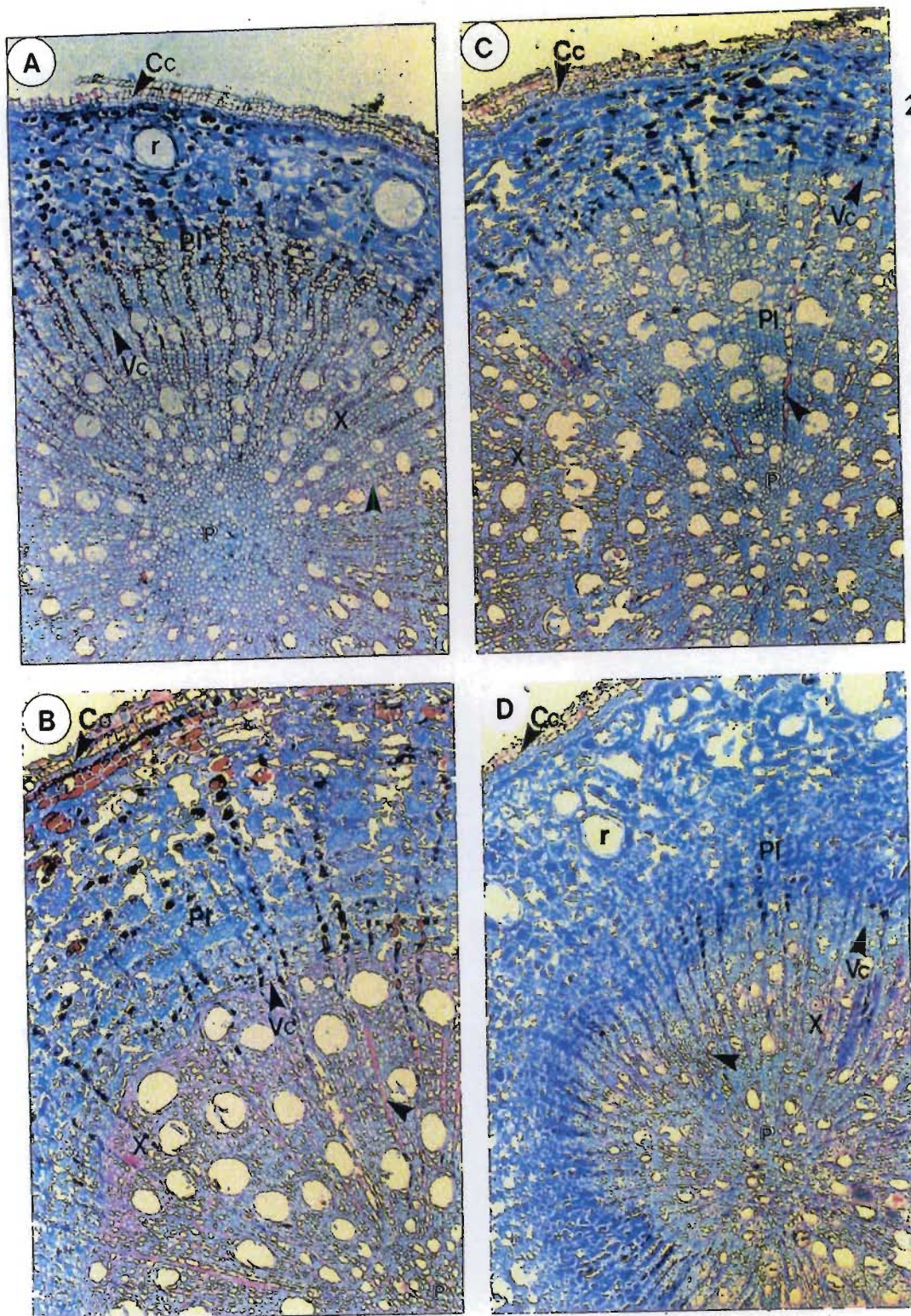
The pith (P) of roots from an *E. grandis* control plant, gc<sub>1</sub><sup>a</sup>, contained clearly visible parenchyma cells. The xylem (X) with clearly visible rays and some secondary growth was visible. The vascular cambium (Vc) was also clearly visible. Phloem (Pl) fibres were distinct (Figure 89A). In the case of the *E. grandis* chimeric plant, gl<sup>a</sup>, the pith (P) was discernable (Figure 89B). Large xylem vessel elements were present in the secondary tissue (Figures 89A and B). The rays were more clearly discernable in transformed (Figure 89B) than non-transformed (Figure 89A) roots. The vascular cambium (Vc) was however, less discernable in transformed (Figure 89B) than non-transformed (Figure 89A) roots. The phloem was well defined, but appeared more compact than that of the control roots. Although resin canals (r) were present in both transformed and non-transformed tissue they appeared to be better developed in the non-transformed tissue (Figure 89A and B). Parenchyma cells were not visible in the pith (P) region of *E. grandis* g2<sub>1</sub><sup>a</sup> (Figure 89C). Secondary xylem (X) growth was also visible in these roots but xylem vessel elements appeared to be somewhat irregularly shaped and occasionally paired. As was the case in *E. grandis*

g1<sup>a</sup> (Figure 87B) the vascular cambium (VC) in the roots of this chimeric eucalypt plant (g2<sub>1</sub><sup>a</sup>) was well defined (Figure 89C). The secondary phloem (Pl) contained many fibres and collapsed parenchyma cells. Parenchyma cells were visible in the pith (P) region of roots from *E. grandis* g3<sup>a</sup> but they were interspersed with primary xylem elements and large, tannin-filled cells (Figure 89D). The secondary xylem (X) vessels were discernable but the region of growth was neither circular nor regularly shaped. Rays were present and distinct, but they were also irregularly arranged. The vascular cambium (Vc) of these roots was not abundant, and was restricted to a single site of 2 to 3 cells. Few phloem fibres were visible in the secondary phloem (Pl) which was irregularly arranged (Figure 89D). Resin canal (r) cells were discernable in the cortex. The cork cambium (Cc) was distinct in all tissue (Figures 89A to 89D).



**Figure 89.** Light micrographs of transverse sections through non-transformed ( $gc_1^a$ ) (A) and transformed (arising from transformations with *A. rhizogenes* strains, LBA9402 ( $g1_1^a$ ) (B), R1601 ( $g2_1^a$ ) (C) and TR8,3 ( $g3^a$ ) (D)) roots of one-year-old acclimatized *in vitro* propagated control and chimeric *E. grandis* plants. Abbreviations: pith (P), xylem (X), vascular cambium (Vc), phloem (Pl) and resin canal cells (r), cork cambium (Cc). Magnification X25.

Parenchyma cells were not clearly defined in the pith (P) region of roots from *E. dunnii* control plants, dc<sup>a</sup> (Figure 90A). The xylem (X) in the control roots of *E. dunnii* was well defined with secondary growth and clearly discernable, regularly arranged rays. Outside the vascular cambium (VC) was a well-developed secondary phloem (Pl) with fibres. In the xylem (X) region of the *E. dunnii* d1<sup>a</sup> roots large vessels and well-developed rays were seen in the secondary tissue (Figure 90B). The vascular cambium (Vc) was also discernable, the phloem (Pl) fibres were extensively represented and the region of secondary phloem was extensive. Clearly visible parenchyma cells interspersed with primary xylem elements were found in the pith region (P) of *E. dunnii* d2<sup>a</sup> (Figure 88C). The xylem vessel elements were smaller and irregularly shaped compared to *E. dunnii* d1<sup>a</sup> (Figure 90B). Xylem rays were discernable and distinct (Figure 90C). The vascular cambium (Vc) was less distinct in this transgenic (d2<sup>a</sup>) root specimen than in the control (dc<sup>a</sup>) roots (Figures 90C and A). Well-defined secondary phloem (Pl) was discernable but considerably compressed. Resin canals (r) were detected in the cortex region (Figure 90C). The pith (P) region of *E. dunnii* d3<sup>a</sup> appeared to contain primary xylem and few parenchyma cells were visible (Figure 90D). Secondary xylem vessels were discernable but irregularly arranged, as were the xylem rays. The vascular cambium (Vc) was not as well defined as in the non-transformed roots. The phloem (Pl) fibres were visible and the cortex region contained resin cells (r). The cork cambium was discernable in all tissue examined (Figures 90A to D).



**Figure 90.** Light micrographs of transverse sections through non-transformed ( $dc^a$ ) (A) and transformed (arising from transformations with *A. rhizogenes* strains, LBA9402 ( $d1^a$ ) (B), R1601 ( $d2^a$ ) (C) and TR8,3 ( $d3^a$ ) (D)) roots of one-year-old acclimatized *in vitro* propagated control and chimeric *E. dun nii* plants. Abbreviations: pith (P), xylem (X), vascular cambium (Vc), phloem (Pl), resin canal cells (r) and cork cambium (Cc). Magnification X25.

