The actions of, and interactions between, auxins and cytokinins and their effect on *in vitro* rooting of selected *Eucalyptus* clones

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Authentication:

As the candidate's supervisor I have approved this thesis for submission.

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ABSTRACT

Clonal propagation of *Eucalyptus* spp. and its hybrids allows for competitiveness in the commercial forestry industry through the propagation and preservation of superior/elite genotypes. Vegetative propagation through rooted cuttings is the industry's standard and the choice of clones selected for plantations are determined by their rooting ability. However, as many potentially valuable genotypes are recalcitrant to adventitious rooting, micropropagation is the only effective means of propagating them. Micropropagation results in high plantlet yields, achieved primarily through the empirical use of the key plant growth regulators (PGRs) cytokinins and auxins, for shoot and root production, respectively. Their selection for use *in vitro* is driven by their effects on percent rooting rather than root quality. Little is known regarding the quality of the roots of the plantlets *ex vitro*, but there is some evidence that they are different from those of seedlings and cuttings. It was therefore hypothesized that the properties of exogenous PGRs and their interaction with other exogenous and endogenous PGRs, influenced root development and subsequent root quality. This was tested *in vitro* using a good-rooting *E. grandis* (TAG31) and two poor-rooting *E. grandis* x *nitens* hybrid clones (GN155 and NH58). In the former, the auxins supplied during the pre-rooting culture stages (multiplication and elongation) were sufficient for 100% rooting in an auxin-free rooting medium. Different combinations of PGRs in the two pre-rooting stages, followed by rooting without auxins, revealed a direct relationship between the stability of the supplied auxin and the rooting ability of TAG31. Gas chromatography-mass spectrometry (GC-MS) analyses indicated that endogenous shoot levels of indole-3-acetic acid (IAA) influenced graviperception. Also, low IAA content was associated with atypical starch grain accumulation or its absence from root tips (53.1 nmol IAA g^{-1} DW compared with 325.7 nmol IAA g^{-1} DW in gravisensing roots). The specific roles of the natural auxins IAA and IBA on root morphogenesis were then investigated using 2,3,5-triiodobenzoic acid (TIBA; inhibits IAA transport), ρ-chlorophenoxyisobutyric acid (PCIB; inhibits auxin signal transduction), and the auxin antagonist kinetin in the rooting medium, following root induction. After 3 weeks, the mean root diameter was significantly reduced from 552.8µm (control) to 129.2µm (with PCIB) and 278.6µm (with kinetin). TIBA increased root diameter to 833.4µm, decreased Δ root length,

increased root vasculature and resulted in agravitropism. Hence, whereas rooting could be induced by IBA, IAA was necessary for the maintenance of vascular integrity and graviperception. This critical role of IAA in root development is of importance as IBA, owing to its higher stability, has been traditionally relied upon for root induction in the majority of micropropagation protocols.

The potential of incorporating IAA into the media formulations of *in vitro* protocols for poor-rooters that do not respond well to IAA was then investigated, using GN155 and NH58. While PCIB in the rooting medium of GN155 completely inhibited rooting, the addition of dihydroxyacetophenone (DHAP), an inhibitor of auxin conjugation, to the rooting medium, did not significantly increase % rooting in the presence of 0.1 mg l^{-1} IBA (i.e. 50% rooting with 2mM DHAP and IBA, compared with 45% with IBA alone). The results suggested that the inability of some eucalypts to induce roots easily *in vitro* was not due to a deficiency in auxin signal transduction or to auxin conjugation. Instead, rooting was inhibited by an accumulation of kinetin within shoots during the pre-rooting culture stages. The endogenous levels of PGRs in shoots of GN155 and NH58 showed a strong relationship (R^2 = 0.943) between the shoot kinetin:auxin and shoot rootability. Substituting kinetin with the relatively less stable natural cytokinin *trans*-zeatin in the elongation stage resulted in a significant increase in % rooting in both clones, from 19% to 45% (GN155) and from 31% to 52% (NH58), with 0.1 mg l^{-1} IAA in the rooting medium. However, omitting all cytokinins from the elongation medium, resulted in over 95% and 75% rooting of shoots of GN155 and NH58, respectively, with 0.1 mg l^{-1} IAA.

These results suggest that IAA is a requirement for root development and cannot be substituted by its analogues in certain root developmental events. Hence, IAA should be the preferred auxin for eucalypt micropropagation. As fundamental research, the approach taken in this study circumvents the empirical method used in improving micropropagation protocols. The importance of the properties and the interactions between endogenous and exogenous PGRs in regulating root morphogenesis, and the practical implications of these findings is emphasised.

DECLARATIONS

Declaration 1: Plagiarism

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Declaration 2: Publications

Publication 1: Nakhooda M, Watt MP, Mycock D (2011) Auxin stability and accumulation during *in vitro* shoot morphogenesis influences subsequent root induction and development in *Eucalyptus grandis*. Plant Growth Regulation 65: 263-271.

Contributions: MN carried out the experimental work, recorded data and wrote the manuscript. MPW and DM were supervisors and edited the manuscript.

Publication 2: Nakhooda M, Watt MP, Mycock D (accepted February 2012) The properties and interaction of auxins and cytokinins influence the rootability of *Eucalyptus* clones *in vitro*. Plant Cell, Tissue and Organ Culture.

The corrected manuscript, as per referees' request, has been accepted by Plant Cell, Tissue and Organ Culture for publication.

Contributions: MN carried out the experimental work, recorded data and wrote the manuscript. MPW and DM were supervisors and edited the manuscript.

Signed: Date:

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LIST OF ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
AG	Altered graviperception
BAP	Benzylaminopurine
DHAP	Dihydroxyacetophenone
FAP	6-Furfurylaminopurine
GA_3	Gibberellic acid
GC-MS	Gas chromatography-mass spectrometry
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
KI	Potassium iodide
MS	Murashige and Skoog (1962)
NAA	α-Naphthalene acetic acid
PAST	Paleontological statistics
PCIB	ρ-Chlorophenoxyisobutyric acid
PGR	Plant growth regulator
SD	Standard deviation of the mean
SE	Standard error of the mean
TIBA	2,3,5-Triiodobenzoic acid

CHAPTER 1: INTRODUCTION

1.1 The importance of *Eucalyptus*

When the French botanist Charles Louis L'Heritier De Brutelle adopted the term "*Eucalyptus*" for an Australian tree in 1788, few would have predicted the impact that this genus would have on the forestry industry and subsequently, the global economy. The genus is native to Australia and its neighbouring northern islands, and contains more than 500 species (Turnbull 1999). Originally considered exotic, eucalypts were planted in botanical gardens across Europe before their potential as forestry products was recognised. This led to the dissemination of eucalypts to many parts of the world by travellers, gold miners, traders, priests, soldiers and botanists (Zacharin 1978; Eldridge *et al*. 1994; Turnbull 1999), where they continue to serve a range of industries. During the early period of eucalypt forestry, they were seen as a valuable fuel source and were therefore planted along railway lines in South Africa and Brazil to supply wood for locomotives and were also established for leaf-oil production, land reclamation, hardwood timber, and as windbreaks (Eldridge *et al*. 1994, Turnbull 1999). Since then, eucalypts have emerged as the most prevalent forestry crop worldwide, being the most widely-planted hardwood species (Merkle and Nairn 2005). Their uses extend beyond those initially envisaged, and eucalypt plantations now serve and provide material for mine props, poles, firewood, essential oils, charcoal, honey, paper pulp, and many other industries (Eldridge *et al*. 1994; Turnbull 1999). The most recent industry to take advantage of this short-rotation woody crop is the energy industry, as the interest for renewable, sulphur-free and carbon neutral materials mounts (Rockwood *et al*. 2008).

A perusal of the literature on eucalypt plantations reveals that one of the key contributors to the worldwide success of the genus is its adaptability. The acquisition of this property can be traced back to the evolution of eucalypts alongside the changing environmental conditions in Australia. The *Eucalyptus* lineage can be traced back to over 70 million years ago, during a period when the formation of continental ice in Antarctica led to the drying of the continent (Hill *et al*. 1999; Myburg *et al*. 2007). Since then, the Australian climate has undergone a series of glacial and interglacial

phases which has resulted in vegetation, soils and landscape instability. Thus, the Eucalyptus progenitor/s can be assumed to have possessed remarkable adaptability in order to keep pace with these environmental changes. Eucalypts have, therefore, adapted to conditions such as dry climates and low nutrient soils, and exhibit traits like high growth rates and being relatively pest-free. Under present climatic conditions, eucalypts can extend from the cool temperate highlands, to the hot and humid lowlands of the tropics, but can also survive outside of this range if the local climatic conditions are favourable and the incidence of pathogens and insect pests are low (Eldridge *et al.* 1994).

Major industrial eucalypt plantations can now be found in Brazil, India, China, South Africa, Spain and Portugal, with relatively smaller-scale operations in Morocco, Thailand, Indonesia, Vietnam, Congo, Australia and Chile. According to available data, the largest plantation area exists in Brazil, with an excess of 4 million hectares (Couto *et al.* 2011), primarily due to government incentive policies between 1965 and 1985 (Stape *et al.* 2001), followed by China with an excess of 3 million hectares (www.globalwood.org, 2011). Eucalypt plantations in South Africa currently cover over 500 000 hectares (Godsmark 2010) and plantation areas are projected to increase as the demand for forestry products increase.