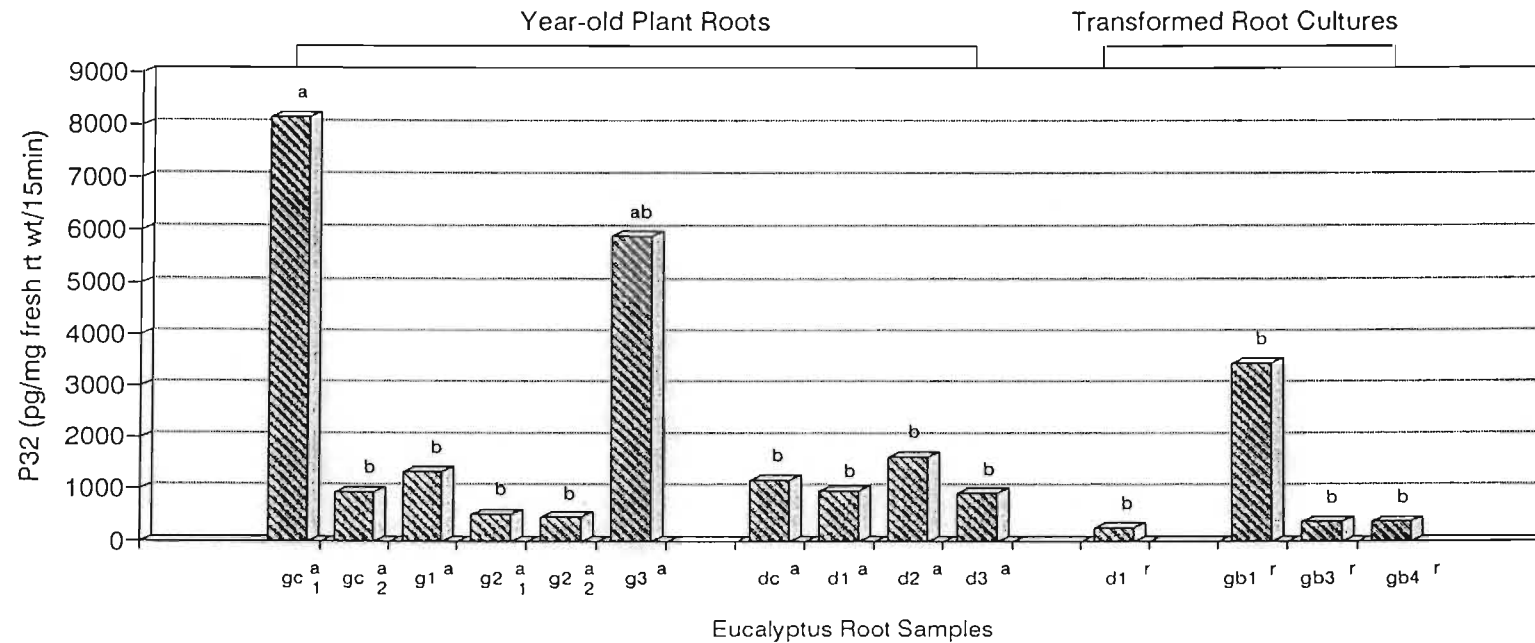
### 6.3.2 Nutrient Uptake Ability of Transformed and Non-transformed Roots

The demand for phosphorous in the one-year-old *E. grandis* control plant  $gc_1^a$  was significantly higher than that of control plant  $gc_2^a$  and for the chimeric plants,  $g1^a$ ,  $g2_1^a$  and  $g2_2^a$ , but was not significantly different from chimeric *E. grandis*  $g3^a$  which had a higher phosphorous demand than the other transformed plants. The difference however, was not significant (Figure 91). No significant difference in phosphorus demand was detected between roots taken from chimeric and control *E. dunnii* plants. There was also no significant difference in phosphorous demand of transformed *E. dunnii* ( $d1^r$ ) and *E. globulus* ( $gb1^r$ ,  $gb3^r$  and  $gb4^r$ ) roots grown in culture.

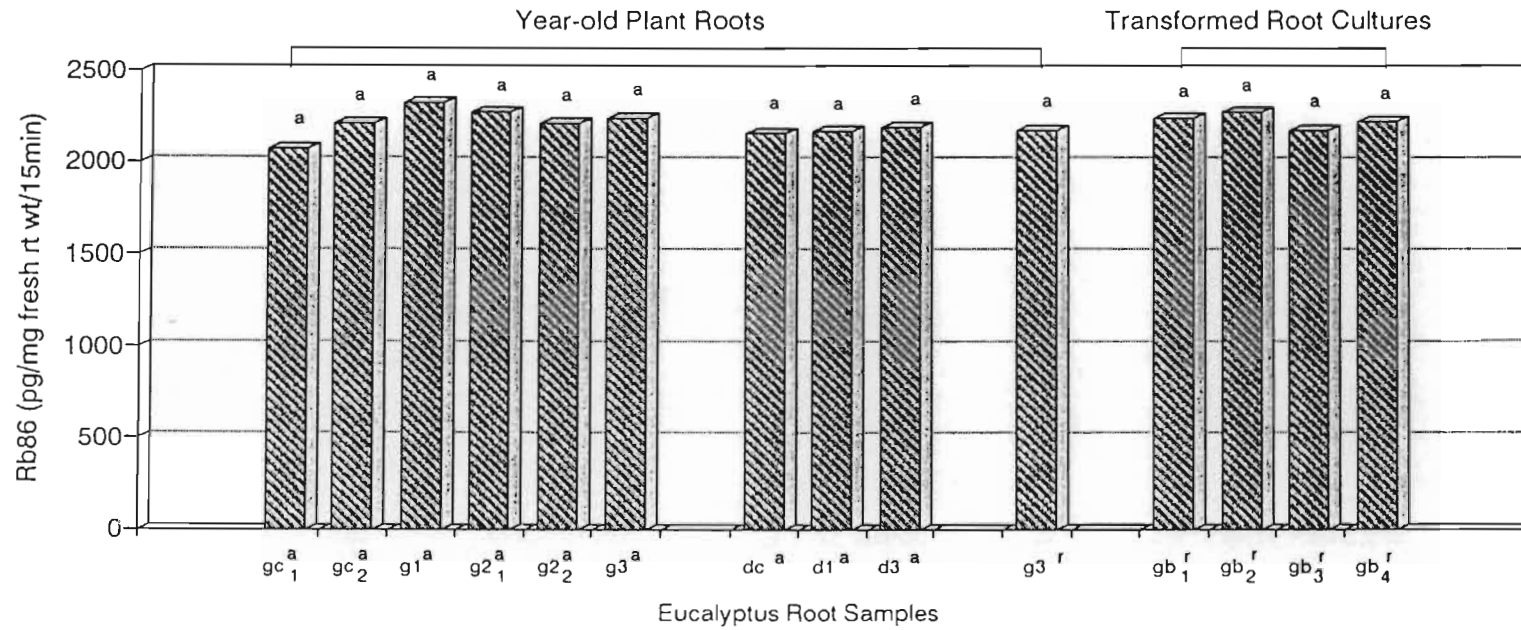
When  $^{86}Rb$  was used as a measure of potassium uptake, no significant differences were observed in the demand for potassium by roots from control and chimeric plants of *E. grandis* and *E. dunnii* and from transformed root cultures of *E. grandis* and *E. globulus* (Figure 92).