Although Brazil is recognised as having the largest scale eucalypt forestry industry in the world, South Africa is considered a good example of a well-managed forestry establishment and of the economic flows generated from this industry (Denison 2001). According to the latest available forestry report on South Africa (Godsmark 2010), forestry plantations account for approximately 1% (1 274 869 ha) of the total land area, the largest being situated in Mpumalanga, followed by KwaZulu-Natal (6.4% and 5.5% of the total land areas, respectively). Of the current forestry crops, which include pine, wattle and others, eucalypts account for the highest percentage (53.4%) in the province of KwaZulu-Natal, followed by 37.9% in Mpumalanga. Economic and management strategies for these products meant that 82.5% was used for pulpwood in 2009, by far the largest sector of the South African eucalypt material industry, which totalled 6.7 billion rand in that year (Godsmark 2010). In addition, this industry contributes

substantially to South Africa's employment sector, with close to 170 000 direct and indirect employees, according to the latest released report (Godsmark 2010). Apart from industrial eucalypt forests, non-industrial plantations also exist, established by rural farmers for local consumption. These are used for posts and poles, furniture, essential oils and to support honey production.

It should be noted that the extensive establishment of eucalypt plantations has also been met with contention. Eucalypt forests have been described as soil degraders and natural forest destroyers (Lohmann 1990; Lang 2008; Liu and Li 2010). The pulp and paper industry in Thailand, for example, has been accused of indiscriminate destruction of arable land, leading to a decline in ground water availability, loss of local food sources and permanent damage to soil (Lang 2008). The reduction in water yields and stream flows have been linked to the high rates of evapotranspiration in certain eucalypt species (Langford 1976; Roberts *et al*. 2001). Yet, in some parts of Australia, the establishment of salt-tolerant eucalypts is commonly used as a management strategy to discharge shallow saline groundwater from agricultural lands (Cramer *et al*. 1999) and re-establish water balance in catchments (Dale and Dieters 2007). High transpiration rates and salt-tolerance of certain eucalypts in these instances have been advantageous in remediation of land with dryline seepage salinity (Benyon *et al*. 1999; Benyon *et al*. 2001). Sustainable forestry practices such as mixed *Eucalyptus* and *Acacia* plantations have been shown to enhance water-use efficiency (Forrester *et al*. 2010).

While ecological concerns around eucalypt plantations do exist, one cannot deny the usefulness of the crop in meeting the increasing global demand for forestry-related material. Foresters have responded by showing a growing emphasis on sustainable land use practices, where plantations are considered beyond their use as timber products. This extends to protection of the environment along with the adjoining crops and soil, and by selecting particular species and matching these to suitable sites to create favourable ecological and subsequently socio-economic conditions (Ball 1995; Turnbull 1999; Chipeta 2010). A number of incentives have since been made available in many parts of the world to encourage private sector participation in eucalypt plantation programmes. These include supplying seedlings without charge or at a subsidised rate,

various tax concessions and plantation monitoring and management advice (Ball 1995). In South Africa, commercial forestry is recognised as both an important part of the economy and as a contributor to the substantial loss of biodiversity and groundwater. Consequently, a shift in forestry policies in line with those described above has been suggested (Tewari 2000) and implemented (Chipeta 2010) to add to the sustainability and wider benefit of the industry. In this way, and depending on the species and related management practices, eucalypts can be described as multipurpose trees that serve social, economic and political capacities (Turnbull 1999). To this end, land use planning and proper eucalypt site matching is crucial.

Since the mid 1990s, matching eucalypt species with provenance has become more precise with the development of scientific methods in this field (Eldridge *et al*. 1994; Criddle *et al*. 1995). Parameters such as location of the planting site, local climate, existing vegetation, soil properties, plant physiology and metabolism (Criddle *et al*. 1995) and the proposed management regime are considered before land is made available for eucalypt planting (Eldridge *et al*. 1994). Further, careful trials and screening procedures are subsequently undertaken to ensure the success of the selected eucalypt species at the designated site. In order to be able to screen and select clones that combine site-suitability with preferred growth form and wood characteristics, eucalypts should be selected from a wide genetic base.

1.2 Propagating and improving plantations

1.2.1 Propagation by seeds vs. vegetative propagation

Natural eucalypt forests and planted eucalypt stands with wide genetic bases represent the initial sources of eucalypt material for commercial activities and breeding programmes. These are essential as gene resource and conservation stands and serve as a means of meeting future demands of forestry establishments (Eldridge *et al*. 1994; Harvett 2001). Substantial genetic variation exists in these natural eucalypt forests and conservation stands, which confers advantages in forestry practices with respect to

selecting superior eucalypt genotypes in tree improvement programmes. Once trees with outstanding qualities are identified, large quantities of seeds are collected from these and planted out to establish „sæed orchards', from which inferior trees are identified early and removed. However, the establishment of eucalypt plantations by seed has been viewed with caution, since the possibility of unsuitable provenance and undesirable genetics is high, given the genetic variability (Eldridge *et al*. 1994). Also, many eucalypts exhibit irregular flowering and high abortion rates (Jones *et al*. 2000), which result in unreliable and often limited seed supply (Hung and Trueman 2011). In order to maintain competitiveness in the forestry industry, more efficient and reliable methods were necessary to supply the large amounts of planting material required, while preserving superior genotypes.

The successful rooting of stem cuttings in the mid 1950s (Eldridge *et al*. 1994), and its recognised potential in supplying clonal material to the forestry industry, saw the implementation of this technology gain momentum in subsequent years, with its introduction into commercial forestry in the 1970s (Zobel 1993; de Assis *et al*. 2004; Saya *et al*. 2008; Stape *et al*. 2010). Propagation by seed and vegetative propagation are often integrated in the management of forestry establishments, each with its advantages and disadvantages. Propagation by seeds is favoured when genotype-site matching has not been well established, when sites are potentially highly variable, when rooting of superior selected genotypes proves difficult, and owing to the relatively low technological requirement, when cheap propagation is required (Zwolinski and Bayley 2001). However, propagation by seed is a relatively slower process compared with vegetative propagation. Seedlings typically take four months before they are ready to be transferred to the forest (Meadows 1999), while vegetative propagation via rooted cuttings, although variable in the time taken to prepare shoots for deployment into forests, represents a significantly shorter time-frame than seed propagation (Eldridge *et al*. 1994; de Assis *et al*. 2004). Clonal forestry plantations display greater uniformity in crop height, diameter and wood properties, which subsequently translate to a reduction in management costs (Eldridge *et al*. 1994; Watt *et al*. 2003). Being superior to sexual regeneration through the preservation of superior genetic potential, vegetative or clonal propagation was recognised by foresters as the most favourable option in meeting the

objectives of crop improvement programmes (Denison and Kietzka 1993a; Watt *et al*. 2003; de Assis *et al*. 2004; Saya *et al*. 2008).

1.2.2 Tree improvement programmes

While good potential for the expansion of forestry establishments through prudent site-matching strategies exists in countries with suitable rainfall, climate and soils, drier countries such as South Africa have seen the expansion of forests on more marginal areas (Denison and Kietzka 1993b). In an effort to remain sustainable and competitive, forestry establishments need to either expand into newer sites, or seek from existing stands better clone- site suitability, and increased growth rates and yields, while minimising costs. This challenge has been addressed through tree improvement programmes and are epitomised by the development of hybrids (Denison and Kietzka 1993b; de Assis 2011).

Hybridisation is integral to improvement programs for many crop species. Many eucalypt species (within, but not between the major subgenera) can be readily crossed (Potts and Dungey 2004), conferring properties generally midway between both parent species (Denison and Kietzka 1993b). These should ideally display superior performance traits than those of the parent species if they are to be economically viable. Some outstanding benefits that hybrids are selected for are hybrid vigour, increased disease resistance, superior wood, growth and maintenance properties, and site adaptability, the latter allowing for plantation in marginal areas where pure species cannot be easily grown (Denison and Kietzka 1993b). An initial drawback to the implementation of large-scale hybrid forestry was the difficulty in attaining sufficient quantities of hybrid seed (Denison and Kietzka 1993b; Potts and Dungey 2004). For this reason, vegetative propagation has found favour and has since been extended to include the propagation of pure species (Eldridge *et al*. 1994; Watt *et al*. 2003).

Since 1983, Mondi Forests, a division of the then Mondi Paper Company Limited (now Mondi Business Paper of Mondi Group), has been actively involved in tree improvement programmes in South Africa (Denison and Kietzka 1993a; Harvett 2001;

www.mondigroup.com, 2011), of which the present study forms a part. The focus of these programmes has been on maintaining sustainability while improving the quality, wood properties and yield of forestry crop.