The nitrogen uptake ability of the roots from one-year-old *E. grandis* ( $gc_1^a$ ) and *E. dunnii* ( $dc^a$ ) control plants was not significantly different from that of any of the chimeric plants developed from these species, using *A. rhizogenes* strains, LBA9402,

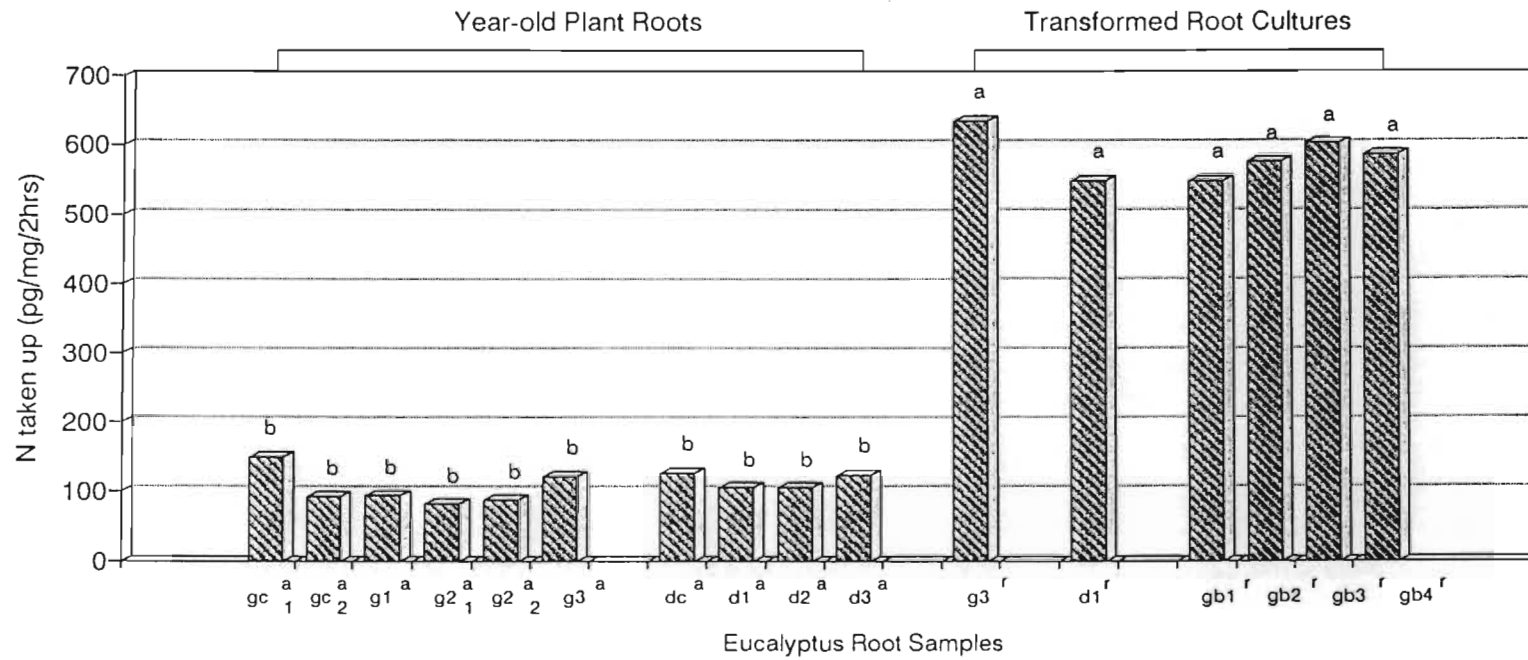
R1601 and TR8,3 (Figure 93). The demand for nitrogen in the transformed root cultures was significantly higher, up to 85 %, than that of the control and chimeric plants grown in the soil. There was, however, no significant difference in the nitrogen uptake ability of the transformed roots from *E. grandis*, *E. dunnii* and *E. globulus* root cultures (Figure 93).



**Figure 91.** Uptake of phosphorous ( $^{32}\text{P}$ ) by excised roots of chimeric and control *Eucalyptus* species grown in pots out doors and of transformed root cultures (Table 18). Based on the Duncan Multiple Range Test any two means having a common letter are not significantly different from each at the 95 % confidence level.



**Figure 92.** Uptake of potassium ( $\text{K } ^{86}\text{Rb}$ ) by roots of chimeric and control *Eucalyptus* species grown in pots out doors and of transformed root cultures (Table 18). Based on the Duncan Multiple Range Test any two means having a common letter are not significantly different from each at the 95 % confidence level.



**Figure 93.** Rates of nitrogen ( $^{15}\text{N}$ ) uptake by excised roots of chimeric and control *Eucalyptus* species grown in pots out doors and of transformed root cultures (Table 18). Based on the Duncan Multiple Range Test any two means having a common letter are not significantly different from each at the 95 % confidence level.

## 6.4 DISCUSSION

Roots taken from control *E. grandis* seedlings propagated *in vitro* exhibited a normal primary structure with the exception of the cortex which was not well defined. Roots from transformed root cultures appeared to be similar in structure except that the vascular bundles were slightly less defined. This may be due to culture conditions, as the transformed roots were growing in liquid culture. The fact that cytological differences were not detected by transmission electron microscopy between non-transformed and transformed roots taken from control and chimeric *in vitro* propagated *E. grandis* and *E. globulus* seedlings is of importance.

The anatomy of roots from one-year-old control (non-transformed) plants of both *E. grandis* and *E. dunnii* exhibited normal secondary growth. Transformed roots from chimeric plants of both these *Eucalyptus* species produced after inoculation with *A. rhizogenes* strains LBA9402 (g1<sup>a</sup> and d1<sup>a</sup>) and R1601 (g2<sup>a</sup> and d2<sup>a</sup>) exhibited similar anatomical characteristics to non-transformed roots. Sometimes slightly irregularly shaped xylem vessel elements were detected in roots developed after *A. rhizogenes* R1601 transformation. The anatomy of roots from chimeric plants (g3<sup>a</sup> and d3<sup>a</sup>) derived after inoculation with *A. rhizogenes* TR8,3 differed somewhat from the non-transformed control roots. Although the roots contained all the individual components of the vascular bundle, the vascular tissue appeared to be somewhat deformed.

These slight anatomical differences detected in certain transformed roots did not markedly influence their nutrient uptake ability. Very few differences were observed between the phosphorous, potassium or nitrogen uptake abilities of both control and transformed roots taken from one-year-old *E. grandis* and *E. dunnii* plants and those of transformed roots from *in vitro* root cultures of *E. grandis*, *E. dunnii* and *E. globulus*. This suggested that the uptake mechanisms for these nutrients had not been adversely affected by the insertion of a section of the Ri plasmid into the plant genome.

The higher phosphorous demand observed in one of the control plants, gc<sup>a</sup><sub>1</sub> and a chimeric plant, g3<sup>a</sup> (with roots transformed by *A. rhizogenes* TR8,3) is probably due to a depletion of phosphorous in the soil in which these plants were growing. Similarly, the high nitrogen demand in *E. grandis*, *E. dunnii* and *E. globulus* root cultures probably resulted from a depletion of nitrogen in the liquid medium in which they were growing as samples were taken just prior to the three weekly subculture period. A period of 20 days had elapsed since the last subculture.

The significantly higher demand for phosphorous by one of the one-year-old *E. grandis* control plants, gc<sup>a</sup><sub>1</sub>, compared to that of the other control plants, gc<sup>a</sup><sub>2</sub>, and the chimeric plants, g1<sup>a</sup>, g2<sup>a</sup><sub>1</sub> and g2<sup>a</sup><sub>2</sub> could possibly be due to greater microbial activity in the rhizosphere of this plant and/or presence of weeds which would

compete for nutrients. This hypothesis could also be used to explain the greater (although not significant) demand for phosphorus by the chimeric *E. grandis* plant, g3<sup>a</sup>, compared to the control plant, gc<sub>2</sub><sup>a</sup>, and the chimeric plants, g1<sup>a</sup>, g2<sub>1</sub><sup>a</sup> and g2<sub>2</sub><sup>a</sup>.

The present results indicate that the integration and expression of part of the T-DNA of the Ri plasmid present in *A. rhizogenes* in plant cells may result in slight anatomical changes depending on the strain of *Agrobacterium* used. These changes did not adversely affect the functionality (nutrient uptake ability) of the root system of the chimeric plants. They should therefore be able to function in a normal way when chimeric plants are grown in the field.

## CHAPTER 7

### APPLICATION OF *AGROBACTERIUM*-MEDIATED ROOTING FOR THE PROPAGATION OF OTHER TREE SPECIES

#### 7.1 INTRODUCTION

The success achieved with *Agrobacterium*-mediated rooting of certain *Eucalyptus* clones lead to a preliminary investigation into the potential use of this technique for rooting two other tree species which are difficult to propagate vegetatively, namely, *Anacardium occidentale* L. (cashew), and *Pinus* (pine hybrids in particular).

Numerous attempts have been made to propagate *Anacardium occidentale* L. by cuttings (NAGESWARO RAO *et al.*, 1988), by air layering (NAGABHUSHANAM and MURTHY, 1979), by grafting (VENKETARAS, 1981; NAGABHUSHANAM, 1983; SESHADRI and RAO RAMA RAO, 1986; SWAKE *et al.*, 1986), and by tissue culture (PHILIP, 1984; LIEVENS *et al.*, 1989). However, none of these techniques has provided a reliable, simple and economic means of vegetative propagation. Rooting and root quality are undoubtedly the limiting factor in vegetative propagation of this species. The cashew tree has an extensive lateral root system and a tap root which penetrates deep into the soil. As *A. rhizogenes* strains

are able to induce numerous secondary functional roots on a range of dicotyledonous plants, the potential use of a broad host range strain, LBA9402 to induce rooting on soft wood cashew cuttings was investigated.