A number of forestry establishments throughout the world have reported successes in tree improvement programmes based on interspecific hybridisation. Worldwide, *E. grandis* is often encountered as either a pure species or as a hybrid (Denison and Quaile 1987; van Wyk 1990; Eldridge *et al*. 1994; Harvett 2001; Potts *et al*. 2001; Potts and Dungey 2004), the latter resulting in an extension in its plantation range. The choice of parent species in producing hybrids depends largely on the respective climatic conditions and the properties of the parent species. For example, in more temperate regions, one may find *E. grandis* x *E. nitens* (Denison and Kietzka 1993b; Harvett 2001; Potts and Dungey 2004), combining the fast growth rates of *E. grandis* with the cold-tolerance of *E. nitens* (Denison and Kietzka 1993a). Further, in subtropical climates, suitable hybrids may be produced from *E. grandis* and *E. urophylla* (Denison and Kietzka 1993b; Harvett 2001; Potts and Dungey 2004), which seeks to combine the preferred growth rates of *E. grandis* with the greater coppicing ability and disease tolerance of *E. urophylla* (Eldridge *et al.* 1994). Other examples of large, successful hybrid propagation programmes include *E. tereticornis* x *grandis* at Pointe Noire in Congo and *E. grandis* x *urophylla* at Aracruz in Brazil (Eldridge *et al.* 1994; Turnbull 1999; Potts and Dungey 2004). One of the objectives of the tree improvement programmes is to reduce wood specific consumption (WSC – the amount of wood needed in the production of a ton of pulp) while simultaneously improving pulp yield (Grattapaglia and Kirst 2008). Hybrids of *E. grandis* had initially reduced the WSC by 20% in the 1980s (Ikemori *et al.* 1994). Further reductions were subsequently achieved through hybrids with *E. globulus*, known for its superior wood properties (de Assis *et al.* 2005; de Assis 2011).

More recent developments in eucalypt improvement have been in the application of transgenic technologies. A number of genes related to wood formation in eucalypts (and other commercially important forestry trees) have been identified and manipulated to improve genotypes (Boerjan 2005; Harfouche *et al.* 2011). Despite the ongoing debate,

genetic modification is regarded as an important tool in potentially improving forestry crop and meeting future demands for wood products, while easing the pressure on native forests and biodiversity. Some of the key advantages of genetic modification include the potential to substantially reduce the long generation times of forest trees compared with classical tree-breeding programmes, and the reduced risk of elite clones losing their superior genetic composition, compared with hybrid crossing. Studies on *E. globulus* have already shown a reduction in allele richness as a result of forestry breeding programmes (Jones *et al.* 2006).

Perpetuating genetically improved clones relies almost exclusively on vegetative propagation, as several physiological and structural barriers hinder the production of hybrid seed (Potts and Dungey 2004). Hence, advancements in clonal propagation technology must accompany genetic improvements of forestry crops. Improvements in vegetative propagation should see more reliable and efficient ways of generating whole plants that are of suitable quality in terms of above- and below-ground development. The current methods of vegetative propagation, although successful in many respects, do have their limitations, the most critical of which is root production. There exists a need, therefore, to understand and refine current techniques of vegetative propagation in order to ensure clone quality, and meet the requirements of industry as well as for fundamental research.

1.3 Eucalyptus vegetative propagation

1.3.1 Propagation by cuttings

The ability to propagate plants vegetatively via rooted cuttings was recognised as far back as the 4th century B.C.E, as the writings of Aristotle and Theophrastus suggests (Haissig and Davis 1994). However, the Chinese and later the Japanese, are credited with the first application of this technique on a commercial scale for timber, with the Chinese propagating *Cunninghamia lanceolata* (Chinese fir) over a thousand years ago, and the Japanese for *Cryptomeria japonica* (sugi) over 500 years ago (Ritchie 1994;

Hartmann *et al.* 1997). However, the usefulness of rooted cuttings in forestry applications in the West was only recognised at the end of the 19th century (Zobel 1993; Ritchie 1994). Initially applied to the propagation of *Populus* spp. and *Salix* spp., this method of vegetative propagation has since grown in popularity to include almost all forestry crops, as its potential in maintaining and propagating superior genotypes to yield more efficient and uniform forests was recognised (Zobel 1993; Ritchie 1994). The proportion of eucalypt forests propagated via rooted cuttings in terms of global forestry establishments has and continues to increase steadily, helped in no small part by the huge initial successes observed in Aracruz in Brazil and Pointe Noire in Congo (Zobel 1993; Eldridge *et al*. 1994; Turnbull 1999), which continue to-date (Saya *et al*. 2008; Stape *et al*. 2010).

Vegetative propagules for stem cuttings in commercial forestry applications were initially sourced from field plantations, which meant reserving substantial land areas solely for this purpose (de Assis *et al*. 2004). In order to overcome this and, in the process, increase the efficiency in commercial plantations, the clonal hedge-based concept was developed. These hedges required significantly less land area and provided large numbers of shoots for vegetative propagation (Denison and Kietzka 1993a; Meadows 1999; de Assis *et al*. 2004; Titon *et al*. 2006; Saya *et al*. 2008). The nursery practice entailed collecting macro-cuttings of 8 to 10 cm with basal diameters of 2 to 5 cm from 30- to 60-day-old shoots from coppicing stumps of superior selected clones (Stape *et al*. 2001). Although initially, the macro-cuttings practice cost more than propagating eucalypts by seed, due to higher labour and infrastructure investments, the benefit gained by the industry from uniform plantations of superior clones (Eldridge *et al*. 1994; Campinhos 1999) was seen as a long-term advantage. However, since macro-cuttings were sourced from relatively mature material, a major problem encountered was in the success rates of adventitious root formation (Stape *et al*. 2001; López *et al*. 2010). In order to minimise the effects of maturation, the mini-cuttings system was introduced, whereby cuttings of 2 to 3 cm height and 0.4 to 1 cm basal shoot diameters were used as vegetative propagules (Stape *et al*. 2001). Compared with macro-cuttings, mini-cuttings conferred the advantages of lower production costs and higher rooting ability of cuttings, with superior root systems (Stape *et al*. 2001; de Assis *et al*. 2004;

López *et al*. 2010). Further, mini-cuttings (of good-rooting clones) were able to root with minimal or no root enhancer treatments and still produce roots of a good quality and morphology over those produced from macro-cuttings (Yang *et al*. 1995). Mini-cuttings also represented a substantial reduction in operational costs brought about by a reduction in intensive cuttings and hedge management systems that were characteristic of macro- or stem-cuttings practices (de Assis *et al*. 2004). The speed of rooting of mini-cuttings subsequently resulted in a reduction in the turnaround time of plantation programmes and a reduction in the exposure-time of mini-cuttings to pathogenic fungi, leading to minimal fungicide applications (de Assis *et al*. 2004).

Clonal hedges for macro-cuttings are typically maintained in the ground, outdoors. Hence, they are susceptible to nutrient leaching during periods of adverse climate, such as excessive rainfall (de Assis *et al*. 2004). In an attempt to manage the nutritional status of mother plants better and increase the number of cuttings that could be harvested, indoor clonal hedges were developed and intensely managed. These were often maintained in containers as drip irrigation sand-bed systems or hydroponics (Denison and Kietzka 1993a; de Assis *et al*. 2004; López *et al*. 2010). This system allowed better management of mother plants, which translated to economic advantages due to better productivity of mini-cuttings, and lower chemical and water demands (de Assis *et al*. 2004). While mini-cuttings provided many advantages over macro-cuttings, a number of commercially-important clones still proved difficult-to-root via mini-cuttings. Many potentially valuable eucalypts are not targets for mass vegetative propagation through cuttings owing to their difficulty in producing adventitious roots. The rooting ability of mini-cuttings is dependent on the maturation state of the mother plant, and decreases with the age of the parent plant (Eldridge *et al*. 1994; Watt *et al*. 2003; Yasodha *et al*. 2004). To this end, investigations using adult tissues of *E. grandis* have indicated the existence of possible rooting inhibitors (Paton 1970), probably alluding to auxin antagonists (see later). A reversal of the maturation state (rejuvenation) of the parent plant allows for the restoration of rooting ability of cuttings (Eldridge *et al*. 1994; Yasodha *et al*. 2004).

Rejuvenation in eucalypts can be achieved by coppicing, repeated grafting, serial production of cuttings over a number of generations, or through micropropagation, each with varying levels of success depending on the clone concerned (Eldridge *et al*. 1994; de Assis *et al*. 2004). Of these methods, micropropagation potentially serves the widest range of forestry needs. Not only can it restore juvenility, but it also serves as a source of material for clonal plantations, allowing for rooting of difficult-to-root genotypes (Yasodha *et al*. 2004). Micro-cuttings, sourced from micropropagated mother plants, are more juvenile than their macro- and mini-cutting counterparts and, therefore, result in further gains in rooting ability (Denison and Kietzka 1993a; de Assis *et al*. 2004; Yasodha *et al*. 2004). In addition, micropropagated mother plants provide significantly higher numbers of shoots for micro-cuttings (Yasodha *et al*. 2004). Nevertheless, the juvenility achieved through micropropagation does gradually erode from mother plants maintained *ex vitro*, with a consequent reduction in the rooting ability of micro-cuttings harvested from them (de Assis *et al*. 2004). However, the high multiplication rates achieved through micropropagation, and the maintenance of juvenility *in vitro*, can potentially circumvent most of the shortcomings of the vegetative propagation via cuttings, if plantable units of suitable quality can be produced. *In vitro* propagation of eucalypts can not only meet the demands for propagules in industry, but this technology also serves as a tool in further genetic modification by providing a means of cloning transformed cells.