*Pinus* species (softwoods) occupy 648 568 hectares of the land under forestry in South Africa. This represents 50.1 % of the total afforested land. Although pines are predominantly propagated from seed, vegetative propagation by cuttings is also being used. The limiting factor for the propagation of pines and pine hybrids from cuttings is once again the rooting ability of the selected genotypes. Based on the success achieved with *Agrobacterium*-mediated rooting of certain eucalypts a preliminary trial was established to determine if this means of rooting would be useful for propagation of pines under nursery conditions.

## 7.2 MATERIALS AND METHODS

### 7.2.1 Bacteria and Culture Conditions

*Agrobacterium* strains used are listed in Table 13 and their culture conditions are detailed in section 4.2.2.

### 7.2.2 Plant Material

The plant material used in this study is listed in Table 19.

**Table 19.** *Anacardium occidentale* L. and pine hybrid plant material used in this study.

Plant Material	Source
<i>Anacardium occidentale</i> L. (Cashew)	KFC <sup>1</sup> , Durban and IDC <sup>2</sup> , Johannesburg
Pine Hybrid Clones	Mr Andre Nel, CSIR, FORESTEK, Futululu
E862xP. cubensis (P1)	
E26xP.C.N. (P2)	
E38xC6xC15 (P3)	
E1xC58xC5 (P4)	
E589xP.car.hon (P5)	
E17xPch (P6)	
AE218xC162 (P7)	
AE218xC167 (P8)	
E163xC167 (P9)	
E768xP.car.hon (P10)	
E591xP.car.hon (P11)	
E587xP.car.hon (P12)	
Plot104 (P13)	
Plot88 (P14)	
AE134xP.tec.mix (P15)	
E790xP.car.hon. (P16)	

<sup>1</sup> KFC, Kwa Zulu, Finance Corporation

<sup>2</sup> IDC, Industrial Development Corporation

<sup>3</sup> DWAF, Department of Water Affairs and Forestry

### 7.2.3 *In Vivo* Rooting of *Anacardium occidentale* L. (Cashew) Cuttings by Three *Agrobacterium* Strains

Black plastic bags, 12 x 17 cm were filled with sand:soil (2:1) shaken until just firm and placed in the shade tunnel under mist irrigation (10 seconds on/20 minutes off during the day and 10 seconds on/30 minutes off during the night). Softwood apical cuttings, generally one to two leaf pairs plus apex and between 2 to 8 mm in diameter and 6 to 10 cm in length were harvested from potted cashew plants grown outdoors at CSIR, Durban. The larger leaves were cut in half. The cuttings were treated with Benlate and inoculated with *A. rhizogenes* strain LBA9402 as described in section 5.2.4. The cuttings were set in the prepared sand:soil medium to a depth of 2 to 3 cm. They were maintained in the shade tunnel at ambient temperature under mist irrigation (as detailed above) for the 90 day propagation period. Thereafter the number of cuttings that rooted was assessed and expressed as a percentage of the number of survivors. All rooted cuttings were then repotted into large containers.

#### 7.2.4 *In Vivo* Rooting of Cuttings of Sixteen Pine Hybrid Clones by two *Agrobacterium* cocktails

##### Experimental Design

The trial was set up in a randomised block design with one replication as follows:

- 16 Pine hybrid clones (see Table 19)
- 3 Treatments (T1 and T2 (see section 5.2.1), and T3 - Seradix No. 2)
- 8 Cuttings per row plot
- 384 Cuttings

Unigro Speedling trays were filled with pine bark medium and paced in the shade tunnel under mist irrigation (10 seconds on / 20 minutes off during the day and 10 seconds on / 30 minutes off during the night).

*Agrobacterium* bacterial cocktails, T1 and T2 were prepared as described in section 5.2.1.

Apical stem cuttings 10 cm in length were taken from the pine hybrid clone bank at Futululu and transported to Durban in a cooler bag. The cuttings were inoculated by dipping into the bacterial cocktail suspensions for at least 5 to 10 seconds. The control was uninoculated (T3). They were then set in the prepared pine bark medium

and placed in the shade tunnel under mist irrigation as described above. After 60 days the mist irrigation regime was changed to 30 seconds on every hour during the day and 15 seconds on every hour during the night for the 30 days. After 90 days the cuttings were dug up and the percentage survival, percentage rooting, root number and root wet and dry masses were determined. Percentage rooting was expressed as the percentage of survivors that rooted.

### 7.3 RESULTS

#### 7.3.1 *In Vivo* Rooting of *Anacardium occidentale* L. (Cashew) Cuttings by Three *Agrobacterium* Strains

In order to establish whether *Agrobacterium*-mediated rooting could be effective with softwood cashew cuttings, a small preliminary trial was set up to compare the rooting induced by the broad host range *A. rhizogenes* strain, LBA9402, with that induced by Seradix.

Fifty-two percent of the cuttings inoculated with *A. rhizogenes* LBA9402 rooted, while only 4 % of the cuttings treated with Seradix rooted. The chimeric cashew

plants were repotted and appeared to grow normally (Figure 94).



**Figure 94.** Three-month-old chimeric cashew plant.

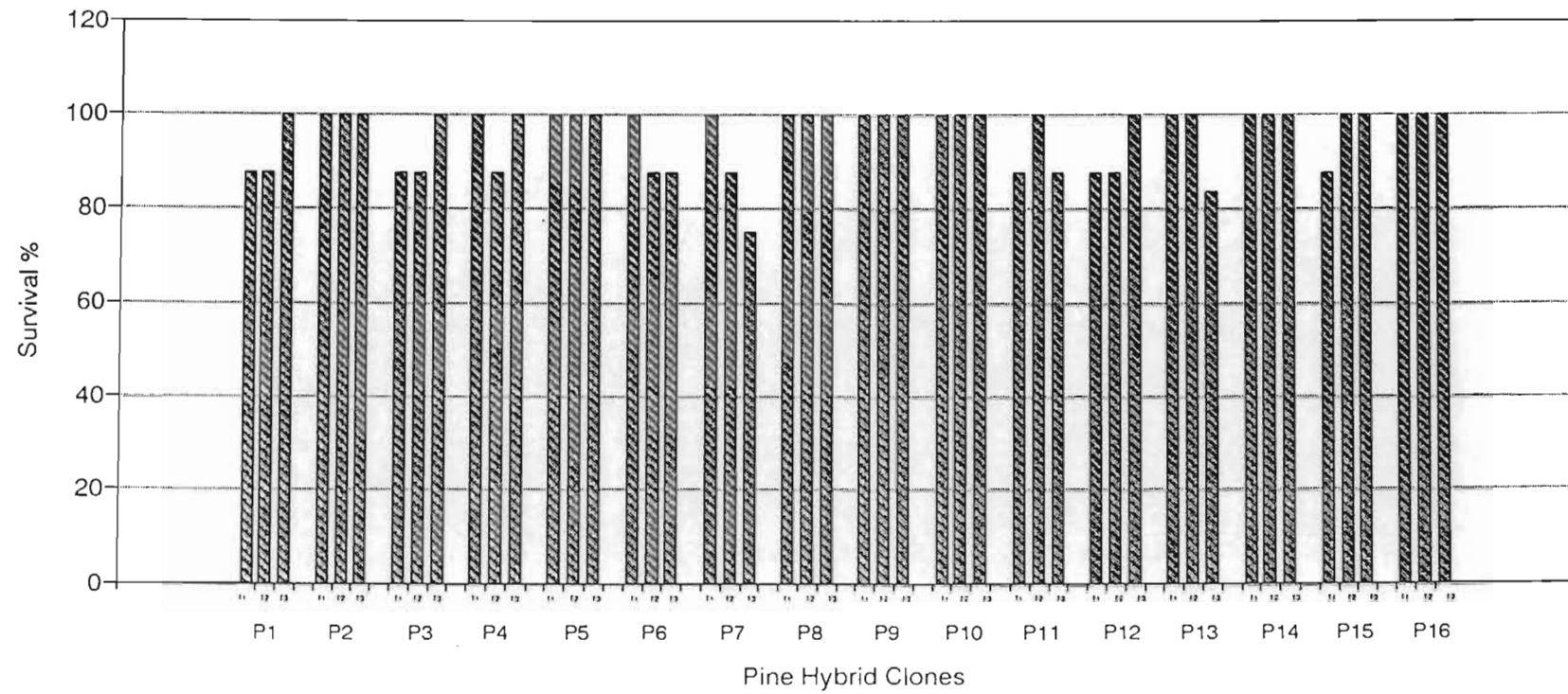
### 7.3.2 *In Vivo* Rooting of Cuttings of Sixteen Pine Hybrid Clones by two *Agrobacterium* cocktails

In order to establish whether *Agrobacterium*-mediated rooting could be effective for pine hybrid cuttings, a small preliminary trial was set up to compare the inherent rooting ability of sixteen pine hybrids with rooting by two *Agrobacterium* cocktails. Pine cuttings are not normally rooted with Seradix, hence a Seradix treatment was not included.