1.3.2 *In vitro* propagation

1.3.2.1 Basic principles and routes of morphogenesis

This aseptic vegetative propagation technique exploits the property of totipotency, a biological principle which states that since every plant cell possesses all the necessary genetic information, it has the potential to reproduce the entire organism, given the correct stimuli and environmental conditions (Hartmann *et al*. 1997). Totipotency in whole plants applies to the zygote and to meristematic cells of the shoot and root

(Hartmann *et al*. 1997). In a plant tissue, cell, or organ culture application, this cellular competency is used to induce somatic embryos, adventitious shoots or adventitious roots, following cell dedifferentiation to attain the meristematic state, often under the influence of plant growth regulators (PGRs) (Hartmann *et al*. 1997). A range of explant material can therefore be used, depending on the purpose of the proposed culture, the plant species in question and the kind of culture that needs to be initiated (George *et al*. 2008). Plantlet regeneration protocols have been achieved for a plethora of species and hybrids of eucalypts, with explants ranging from seeds, shoots and callus, to protoplasts and somatic embryos (reviews by Jones and van Staden 1997; Watt *et al*. 2003). Each *in vitro* stage of morphogenesis contains a suitable combination of micro- and macro-nutrients and vitamins for the explant's metabolism and growth, together with the appropriate PGR (where necessary) to direct and regulate morphogenesis.

Both somatic embryogenesis and shoot and root morphogenesis (organogenesis) can proceed via direct or indirect pathways, the latter involving an intervening callus stage. With regards to somatic embryogenesis, the callus that forms during the indirect pathway can contain either or both embrogenic or non-embryogenic callus, which makes the direct and indirect pathways difficult to delineate (Blakeway *et al*. 1993; George *et al*. 2008). Embryogenic cultures are initiated by culturing the explant on a medium containing a high concentration of auxins, usually 2,4-dichlorophenoxy acetic acid (2,4-D). The embryogenic callus that develops is composed of proembryogenic masses, which continue to proliferate in the high auxin environment until transferred to a medium lacking PGRs. Here, proliferation is halted and the embryogenic callus cells are stimulated to form somatic embryos, following a morphogenic path similar to that of zygotic embryos (Hartmann *et al*. 1997). Somatic embryos are subsequently matured through culture on a medium often characterised by reduced osmotic potential (through the addition of osmotic agents such as organic salts or polyethylene glycol – PEG), or containing abscisic acid (ABA). Plants are then regenerated through embryo „germination' and seedling establishment on a medium lacking PGRs (Hartmann *et al*. 1997; George *et al*. 2008). By virtue of forming from the embryon meristems, roots produced from a somatic embryo are expected to more closely resemble those of zygotic seedlings than those formed adventitiously (Watt *et al*. 1991).

While several routes of morphogenesis are possible, the *in vitro* mass vegetative propagation of *Eucalyptus* plantlets for the forestry industry generally follows plantlet regeneration through shoot and root morphogenesis. This involves directing organogenesis via an induced meristematic group of cells. The number of organogenic stages usually includes culture establishment, shoot multiplication, root formation, and acclimatisation (Hartmann *et al*. 1997). For many plant species, including the eucalypts (Jones and van Staden 1997), a shoot elongation stage that precedes the rooting step, is sometimes necessary to produce plantlets that are suitably robust for an industrial or *ex vitro* application (Warrag *et al*. 1990; Jones and van Staden 1997; Arya *et al*. 2009).

The exogenous supply of phytohormones in micropropagation systems is integral to the culture medium and subsequent organogenesis. These compounds are naturally-occurring, and are generally active at low concentrations (George *et al*. 2008). Since the extraction and isolation of the first PGR ethylene by Gane (1934), more compounds with plant regulatory activity have been extracted and isolated, with the auxins (Haagen-Smit *et al*. 1942), and cytokinins (Letham 1963) soon following. Many more natural and synthetic compounds have since been identified (Barciszewski *et al*. 1999; Weyers and Paterson 2001; Gaspar *et al*. 2003; de Rybel *et al*. 2009; Santner and Estelle 2009). Although many classes of plant growth substances are known to exist, five of these have received most of the attention, i.e. auxins, cytokinins, gibberellins, ethylene and abscisic acid. The most relevant of these classes to the micropropagation of hardwood species such as the eucalypts, are the auxins and cytokinins, which direct and regulate root and shoot organogenesis, respectively. One or more type of cytokinins and auxins is therefore added to the culture medium, depending on factors such as the plantlet regeneration approach, the explant used, and the plant species in question. The roles and interactions of these phytohormones are discussed later.

1.3.2.2 An account of *Eucalyptus* micropropagation

Eucalypt regeneration using tissue culture approaches was first reported in the 1960s, where eucalypts were cultured from lignotubers (Aneja and Atal 1969). Substantial developments were made in the following years, with successful plantlet regeneration from almost all organs (reviews by Le Roux and van Staden 1991; Jones and van Staden 1997; Watt *et al*. 2003). Examples include shoot tips (e.g. Gomes and Canhoto 2003), axillary buds (e.g. Jones and van Staden 1994; Mokotedi *et al*. 2000); nodes (e.g. Gomes and Canhoto 2003; Arya *et al*. 2009; Hung and Trueman 2011), and cotyledons (e.g. Bandyopadhyay *et al*. 1999; Nugent *et al*. 2001a). Plantlet regeneration via somatic embryogenesis has also been reported (e.g. Watt *et al*. 1991; Termignoni *et al*. 1996; Bandyopadhyay *et al*. 1999; Pinto *et al*. 2002) (Table 1.1), albeit with limited success.

While significant advances have been achieved in vegetatively propagating clones *in vitro*, the implementation of some routes of plantlet regeneration into the forestry industry has been hampered by certain limitations. Poor acclimatisation of plantlets and the risk of somaclonal variation has largely stopped efforts towards micropropagation of superior genotypes via indirect organogenesis (Bandyopadhyay *et al*. 1999; Watt *et al*. 2003), while the low frequency of converting somatic embryos into established plants has limited the application of somatic embryogenesis (Watt *et al*. 2003; Moyo *et al*. 2011) (Table 1.1). Other perceived disadvantages of somatic embryogenesis include variations in the induction of somatic embryos across family and over the years of seed production, as reported for *E. globulus* (Pinto *et al*. 2008). Fluctuations in reserve accumulation between somatic embryos and zygotic embryos have also been suggested to contribute to the low frequency of plantlet regeneration via somatic embryogenesis (Pinto *et al*. 2010). However, it still holds potential as a means of regeneration of transgenic plants in crop improvement programmes (Watt *et al*. 2003; Merkle and Nairn 2005; Moyo *et al*. 2011), but further studies are needed to understand and optimise this route of regeneration. In the meantime, mass vegetative propagation via direct organogenesis (through axillary bud proliferation) is the preferred method to supply large numbers of elite clonal material to the industry. This is evidenced in the number of successful regeneration protocols reported for a range of explant types (reported from

the year 2000 to present) (Table 1.2), compared with the number of reports on somatic embryogenesis, and their plantlet regeneration rates (Table 1.1).

The preferred route of vegetative propagation in the majority of industrial applications that employ *in vitro* technology is through harvesting coppice from clonal hedges, followed by *in vitro* shoot proliferation and subsequent rooting. In line with the objectives of reducing costs in commercial applications, several standard media compositions (standard protocols) have been devised for the micropropagation of suites of eucalypt clones, which essentially comprise MS nutrients (Murashige and Skoog 1962) supplemented with vitamins and the appropriate PGR for shoot and/or root development (see earlier). The essential difference amongst these protocols is nested in the nature of the clone (with regard to its ease of propagation) and the required route of morphogenesis, which is dictated by the exogenous PGRs. As the literature suggests, the type and concentration of PGRs used (within a given class) is often determined empirically for a given clone (Table 1.2). In the micropropagation of eucalypts for the forestry industry, the PGR combinations are selected based on their ability to yield the highest number of shoots following multiplication, and eventually the highest number of rooted shoots. These combinations are used to establish complete *in vitro* regeneration protocols. A summary of those reported from the year 2000 is presented in Table 1.2, following from earlier reviews of Le Roux and van Staden (1991), Jones and van Staden (1997), and Watt *et al*. (2003).

The ultimate aim of the various vegetative propagation options discussed above is the production of fully functional plants. In this regard, the production of adventitious roots is critical (de Assis *et al*. 2004), as they need to develop adequately to serve the nutritional and supportive roles of the plant. The success of vegetative propagation programmes is determined by root production, and as mentioned, the difficulty in producing adventitious roots through vegetative propagation has hampered the establishment of a number of potentially important eucalypt clones (Eldridge *et al.* 1994; de Assis *et al*. 2004). While adventitious root production *in vitro* may be regarded as favourable based on quantitative (assessed by root number) or qualitative (by

appearance) analyses, careful trials are necessary to assess the development and function of *in vitro* produced roots in the field.

1.3.2.3 From *in vitro* to the field: assessments of plants post-acclimatisation

Despite their potential in supplying large numbers of rooted shoots to the South African forestry industry, micropropagated plantlets are currently not directly deployed into forestry establishments due to the lack of reliable information regarding their long-term performance. In a review by Gupta *et al*. (1991), several commercially important micropropagated forestry species, including eucalypts, were shown to have increased biomass production, greater uniformity, early flowering and maturation traits, and superior yield, compared with their seedling-derived counterparts. Greenhouse studies, with *in vitro* and seedling-derived *E. grandis*, showed that while some growth differences may be initially observed between *in vitro* plantlets and seedlings, neither propagatory method conferred a nett advantage in terms of photosynthetic ability (Warrag *et al*. 1989a) or dry matter accumulation and distribution (Warrag *et al*. 1989b). Field trials on micropropagated and macropropagated *Eucalyptus* hybrids over 36 months indicated that for the majority of the tested clones, micropropagated plantlets fared significantly better in terms of survival, tree height and growth parameters, and uniformity (Watt *et al*. 1995). Following at least 14 months acclimatisation, micropropagated *E. grandis* x *nitens* were as efficient at leaf gas exchange compared with macropropagated *E. grandis* x *nitens* and seed-propagated *E. grandis* and *E. nitens* (Mokotedi *et al*. 2009a).

Table 1.1 Examples of reports on somatic embryogenesis and plantlet regeneration for eucalypts

Species	**Details**	**Plantlet conversion rate**	**Reference**
E. grandis	Callus from leaf explants	30%	Watt *et al*. (1991)
E. globulus	Callus from cotyledon pieces and hypocotyls	13% of embryos showed signs of germination, but no plantlet development was reported	Nugent *et al*. (2001b)
		Embryos displayed abnormal shoot apex development and poorly-developed cotyledons	
E. globulus	Callus from cotyledons, hypocotyls, leaves and stem explants	21%	Pinto *et al*. (2002)
E. tereticornis	Callus from mature zygotic embryo explants	54% frequency of embryo development, with a subsequent conversion rate of 80%	Prakash and Gurumurthi (2004)
E. camaldulensis	- Direct: from hypocotyl segment explants	10% frequency of embryo development	Prakash and Gurumurthi (2010)
	- Callus from zygotic embryo explants	Highest frequency of embryo development = 63%	
		In total, less than 10% of the somatic embryos reached the germinating stage	

Table 1.2 Examples of successful *Eucalyptus* micropropagation protocols reported from the year 2000, and the PGRs* (mg l^{-1}) used in the reflected *in vitro* stages. NR = Not Reported

Species	Explant	Multiplication PGRs	Elongation PGRs	Rooting PGRs	Reference
E. grandis x *E. nitens*	Nodal segments	BAP (0.1) + NAA (0.01)	IBA (0.01) + NAA (0.01) + Kinetin (0.2)	IBA (20.0) for 24 hrs or IBA (2.5) for 72 hrs, or IBA (0.1, 2.5) for 28 days	Mokotedi *et al*. (2000)
E. nitens	Seedlings and 1 yr-old shoot tips and nodes	BAP (0.1, 0.2)	GA_3 (0.1)	IBA or IAA (1.0, 2.0, 3.0)	Gomes and Canhoto (2003)
Eucalyptus tereticornis x *E. grandis*	Mature Shoots	BAP (1.0) + NAA (1.0)	None	IBA (1.0)	Joshi *et al*. (2003)
E. globulus	*In vitro*-derived meristematic nodules	Shoot regeneration with ABA alone, or with NAA	NR	IBA (0.5)	Trindade and Pais (2003)
E. grandis	Nodal segments	BAP (200-600), pulse for 1-3 hours			de Andrade *et al*. (2006)
E. erythronema x *E.stricklandii*	Axillary shoots of seedlings	BAP (1.0) + NAA (0.2)	BAP (1.0) + NAA (0.2) + GA_3 (0.5)	IBA	Glocke *et al*. (2006a)

Table 1.2 continued

Species	**Explant**	**Multiplication**	**Elongation**	**Rooting**	**Reference**
E. erythronema	Apex and leaf	BAP (0 – 1.0)	NR	NR	Glocke *et al*. (2006b)
E. stricklandii	Apex and leaf	BAP (0 – 1.0)	NR	NR	Glocke *et al*. (2006b)
E. grandis x *E. urophylla*	Shoots	NAA (0.01) + BAP (0.2)	Callus induction using IAA (5.0) + BAP (0.25), followed by shoot initiation	IBA (0-1.0)	Hajari *et al*. (2006)
E. grandis	Shoots	NAA (0.01) + BAP (0.2)	Callus induction using IAA (5.0) + BAP (0.25), followed by shoot initiation	IBA (0-1.0)	Hajari *et al*. (2006)
E. camaldulensis x *E. tereticornis*	Nodal segments	BAP (1.0) + IBA (0.1)	Along with multiplication	IBA (0.1-2.0) alone, or with NAA (0.1-2.0)	Arya *et al*. (2009)
Corymbia. torelliana x C. *citriodora*	Nodal segments	BAP (1.0)	Along with multiplication	IBA (0.1-2.0) alone, or with NAA (0.1-2.0)	Arya *et al*. (2009)
E. benthamii x *E. dunnii*	Nodal segments	BAP (0 – 1.0)	NAA (0 – 1.0) + BAP (0.05)	NR	Brondani *et al*. (2009)

Table 1.2 continued

Species	**Explant**	**Multiplication**	**Elongation**	**Rooting**	**Reference**
E. urophylla x *E. grandis*	Nodal segments from mature plants	BAP (1.0) + NAA (0.01)	Along with multiplication	IBA and/or NAA	Nourissier and Monteuuis (2008)
E. urophylla x *E. grandis*	Nodal segments	BAP (1.0) + NAA (0.01)	Along with multiplication	IBA and/or NAA	Mankessi *et al*. (2009)
E. tereticornis	Nodal segments	BAP (0 – 2.8), in combination with 2,4-D or NAA (various concentrations)	-	-	Aggarwal *et al*. (2010)
E. globulus hybrids	Nodal segments	BAP (0.5)			Borges *et al*. (2011)
E. benthamii x *E. dunnii*	Nodal segments	BAP (0, 0.05, 0.1)	BAP (0, 0.05, 0.1) + GA_3 (0, 0.1, 0.2, 0.3)	IBA (2.0)	Brondani *et al*. (2011)
C. torelliana x *C. Citriodora* (eucalypts)	Shoots	With or without NAA (0.01)	Along with multiplication	IBA (4.0)	Hung and Trueman (2011)

* 2,4-D: 2,4-Dichlorophenoxy acetic acid; BAP: Benzylaminopurine; IAA: Indole-3-acetic acid; IBA: Indole-3-butyric acid; NAA: α-Naphthalene acetic acid; GA_3: Gibberellic acid

While the above-ground performance of micropropagated plantlets following acclimatisation is encouraging, an analysis of the below-ground performance (i.e. root architecture and development) is necessary to correctly assess the total performance and result of this propagatory technique. Even though root architecture is recognised as crucial in plant stability and productivity (Coutts 1983; Lynch 1995), such studies are relatively scarce, since it is difficult and expensive to obtain reliable and precise data on actual root systems (Lynch 1995; Misra *et al.* 1998). The few studies in the development and architecture of *in vitro* produced roots following acclimatisation have presented somewhat contradictory reports. Some documented no differences in field performance between seed- and vegetatively-propagated eucalypts, while others reported gross differences in root morphology following acclimatisation. In a study comparing the early growth of tissue-cultured and seed-propagated *Eucalyptus camaldulensis*, Bell *et al*. (1993) found little above- or below-ground architectural differences in growth. Both micropropagated and seed-propagated plants displayed a similar ability at penetrating heavy clay soil, although in one clonal line, roots were concentrated in the upper soil profiles (Bell *et al*. 1993). Mokotedi *et al*. (2010) undertook a more comprehensive study on the field performance of vegetatively propagated *E. grandis* x *nitens* and seed-propagated *E. grandis* and *E. nitens*. After 16 months of field growth, micropropagated plants displayed significantly lower uprooting resistance than macro- and seed-propagated plants. This decreased uprooting resistance was attributed to the architecture of the roots. The seed- and macro-propagated plants developed tap roots (T-beam) or tap root-equivalent “tap-sinker” roots, whereas the micropropagated plants developed only I-beam shaped horizontal roots, which were less efficient at anchorage (Mokotedi *et al*. 2010).

Root architecture that compromises tree stability is undesirable, particularly in forestry establishments, and more so in the case of tall and fast-growing eucalypt forests. In the post-acclimatisation studies of Bell *et al*. (1993) and Mokotedi *et al*. (2010) mentioned above, micropropagated shoots were rooted *in vitro* using the auxin indole-3-butyric acid (IBA). Generally, the success of the *in vitro* rooting stage is measured by the number and visual quality of the roots, before shoots are prepared for acclimatisation. Studies such as those above suggest that this kind of assessment is insufficient at

predicting root development and quality post-acclimatisation and, therefore, the parameters that influence the adventitious rooting process require attention, if the potential benefits of micropropagation to forestry productivity are to be realised. Such parameters include the actions and interactions of the PGRs used for root induction *in vitro*, and the subsequent induction and development of the adventitious roots.