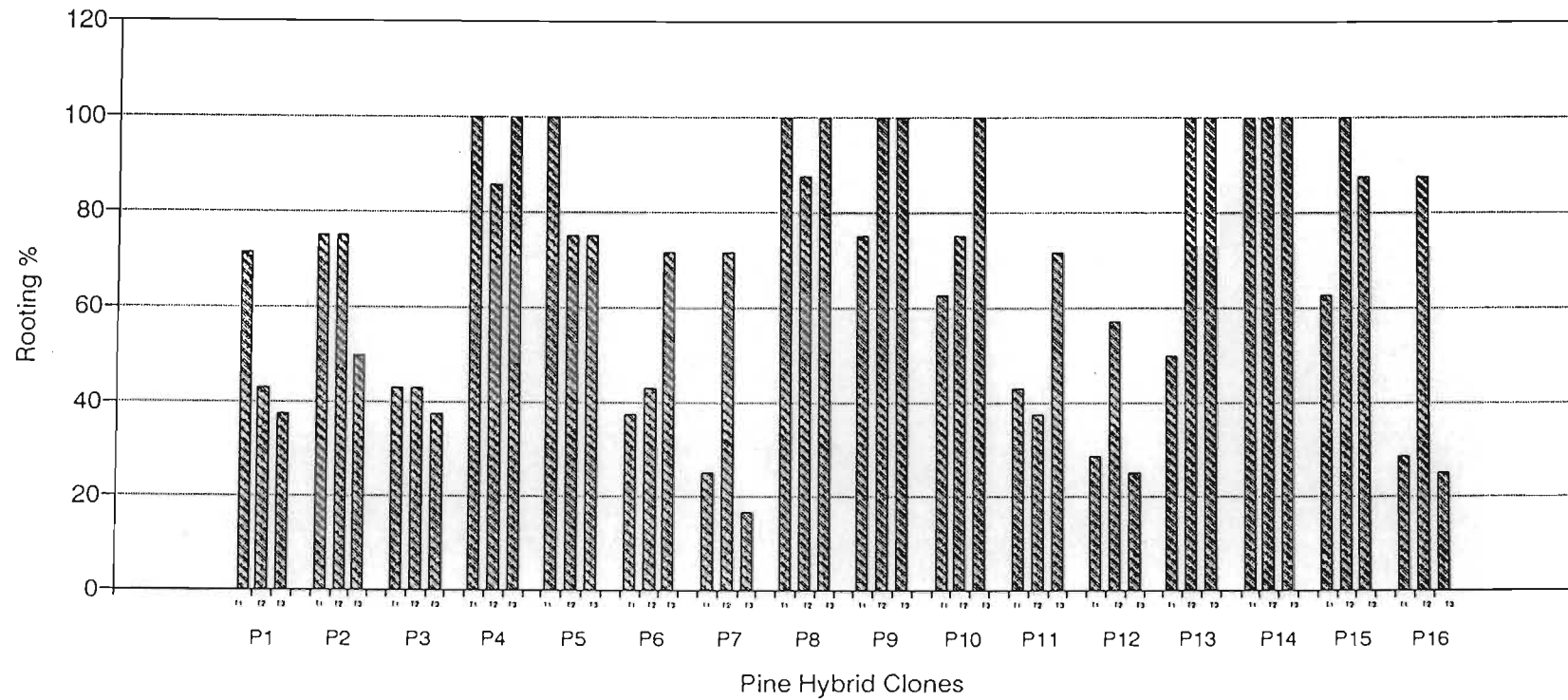
Survival of the six pine hybrid clones was excellent ranging from 75 to 100 % (Figure 95). Survival of cuttings of clones P2, P5, P8, P9, P10, P14 and P16 was not affected by treatment. Survival was reduced slightly, by 10 %, after treatment of clones P1, P3, and P12 with bacterial cocktails T1 and T2. Survival of clone P4 was lower after treatment T2 only and treatment T1 lowered the survival of clone P15. The survival of clone P6 was lower after treatments T2 and T3 compared to treatment T1 and that of clone P11 after treatments T1 and T3 compared to treatment T2. There was no difference in survival of clone P13 after treatments T1 and T2, however, survival of this clone was lower after treatment T3 (the control). The control treatment (T3) also resulted in the lowest survival rate (75%) for clone P7. Eighty-eight percent survival was achieved after treatment T2 and 100 % after treatment T1 for this clone. Whether these differences are statistically significant has

yet to be proven. Further statistical trials will have to be set up before any conclusions can be made on whether the bacterial cocktail treatments could have beneficial or adverse effects on survival of certain pine hybrids.

Treatment of clone P1 with bacterial cocktail T1 resulted in the highest rooting percentage (72 %) compared to bacterial cocktail T2 (43 %) and the inherent rooting ability (T3) (38 %) of this clone (Figure 96). Both bacterial cocktails T1 and T2 improved the rooting ability of clone P2, by 25 % compared to T3. A similar trend was observed for clone P3 but the increase in rooting was only 5 %. There was no difference between the inherent T3- and bacterial cocktail T1-induced rooting abilities of clone P4, however, bacterial cocktail T2-induced rooting was 12 % lower than these. This was also found to be the case in clone P8. Bacterial cocktail T1-induced rooting of clone P5 was 25 % better than this clone's inherent rooting ability (T3) and that achieved with bacterial cocktail T2. Rooting induced by both bacterial cocktails (T1 and T2) were up to 33 % lower than the inherent rooting ability (T3) of clone P6, while for clone P7 the inherent rooting ability and bacterial cocktail T1-induced rooting ability were 58 and 50 % lower respectively, than that achieved with bacterial cocktail T2. The same trend was observed in clones P12 and P16 where rooting by T2 was up to 32 and 63 % greater respectively, than that achieved with the other treatments. In the case of clones P9 and P13, bacterial cocktail T1-induced rooting was lower than that achieved with the other treatments,



**Figure 95.** Survival (%) of stem cuttings of sixteen pine hybrid clones (see Table 19), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 (untreated).