1.4 Role of auxins and cytokinins in root growth and development

1.4.1 Biosynthesis, metabolism and interactions

Historically, the majority of the studies on plant growth regulation have been focussed on auxins and their biochemical and molecular implications in plant development, with relatively few reports on cytokinin biochemistry, other than its gross physiological effect on plants. Recently, however, and probably due to its antagonistic relationship with auxins, there has been a renewed interest in cytokinins. Although the information is still limited, major advances in this field have been steady of late, mainly through molecular and genetic approaches.

Cytokinins are known to occur in the tRNA of most organisms as a bound form, and so it was initially thought that cytokinin synthesis was as a result of the breakdown of tRNA (Mok and Mok 2001). However, in keeping with the low turnover rate of tRNA, this method of synthesis could not account for the levels of cytokinins found in plants (Haberer and Kieber 2002). Subsequent investigations have led to the discovery of a number of genes – *AtIPT* genes – that encode the biosynthesis of the natural cytokinins isopentenyladenine (iP) and zeatin in *Arabidopsis* and in some bacterial species (Takei *et al*. 2001; Kakimoto 2001; Haberer and Kieber 2002). Given that alternative cytokinin biosynthetic pathways have been proposed (Åstot *et al*. 2000), plant tissues can be said to contain several types of cytokinins, each with tissue-specific roles. These may be

found as both free bases or in their corresponding nucleotide and nucleoside forms, with interconversions between these forms mediated by enzymes (Martin *et al*. 2001; Mok and Mok 2001). Cytokinin inactivation and hence turnover is facilitated by cytokinin oxidases, resulting in irreversible cytokinin degradation (Haberer and Kieber 2002). The rapid degradation of the natural cytokinins zeatin and iP by cytokinin oxidases has been suggested to contribute to the ineffectiveness of these regulators in certain plant species (George *et al*. 2008). An enzyme other than cytokinin oxidase has been suggested to be involved in the degradation of certain synthetic cytokinins in some plant species (Forsyth and van Staden 1987). This implies that synthetic cytokinins that are not substrates for these enzymes will persist in plant tissues. While the deeper understanding of cytokinins has only recently received renewed attention, auxins have long been recognised as a major regulator of plant development and, therefore, much more has been documented regarding the auxins.

Although auxin biosynthesis has been reported as occurring mainly via the indole amino acid tryptophan, alternate pathways independent of tryptophan (Trp) have been documented (Bartel *et al*. 2001; Woodward and Bartel 2005). The biosynthesis of the most widely-encountered and studied natural auxin indole-3-acetic acid (IAA) via indole-3-pyruvic acid (IPA) has been oft-reported, in which Trp is transaminated (by tryptophan transaminase) to IPA, which is decarboxylated to indole-3-acetaldehyde (IAAld) (Gibson *et al*. 1972; Schneider *et al*. 1972). Then, IAA is formed either through oxidation or dehydrogenation of IAAld (Woodward and Bartel 2005; Tromas and Perrot-Rechenmann 2010). Similarly, other Trp-dependent biosynthetic pathways have been identified for IAA, e.g. the indole-3-acetamide (IAM) pathway, the tryptamine pathway, and the indole-3-acetaldoxine (IAOx) pathway (Woodward and Bartel 2005; Tromas and Perrot-Rechenmann 2010). Tryptophan auxotrophs of maize and *Arabidopsis* maintained the ability to synthesise IAA, leading to the suggestion that IAA can be synthesised independently of Trp (Wright *et al*. 1991; Normanly *et al*. 1993; Östin *et al*. 1999). However, the majority of IAA biosynthesis is still thought to occur via Trp-dependent pathways (Eckardt 2001). The biosynthesis of the other natural auxin found in plants, indole-3-butyric acid (IBA) is in many ways analogous to that of IAA. This can occur through Trp, but with a longer side chain (Epstein and Ludwig-

Müller 1993; Ludwig-Müller 2000); via β-oxidation in chain-elongation reactions, similar to fatty acid synthesis (Epstein and Ludwig-Müller 1993; Ludwig-Müller 2000); or as some evidence suggests, via Trp-independent pathways similar to those described for IAA (Wright *et al*. 1991; Normanly *et al*. 1993; Östin *et al*. 1999).

Since plants utilise auxins for numerous developmental processes, there are advantages to storing the hormone in tissues. IAA can be stored in either conjugated form or IBA, which allows for the availability of free IAA either through hydrolysis to free the conjugated form, or through β-oxidation to convert IBA to IAA (Bartel *et al*. 2001; Woodward and Bartel 2005). Conjugation products have also been reported for IBA, which have been suggested to be a better source of free IAA than conjugation products of IAA (Wiesman *et al*. 1989). IBA and IAA are both rapidly metabolised and conjugated within plant tissues. These conjugates act as „slow release' mechanism for the hormone, from which they are hydrolysed to release free auxins (Ludwig-Müller 2000). Auxin-conjugates play an important role in auxin metabolism and physiology, by facilitating its storage and utilisation, protecting auxins from enzymatic deactivation, and in maintaining a homeostatic concentration of the hormone in the plant (Epstein and Ludwig-Müller 1993). IAA conjugates are said to be more susceptible to oxidative degradation and hence deactivation than IBA conjugates (Epstein and Ludwig-Müller 1993; Woodward and Bartel 2005), again supporting the greater stability of IBA over IAA. No oxidation products have been reported for IBA conjugates. IBA thus remains at elevated levels longer than IAA (Epstein and Ludwig-Müller 1993). Auxin conjugation products need to be hydrolysed in order to avail free auxin to the plant (Epstein *et al*. 1993; Epstein and Ludwig-Müller 1993; Bartel *et al*. 2001). Easy-to-root cultivars of sweet cherry were shown to be able to hydrolyse IBA conjugates to free IBA more successfully than difficult-to-root cultivars (Epstein *et al*. 1993). It was therefore hypothesised that difficult-to-root eucalypts may also be lacking in their ability to hydrolyse auxin conjugation products.

The regulation of both auxins and cytokinins are tightly controlled, with significant cross-talk between their respective metabolic pathways. Investigations into auxin signalling have revealed three main families of auxin response genes, which accumulate

rapidly in response to auxin: *Small Auxin Up RNA* (*SAURs*), *GH3*-related genes (*GH3s*), and *Auxin/Indole-3-acetic acid* (*AUX/IAA*) (reviewed by Hagen and Guilfoyle 2002; Woodward and Bartel 2005; Tromas and Perrot-Rechenmann 2010). Transcripts of the *SAURs* genes are short-lived and highly conserved, being implicated in calmodulin binding (Yang and Poovaiah 2000). Genes of *GH3* code for conjugating enzymes that regulate free auxin levels (Staswick *et al*. 2005). The *AUX/IAA* gene family has been studied in *Arabidopsis*, and comprise at least 29 genes in that species (Overvoorde *et al*. 2005), with homologous genes present in other plant species (Woodward and Bartel 2005). Sequence identity is shared in four conserved domains between proteins of Aux/IAA (Hagen and Guilfoyle 2002). Domain I of the Aux/IAA protein has been identified as a potent transcriptional repressor (Tiwari *et al*. 2004), Domain II is involved in Aux/IAA instability, and domains III and IV elicit dimerisation or multimerisation between of Aux/IAA proteins and heterodimerisation between Aux/IAA and Auxin Response Factor (ARF) proteins. The latter can attach to Auxin Response Elements (AREs), which are ultimately responsible for auxin-induced gene expression (Hagen and Guilfoyle 2002; Woodward and Bartel 2005; Tromas and Perrot-Rechenmann 2010). Auxin perception at the site of action is mediated by receptors of the Transport Inhibitor Response 1 (TIR1) family. The *TIR1* gene encodes an F-box protein subunit which forms part of the ubiquitin ligase complex that targets substrates for degradation by the 26s proteasome (Hagen and Guilfoyle 2002; Woodward and Bartel 2005; Moubayidin *et al*. 2009; Tromas and Perrot-Rechenmann 2010).

As reviewed by several authors (Hagen and Guilfoyle 2002; Woodward and Bartel 2005; Moubayidin *et al*. 2009; Tromas and Perrot-Rechenmann 2010), the control of auxin signalling lies mainly with the ARFs and Aux/IAA protein families, and proceeds as follows: Under conditions of low auxin concentration, the Aux/IAA protein is heterodimerised to the ARFs, thereby preventing ARFs from binding to auxin responsive elements within auxin-responsive genes. When auxin concentration is high, the auxin is perceived by the TIR1 receptors at the site of action. This stimulates and stabilises the interaction between TIR1 and the Aux/IAA proteins, resulting in the ubiquitination and ultimate degradation of Aux/IAA by the 26S proteasome. This

relieves the ARFs from their inhibition, resulting in its interaction with AREs and hence the expression of auxin-responsive genes.