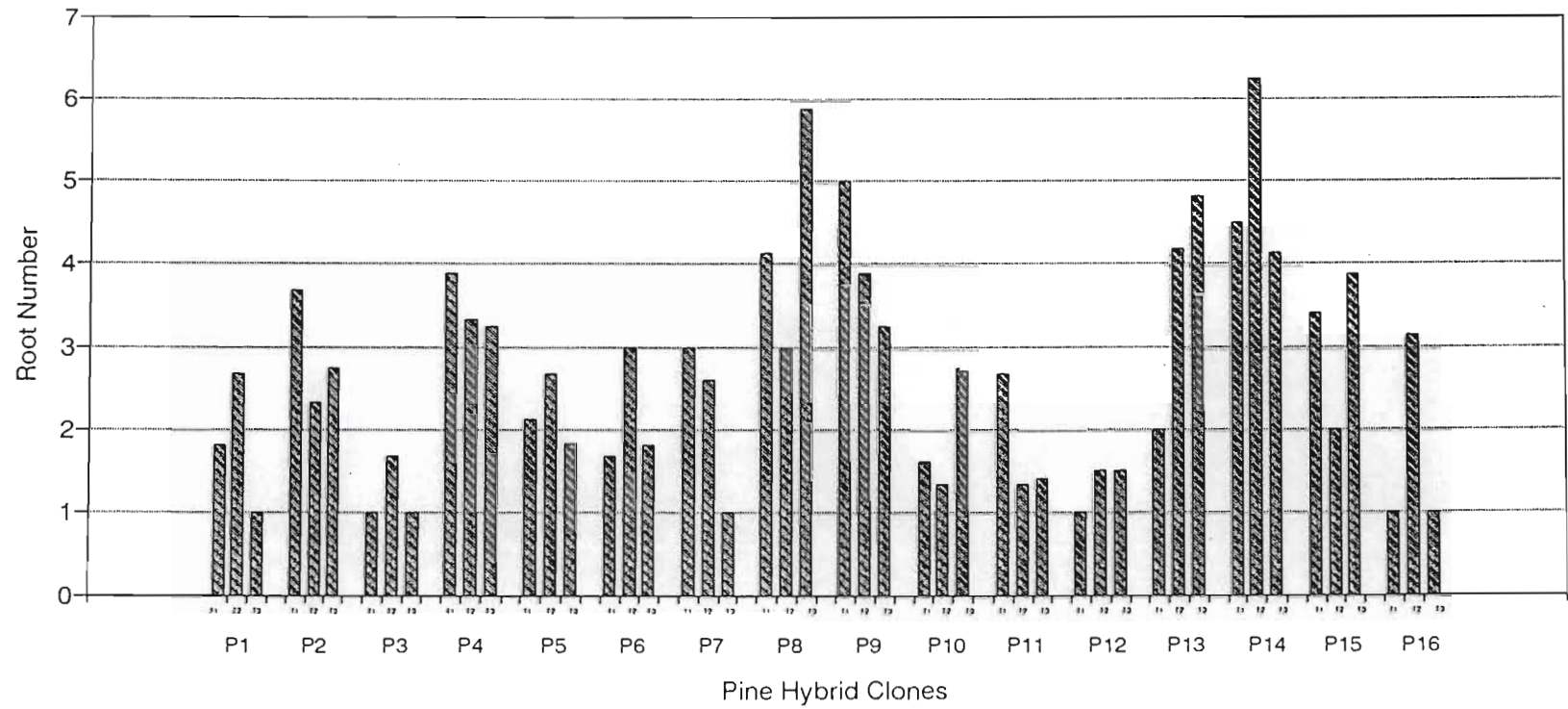


**Figure 96.** Rooting ability (%) of stem cuttings of sixteen pine hybrid clones (see Table 19), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 (untreated).

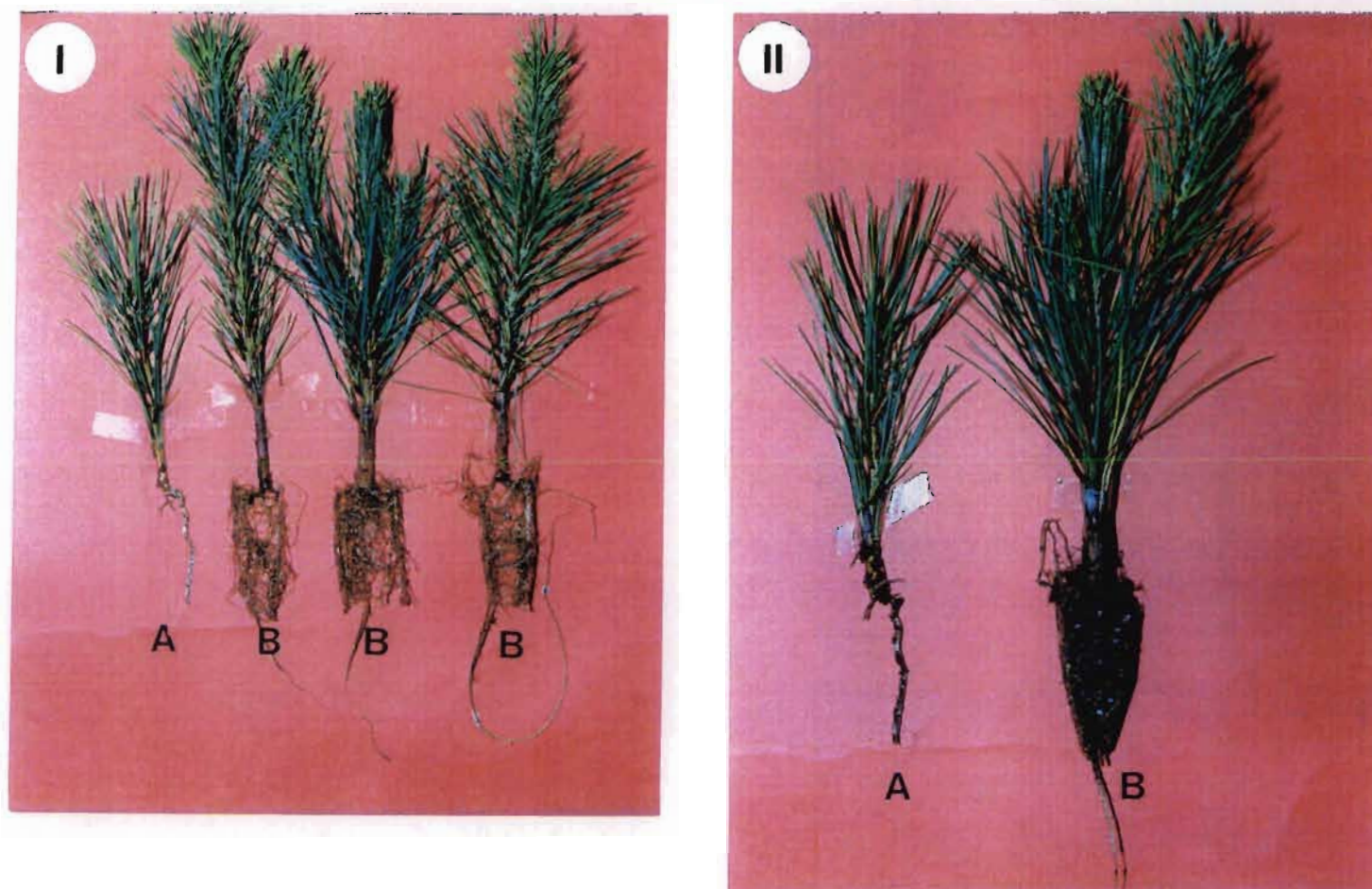
for which 100 % rooting was achieved. Both bacterial cocktail treatments (T1 and T2) appear to reduce the rooting ability of P11 compared to its inherent rooting ability (T3). Rooting ability of clone P14 was unaffected by treatment, with 100 % rooting being achieved throughout. Finally, for clone P15 the best rooting was achieved after treatment T2 (100 %). Rooting after treatments T1 and T3 were 37 and 12 % lower respectively (Figure 96).

The average number of roots per rooted surviving cutting varied from clone to clone and was influenced by the treatment (Figure 97). The average number of roots for clone P1 was greatest after bacterial cocktail treatment T2 (2.7) and this was followed by treatment T1 (1.8) and then treatment T3 (1). This suggests that bacterial cocktail treatments T1 and T2 were able to enhance root formation compared to the inherent rooting ability of this clone. Treatment of clone P2 with bacterial cocktail T1 resulted in the greatest number of roots per rooted cutting (3.7) compared to the other treatments (T2 - 2.3 and T3 - 2.7). A similar trend was found for P11. In the case of clones P3, P6, P14 and P16 similar trends were found with treatment T2 yielding the greatest root number. Where detectable, the differences between the number of roots arising after treatments T1 and T3 were slight. A 3.2 fold increase in root number was found after treatment of clone P16 with bacterial cocktail T2 compared to the other two treatments (Figure 97). Figure 98 I and II shows the marked improvement in root quality and root plug firmness of clone P16 after application of bacterial cocktail T2, compared to this clone's inherent rooting ability (T3). Treatment T1 yielded the greatest number of roots for clone P4 followed by treatments T2 and T3. For clone P5 the greatest root number was obtained after treatment T2. Treatment T1 produced slightly more roots than treatment T3 for this clone. The greatest number of roots were found on clone P7 inoculated with bacterial cocktail T1, however, both bacterial cocktails increased the number of roots per cutting of this clone, by up to 3 fold, compared to that achieved

on control cuttings (T3) (Figure 97). A similar trend was observed for clone P9. These results suggest that the bacterial cocktails could increase root numbers on cuttings of certain pine hybrids. The average number of roots arising on cuttings of clone P8, treated with bacterial cocktails T1 and T2 were less, by 1.4 and 1.9 fold respectively, than that found on untreated (T3) cuttings. The same trend was found for clone P10. The root number on cuttings of clone P12 after treatments T2 and T3 were the same and 1.5 fold greater than that obtained after treatment T1. In the case of clone P13, bacterial cocktail treatment T1 yielded the lowest root number per cutting (2), followed by T2 (4.2) and T3 (4.8), suggesting that these bacterial cocktails may have an inhibitory effect on root development in certain pine hybrid clones. Finally, the greatest root number was achieved on the control uninoculated (T3) cuttings of clone P15. The number of roots arising after treatment of this clone with T1 and T3 were 1.75 and 1.9 fold greater than that obtained after treatment T2 for this clone.