While cytokinins have been shown to modulate auxin-induced organogenesis through its regulation of auxin efflux (Pernisová *et al*. 2009; Růžička *et al*. 2009), the mechanisms of these interactions still remain largely unknown. Investigations using *Arabidopsis*, maize and rice have shown that a two-component signalling pathway is employed (Hwang and Sheen 2001; To and Kieber 2008). In *Arabidopsis*, cytokinin reception is mediated by proteins of the histidine kinase *Arabidopsis* Histidine Kinase (AHK 1, AHK 2 and AHK 3) and the Cytokinin Response 1 families. Two classes of *Arabidopsis Response Regulators* (ARRs) are in turn activated, following the transfer of the cytokinin signal through phosphorelay to the nucleus. Type A-ARRs are negative regulators of cytokinin signalling (To *et al*. 2004), while type-B ARRs are positive regulators which, when phosphorylated, activates the transcription of cytokinin-regulated genes (Mason *et al*. 2005). This results in a negative feedback loop of cytokinin regulation. Some components of cross-talk between auxin and cytokinin signal transduction in root meristems has been uncovered in *Arabidopsis*. The cytokinin-response transcription factor ARR1 activates the gene *SHY2*, which is a member of the *Aux/IAA* family of auxin-inducible genes, which heterodimerise to inactive ARFs (see earlier). Activation of the *SHY2* gene by cytokinin perception therefore results in repression of auxin signalling, particularly of the auxin transport proteins (PIN proteins – discussed later). Being members of the Aux/IAA family of proteins, auxin availability and perception therefore results in the degradation of the SHY2 protein, restoring and sustaining polar auxin transport (Ioio *et al*. 2008; Moubayidin *et al*. 2009). In this way, cell division, differentiation and development are regulated and tightly controlled by the antagonistic relationship and cross-talk between auxins and cytokinins. An understanding of the relationship and properties of phytohormones in *in* vitro applications is invaluable towards increasing the yield and quality of commercially important forestry crops. Their metabolism and interactions within plant tissues are of particular importance, since the addition of one of these groups during a specific propagation stage could potentially impact on the performance of the other group in subsequent stages. In this regard, the efficiency of the final and

most crucial *in vitro* stage in the production of fully-functional plantlets, i.e. rooting, can potentially be affected by the exogenous PGRs used in the preceding stages. Hence, an understanding of the factors influencing adventitious root development is necessary if the quality of *in vitro*-produced plants is to be improved.

1.4.2 Adventitious root induction and development

Plant root systems comprise primary, lateral and adventitious roots. The origins of primary roots can be traced back to the development of the radicle during embryogenesis (Barlow 1986; Casson and Lindsey 2003). However, lateral and adventitious roots are formed post-embryogenically, from differentiated cells (Barlow 1986; Casson and Lindsey 2003; Geiss *et al*. 2009). Lateral roots typically form from existing roots, while adventitious roots form from leaf or stem tissues (Barlow 1986; Geiss *et al*. 2009). The potential for adventitious root development is a natural product of plant evolution that allowed for the diversification and exploration of new environments, as in the case of epiphytes (Barlow 1986). Fossil evidence has even suggested that adventitious root systems precede embryonic or primary root systems (Barlow 1986). Adventitious roots can become specialised to serve numerous functions over and above those of primary roots (i.e. acquisition of nutrients and water, storage of food reserves and anchorage). They can be induced naturally or artificially, through environmental changes, wounding, or phytohormone application (Barlow 1986, George *et al*. 2008), a property which is extensively exploited in the vegetative propagation of most of the commercially important horticultural, agricultural and forestry crops (Ritchie 1994; Hartmann *et al*. 1997). As a result, this has generated much interest in the field of adventitious root formation and the factors influencing their development.

Essentially, adventitious roots arise from a group of cells – the root initials - that are able to dedifferentiate and become meristematic (Hartmann *et al*. 1997; Geiss *et al*. 2009). The location of root initials varies with species and in a number of easily-rooted species, root initials are latent or preformed. These lie dormant until environmental conditions are favourable for their emergence and development as adventitious roots

(Girouard 1967; Smith and Wareing 1972; Hartmann *et al*. 1997). Latent root initials have been observed in species such as willow (*Salix*), currant (*Ribes*) and citron (*Citrus medic*) (Girouard 1967; Hartmann *et al*. 1997). Where root initials are not latent, they can be induced through wounding (*de novo* formation). After a cut is made, a wounding response is triggered which results in apoptosis of the injured cells and eventually leads to the division of cells in the vicinity of the vascular cambium and phloem to initiate adventitious roots (Hartmann *et al*. 1997; Schiefelbein *et al*. 1997; Amissah *et al*. 2008; Millán-Orozco *et al*. 2011). The *de novo* development of adventitious roots is generally divided into successive stages, each with different physiological requirements. Although the number of discrete, but interdependent phases is debated, it is generally accepted that the stages consist of cell dedifferentiation, induction and initiation (when cell division starts and root initials are formed), development of root initials into root primordia, and finally expression (growth of root primordia and emergence) (Hartmann *et al*. 1997; Kevers *et al*. 1997; de Klerk *et al*. 1999; Geiss *et al*. 2009).

As already mentioned, the location of root initials is species-specific. In stem cuttings of *Quercus* sp., adventitious root primordia were found to develop from secondary phloem (Amissah *et al*. 2008). In microcuttings of apple, root primordia were observed from outside the xylem (Hicks 1987) or from phloem parenchyma cells (Harbage *et al*. 1993). Other authors have reported the initiation of adventitious roots from interfascicular cambium adjacent to phloem cells (de Klerk *et al*. 1995; Jásik and de Klerk 1997). Specifically in eucalypts, Baltierra *et al*. (2004) found that adventitious roots *in vitro* originated from either old vascular tissue or from newly-formed xylem. In common, however, is the *de novo* development of adventitious roots adjacent to the central core of vascular tissue, and in woody species, from phloem ray parenchyma cells (Hartmann *et al*. 1997). Whilst histological studies on the cellular origins of adventitious roots in various plant species have been useful in characterising the steps and cells involved in this complex process, studies focussing on the environmental and molecular factors influencing adventitious rooting have revealed some underlying mechanisms governing this process.

Molecular and genetic studies of adventitious rooting have shown that rooting competence is heritable and, therefore, quantifiable (Grattapaglia *et al*. 1995; Marques *et al*. 1999; Geiss *et al*. 2009). In the case of eucalypts (and other woody species of economic importance), clones are classified as difficult- or easy-to-root based on their genetic predisposition. Reports of the existence of quantitative trait loci (QTLs) for root number in cuttings of *Populus deltoides* (eastern cottonwood) by Wilcox and Farmer (1968), led to the search for rooting genes in other commercially important crops. Grattapaglia *et al*. (1995) found four QTLs for the percentage of rooted cuttings in *E. grandis* X *urophylla*, with *E. urophylla* contributing most of the rooting ability in that cross. Similarly, investigations into the vegetative propagation traits in *E. tereticornis* and *E. globulus* revealed nine QTLs associated with adventitious rooting, with a larger portion of the phenotypic variation in adventitious rooting associated with *E. tereticornis*, a known good-rooting clone (Marques *et al*. 1999; Geiss *et al*. 2009). Further, attempts to identify candidate genes associated with adventitious root formation have disclosed a number of genes that are either up-regulated or down-regulated during adventitious rooting (reviewed by Casson and Lindsey 2003; Geiss *et al*. 2009; Li *et al*. 2009). These were also strongly induced by phytohormones, particularly auxins and their affiliated transport proteins.

From some of the earliest studies in this field, auxins emerged as an important factor in the genetic regulation of adventitious rooting (Dhindsa *et al*. 1987). More recent developments towards understanding auxin signalling and perception have provided further evidence in this regard (see reviews by Quint and Gray 2006; Pop *et al*. 2011). Genes identified as promoting adventitious root formation, such as *ROLB* (root loci) in *Agrobacterium*, have been shown to confer increased sensitivity to auxin (Shen *et al*. 1988), which indicates a relationship between root formation and the auxin perception pathway (Quint and Gray 2006; Geiss *et al*. 2009; Li *et al*. 2009; Pop *et al*. 2011). The positive regulator genes of crown root formation in rice, *CRL1/ARL1* (*Crown Rootless1*/*Adventitious Rootless 1*) (Inukai *et al*. 2005), are also auxin-responsive genes (Geiss *et al*. 2009). Genes of the auxin response factors (*ARF*s) have often been reported as being involved in the initiation and control of adventitious rooting (Geiss *et al*. 2009; Li *et al*. 2009; Pop *et al*. 2011). Even during the initiation of root development,

expression of genes such as *Scarecrow* (*SCR*), which are involved in a range of root control and maintenance events, are dependent on auxin availability and distribution (Sabatini *et al*. 2003; Sánchez *et al*. 2007). Although auxin is accepted as being crucial to each step of the adventitious rooting process, a number of other phytohormones and endogenous factors have been shown to work either synergistically or antagonistically with auxin during root formation, the most documented being ethylene and cytokinins.