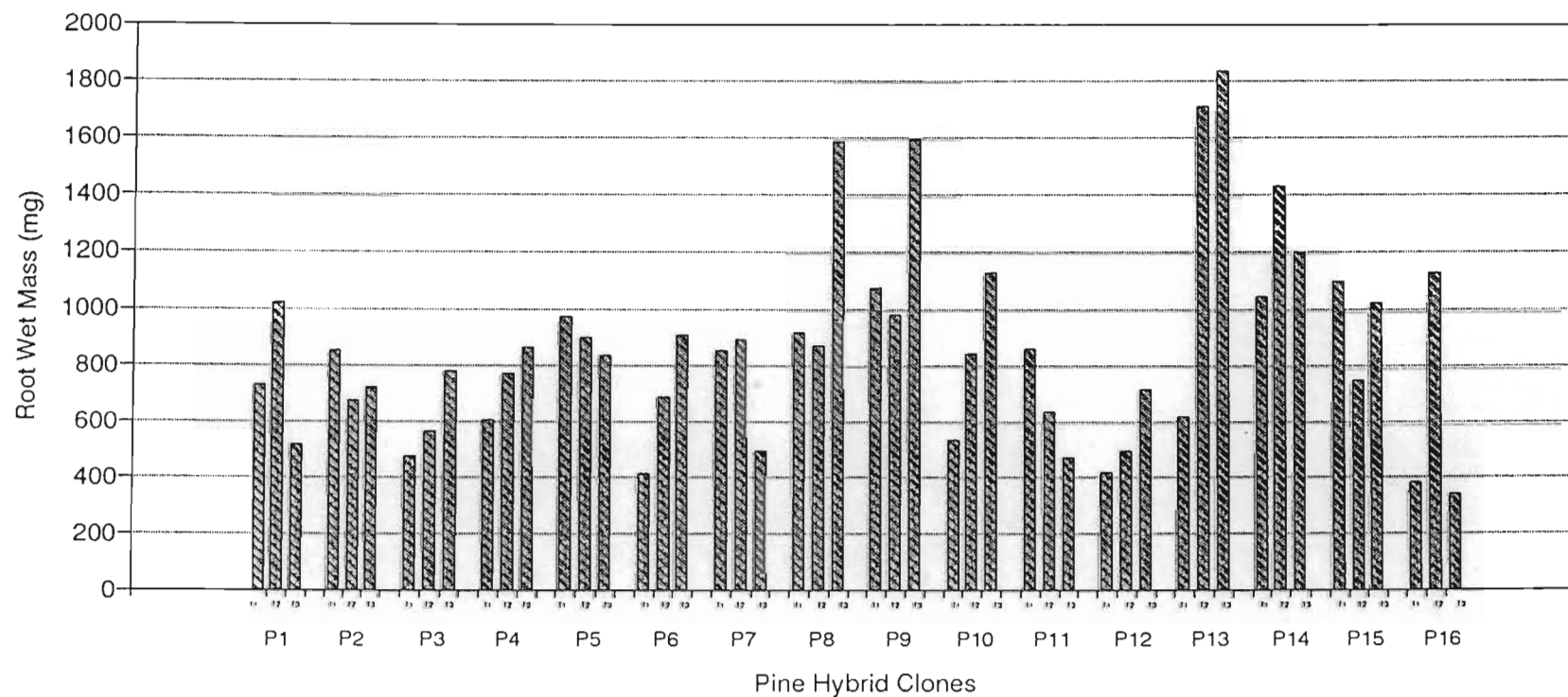


**Figure 97.** Average number of roots on rooted stem cuttings of sixteen pine hybrid clones (see Table 19), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 (untreated).



**Figure 98.** A comparison of the root quality (I) and plug firmness (II) of pine hybrid clone P16 cuttings, 90 days after control treatment T3 (uninoculated, no hormones) (A) and treatment with bacterial cocktail T2 (B) (see section 5.2.1).

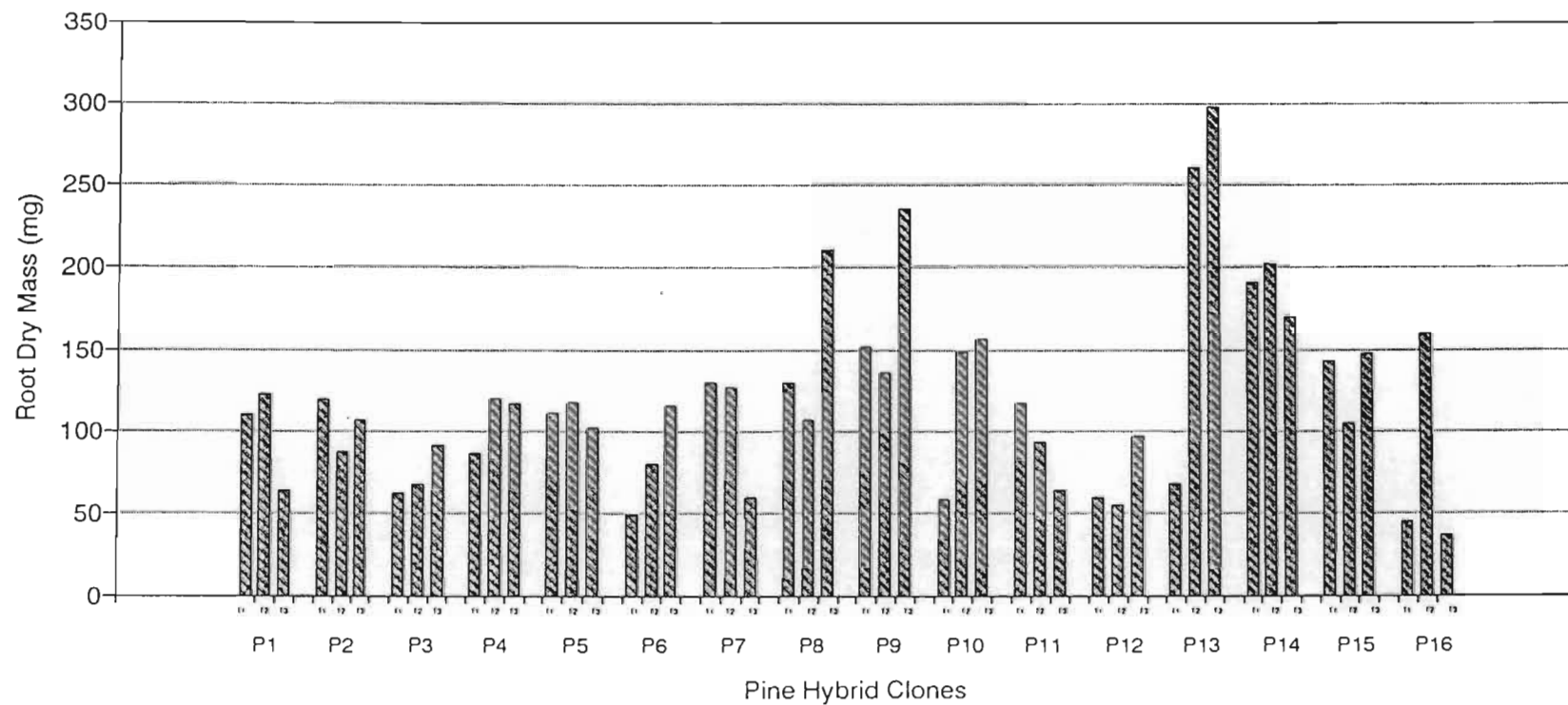
The greatest average wet masses of roots from clones P1 and P16 were achieved after treatment T2, up to 2.4 and 3.3 fold greater respectively, than that achieved after the other treatments. The lowest root wet mass for these clones was achieved after treatment T3. (Figure 99). This suggests that the bacterial cocktails could be instrumental in increasing secondary root formation and therefore root biomass. Bacterial cocktail treatment T1 also yield greater root wet masses for these clones than did treatment T3. In the case of clone P14, treatment T2 also yielded the best root wet mass (1420 mg), however, unlike the above mentioned clones the next best root wet mass was achieved after treatment T3 (1200 mg), while treatment T1 yielded the poorest wet mass of roots (1040 mg). The best root wet masses for clones P2, P5, P11 and P15 were achieved after treatment T1. The differences between root wet masses of clones P2 and P5 after treatments T2 and T3 were however, small (Figure 99). On the other hand, in the case of clone P11, treatment T2 yielded a better root wet mass than treatment T3, while for clone P15 the treatment T3 root wet mass was greater than that achieved with treatment T2. The average root wet masses of clones P3, P6, P10 and P12 after treatments T1, T2 and T3 showed similar trends, with root wet masses after treatment T3 being greater than those obtained after treatment T2, which in turn were greater than those achieved after treatment T1. Treatment T3 also yielded the best root wet masses for clones P8, P9, and P13. In the case of clones P8, P9 and P12 the root wet masses after treatment T3 were up to 1.8 fold greater than those achieved after treatments



**Figure 99.** Average wet mass of roots from cuttings of sixteen pine hybrid clones (see Table 19), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 (untreated).

T1 and T2. The differences between treatments T1 and T2 for these clones were however, small. Compared to treatment T3, the root wet masses of clone P10 decreased by 1.2 fold and 1.9 fold after treatments T2 and T1 respectively. For clone P13 the difference in average root wet mass achieved after treatments T3 and T2 was small. However, they were up to 3 fold greater than that achieved after treatment T1. Finally, the root wet masses of cuttings from clone P7 after treatments T1 and T2 were similar (850 and 880 mg respectively), but up to 1.8 fold greater than that achieved after treatment T3.

Similar root dry mass results were achieved for clone P1 after treatment T1 and T2, and these were up to 1.9 fold greater than that achieved after treatment T3 (Figure 100). A similar trend was found for clones P5 and P14, however, the difference between the two bacterial cocktail treatments (T1 and T2) and the uninoculated control treatment (T3) were smaller (up to 1.1 and 1.2 fold for clones P5 and P14, respectively) than that achieved for clone P1. In the case of clone P16 treatment T2 also yielded the greatest root dry mass, 3.3 and 4.6 fold greater than that achieved with treatments T1 and T3 respectively. The root dry masses of clones P3, P6, P10 and P13 showed similar trends, with treatment T3 yielding the greatest root dry masses, treatment T2 the next greatest and treatment T1 the lowest root dry masses. The differences in root dry masses, between these treatments, did however, differ from clone to clone (Figure 100). For example, the difference between treatments



**Figure 100.** Average dry mass of roots from cuttings of sixteen pine hybrid clones (see Table 19), 60 days after treatment with two bacterial cocktails T1 and T2 (see section 5.2.1). Control treatment T3 (untreated).

T2 and T3 for clone P13 was only 1.3 fold while the difference between treatments T3 and T1 was 4.6 fold. Treatment T3 also yielded the best root dry masses for clones P8, P9 and P12. Treatment T1 produced slightly better root masses than treatment T2 for this clone. The root dry masses of clone P2 were greatest after treatment T1. Treatment T3 gave the next best results for this clone and treatment T2 the lowest root dry mass. In the case of clones P7 and P11 the bacterial cocktail treatments T1 and T2 yielded better root dry masses than the control treatment T3. The difference between the two bacterial cocktail treatments was smaller in the case of clone P7 than clone P11. In the case of clone P4 the average root dry mass after treatments T2 and T3 were similar but greater than that achieved after treatment T1. Similar trends in root dry mass were observed in clones P2 and P15, in that treatment T1 and T3 yielded similar root dry masses although in the case of clone P2 treatment T1 produced slightly better results than treatment T3. For clone P15 however treatment T3 produced slightly better results than treatment T1. In both these clones bacterial cocktail treatment T2 yielded the lowest root dry masses.

#### 7.4 DISCUSSION

The establishment of cashew plantations in South Africa has been initiated by IDC

(Industrial Development Corporation) and KFC (Kwa Zulu Finance Corporation). This led to a preliminary investigation into the potential use of *Agrobacterium*-mediated rooting as a means of propagating cashew cuttings. The broad host range *A. rhizogenes* strain LBA9402, was able to induce rooting on softwood apical cashew cuttings, suggesting that this method of rooting could have a role to play in vegetative propagation of cashews. Research on propagating cashew cuttings using softwood cuttings from both juvenile and mature trees using bacterial (*Agrobacterium*) cocktails (results not shown) has also shown promise. Further research is however, necessary before its value in vegetative propagation of cashews can be assessed.

The results from the preliminary pine hybrid transformation cutting trial showed that as with *Eucalyptus*, the rooting of pine hybrid cuttings is under genetic control. Bacterial cocktails were able to overcome the genetic control on the inherent rooting abilities and improve root quality of some, but not all, of the pine hybrid clones tested. Further statistical trials are necessary before conclusions can be drawn on the value of this method of rooting in pine hybrid propagation. However, from the dramatic improvement in rooting of certain pine hybrid clones, such as clone E790xP.car.hon. (P16), by the bacterial cocktails, it seems likely that this method of rooting could have a valuable role to play in the propagation of pine hybrid clones from cuttings.

## GENERAL DISCUSSION AND CONCLUSIONS

Of the potential biocontrol strains tested, two, *A. tumefaciens* J73 and H6, showed promise as biocontrol agents for crown gall disease of grapevines. The results from the *in vitro* biological control assessment of J73 clearly showed that this strain produces a potent agrocin which has a broad activity spectrum. However, it was unable to control biotype 3 pathogens, which induce crown gall on grapevines, *in vivo*. The poor long term colonisation ability of J73 was found to be the reason for this and was also the reason for ineffectiveness against biotype 1 and 2 pathogens, *in vivo*. *A. tumefaciens* J73 therefore had no value in the control of crown gall disease on grapevine. However, as agrocin J73 was effective against biotype 3 pathogens, it may be worth inserting the agrocin J73 plasmid into a good biocontrol strain (such as H6 or K84) which can colonise plants efficiently. Although H6 produced a narrow activity spectrum agrocin, being effective against biotype 3 pathogens only, it was a good longer term coloniser of the grapevine rhizosphere. H6 therefore has great potential as a biocontrol agent for grapevine crown gall disease and field testing should be carried out to confirm this finding.

The importance of colonisation in the biological control of crown gall disease by non-pathogenic biocontrol strains was clearly demonstrated by the biological control research results. Thus the ability of a potential biocontrol strain to colonise host

plants efficiently should be one of the initial and major criteria in selecting new potential biocontrol strains.

It should however be noted that, as suggested by WEBSTER (1990), there is a possibility that the solution to the problem of controlling crown gall could have become too complicated. BURR *et al* (1989) described a hot water treatment of dormant grape cuttings that eradicated biotype 3 pathogens or at least reduced them to a non-detectable level. Although such a simple, practical and economic solution may not provide full protection against the disease in grapevines, more of this type of solution should be considered and investigated before lengthy and costly research is undertaken to obtain or engineer those elusive new biocontrol agents.

The limiting factor in the vegetative propagation of *Eucalyptus* species from cuttings is the rooting ability of the selected material. The use of tissue culture to rejuvenate and improve rooting of *Eucalyptus* was explored. Results showed that rooting of certain clones/genotypes could be improved in this manner. However, certain clones developed *in vitro* did not root well. The potential use of *Agrobacterium*-mediated rooting was therefore investigated.

Research results showed that despite complex interaction among genetic and physiological variables, *A. rhizogenes* can be used to propagate eucalypts under both

*in vitro* and nursery conditions. The resulting plants are chimeric, having Ri T-DNA in all or some of their roots. Their aerial parts are of normal morphology. This substantiated the finding of LAMBERT and TEPFER (1991) who proposed the use of *A. rhizogenes* for the propagation of apples. The research findings presented in this dissertation however, differ from those of LAMBERT and TEPFER (1991), in that *A. rhizogenes* strains were found to be able to overcome the low inherent rooting ability of certain plant genotypes. No differences were found in the anatomy and nutrient uptake abilities of the transformed and non-transformed roots. This molecular approach to rooting also showed promise for the propagation of cashew and pine hybrid cuttings.

The genotype of both the bacterium and the plant was shown to be critical to the success of *Agrobacterium*-mediated rooting. The limiting factor in *Agrobacterium*-mediated rooting is therefore the host range of the bacterium. As for inherent rooting and auxin-induced rooting, the propagation conditions and condition of the plant material were found to influence the success of this means of rooting. The novel use of bacterial cocktails to overcome the host range limitations of individual *A. rhizogenes* strains showed that bacterial cocktails were able to improve rooting of certain, but not all, *Eucalyptus* genotypes. The host range of the bacterial cocktails will be governed by the host ranges of the individual bacterial strains that make up the cocktail.

The *A. rhizogenes*-induced root system induces a secondary rhizosphere near the surface of the soil, composed of densely packed, horizontally radiating roots. The possible advantages of this secondary root system might be better anchorage, ability to resist anoxia from flooding of the soil, increased drought resistance due to high root density and greater chances of interactions with mycorrhizal soil fungi, which establish exchange organs in the tissue produced by a growing root tip (TEPPER 1983) all of which are important in tree species.

Future research into the use of *Agrobacterium*-mediated rooting of plant species should include a study of the role of colonisation, as the ability of the *A. rhizogenes* strain to colonise the wound site and induce rooting undoubtedly plays a critical role in successful transformation and root induction.

In conclusion the research data presented in this dissertation demonstrate the potential of *Agrobacterium* species as tools in plant improvement. The use of non-pathogenic *A. tumefaciens* strains is a viable means of controlling crown gall disease, while *A. rhizogenes* has great potential as a means of improving rooting and therefore propagation potential of a range of dicotyledonous plant species. Much research is however, still needed, as the precise mechanisms involved in the transfer of DNA from *Agrobacterium* to plant cells is incomplete.

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