The relationship between ethylene and auxin was recognised as early as 1935 by Zimmerman and Wilcoxon. Increased ethylene concentrations have been shown to heighten endogenous IAA sensitivity in *Rumex palustris* (Visser *et al*. 1996). Subsequently, various roles of ethylene on root development have been reported in a number of plant species. These have often appeared contradictory, with ethylene either promoting or inhibiting root development (Geiss *et al*. 2009). What has been established, is that the effects of ethylene on root development is dependent on the synthesis, transport and signalling of auxin (Stepanova *et al*. 2007; Dugardeyn and van Der Straeten 2008). Cytokinins, however, are known to work antagonistically with auxins in plant development, with the inhibitory role of cytokinins on root initiation being well-recognised (Hartmann *et al*. 1997; George *et al*. 2008). The cross-talk between auxins and cytokinins has been the subject of numerous studies on root development (reviewed by Moubayidin *et al*. 2009). As a consequence of its antagonistic relationship with auxins, cytokinins play various regulatory roles during root development, most notably via their regulation of polar auxin transport (Růžička *et al*. 2009). In addition to the primary genetic and phytohormone determinants in adventitious root development, environmental conditions also influence root initiation and development, either directly or in conjunction with each other.

Environmental factors such as temperature, light and nutrient availability have varying effects on adventitious rooting. Corrêa and Fett-Neto (2004) demonstrated that the effect of temperature on adventitious root formation is species-specific, with *E. saligna* more resistant to higher temperatures than *E. globulus* in terms of rooting of *in vitro*-grown microcuttings. Whereas warmer temperatures increased the rooting potential of *E. saligna*, the opposite was true for *E. globulus*. In conifers (Berhens 1988) and

Chrysanthimum spp. (Dreuge *et al*. 2000), cold storage of mother plants was found to influence carbohydrate concentration, leading to an alteration in the nitrogen/carbohydrate ratio, thereby increasing the rooting potential of cuttings. The reliance of adventitious rooting on carbohydrate content has also been demonstrated in other commercially important crops, such as *Tectona grandis* (teak) (Husen and Pal 2007). The rooting potential of explants of *E. sideroxylon* was dependant on sucrose concentration *in vitro* (Cheng *et al*. 1992), starch accumulation was observed prior to root primordium emergence in *Pinus radiata* (Li and Leung 2000), and an adequate supply of carbohydrate was necessary for adventitious root initiation and development in apple microcuttings (Calamar and de Klerk 2002). Moreover, in the presence of auxin, the type of carbohydrate was found to influence the adventitious rooting capacity of *E.saligna* and *E. globulus* (Corrêa *et al*. 2005). Mineral nutrition also appears to modulate adventitious rooting by increasing the root number or influencing root length (Hartmann *et al*. 1997; George *et al*. 2008; Geiss *et al*. 2009). An investigation into the effects of various minerals on *E. globulus* revealed that while root length was affected by phosphorous, iron and manganese, root number was influenced by calcium, nitrogen source and zinc (Schwambach *et al*. 2005), although the effects of specific minerals on adventitious rooting depends on the species (Geiss *et al*. 2009). Calcium plays a particularly important role in this process, as it is involved in cell division and elongation of root primordia (Geiss *et al*. 2009), and acts as a second messenger in key signalling pathways, particularly those of auxin (Schwambach *et al*. 2005; Lanteri *et al*. 2006).

The influence of light on adventitious rooting was investigated by Fett-Neto *et al*. (2001) in two eucalypt species with varying rooting abilities. They found that cuttings of the good-rooting *E. saligna* responded to lower auxin concentrations and were not significantly affected by light, whereas cuttings from the poor-rooting *E. globulus* did not root when exposed to light during the root formation stage, in the absence of auxin. The addition of the auxin IBA reversed this response, indicating the role of irradiance as a factor in adventitious root formation (Fett-Neto *et al*. 2001). A genetic basis of the interactions between auxins and light was established using *Arabidopsis* mutants, wherein a suite of proteins were identified that correlated with adventitious root

formation (Sorin *et al*. 2005). Indications were that some of these proteins were involved in the regulation of light-associated metabolic pathways and auxin homeostasis (Sorin *et al*. 2005; Lao and Deng 2010). The involvement of auxin does not end with adventitious root induction, but it is oft-encountered during almost all aspects of root development and physiology (Hartmann *et al*. 1997; Perrot-Rechenmann and Napier 2005; George *et al*. 2008; Vanneste and Friml 2009).

1.4.3 Root gravitropism

The complete plant gravitropic reaction proceeds broadly in three stages: perception, transduction, and response. The idea that structures in the root tip provide the means for gravity perception in roots was put forward in the late 19th century by Ciesielski (1872) and later by Darwin (1880), who showed that de-capped roots were unable to respond to gravity. It was postulated that the cap of the root tip would sense gravity and a physiological signal is then produced that promotes differential elongation on the upper root surface (away from the gravity vector), so as to create curvature in the direction of gravity (reviewed by Chen *et al*. 1999; Swarup and Bennett 2009). Since then, there have been a number of hypotheses that attempted to explain the mechanism of graviperception, the two most popular being the starch-statolith hypothesis and the protoplast pressure model.

The starch-statolith hypothesis of graviperception was proposed as early as 1886 when Berthold and later Noll (1892) speculated that it was possible for gravitropism to proceed via asymmetric settling of cellular inclusions. A few years later Haberlandt (1900) and Němec (1900) identified the sedimenting inclusions as starch grains (amyloplasts) (Shen-Miller and Hinchman 1974; Staves 1997), contained within the columella cells in the root tip. The sedimentation of these starch grains is thought to then activate a signal transduction pathway that eventually results in root curvature (Evans and Ishikawa 1997). A number of researchers have subsequently presented both direct and indirect evidence to support the starch-statolith hypothesis (Chen *et al*. 1999; Swarup and Bennett 2009).

A strong correlation exists between amyloplast density and root gravity response, as shown by Kiss *et al*. (1996). Through comparisons of various gravity response parameters between starchless mutants, intermediate-starch mutants, and wild type plants, those authors demonstrated that the starch content of *Arabidopsis thaliana* roots affected graviperception. Starchless mutants responded much less to gravity compared with intermediate-starch mutants and wild type plants, indicating that the degree of gravity perception depended on the mass of plastids per cell. Additional support for the starch-statolith theory was provided using a laser-ablation approach, in which *A. thaliana* roots were shown to lose most of their ability to perceive gravity when the central columella cells of the root tip were ablated (Blancaflor *et al*. 1998).

Despite the numerous studies that support the starch-statolith theory, many authors have doubted its validity. Studies by Pickard and Thimann (1966) showed that coleoptiles depleted of starch grains were still able to carry out a geotropic response, albeit much reduced compared with those containing starch. Work on starchless *A. thaliana* mutants by Kiss *et al*. (1989) and Caspar and Pickard (1989) convincingly demonstrated that starch was not an absolute necessity for full gravity perception and response. The protoplast pressure model was proposed as an alternative to the starch-statolith theory, and a number of studies supported this model.

The protoplast pressure model, which suggests that other organelles may be involved in gravity perception, was first alluded to by Pickard and Thimann in 1966. Work by Wayne *et al*. (1990) on internodal cells of the algae *Nitellopsis obtusa* provided support for this model, wherein it was demonstrated that the entire mass of the cytoplasm is involved in gravity perception. However, Sack (1997) and Perbal (1999) attempt to merge the theories by suggesting that multiple mechanisms function to bring about gravity perception in plants. Essentially, receptors within the plasma membrane of columella cells need to sense pressure in order to activate graviresponse. This pressure can be applied by the entire mass of the protoplast, by sedimenting starch grains, or by plastids in general. The difference between these effectors can then be reduced to a matter of efficiency or the area on which they focus pressure (Perbal 1999). Once gravity is perceived, a signal transduction pathway is activated which results in a

response to the gravity stimulus. While this model attempted to explain the variations in gravitropic efficiency through differences in the mass of the cytoplasm, it did not account for the biochemical signal and maintenance of the geotropic response once initiated.

The Cholodny-Went theory of gravitropic bending has traditionally been the most widely accepted model upon which research in gravitropism has been built. This theory proposes that following gravity perception, the response is controlled and maintained by auxin, which is laterally transported in the tissue (either stem or root). An asymmetric differential redistribution of this auxin then results in curvature, depending on the organ in question, since auxin plays very different roles in stems and roots. Auxins have an inhibitory effect on growth in root tissues, and an opposite effect in shoot tissues (Kaufman *et al*. 1995). Although the essential predictions of the theory have been extensively proven experimentally, some criticisms against the theory are that it is an over-simplification of a much more complex process involving (at least) factors such as changing sensitivity to auxin (Trewavas 1992; Davies 1995), as well as participation of other phytohormones together with auxin (Philosoph-Hadas *et al*. 2005).

Building on the Cholodny-Went hypothesis, the „fountain model' (Trewavas 1981; Evans *et al*. 1986) proposes that auxin is transported towards the root tip through the stele and eventually enters the root cap, from where it is transported symmetrically back (basipetally) through the cortex towards the root elongation zone (Wolverton *et al*. 2002). When the root perceives a reorientation with respect to gravity, an asymmetric redistribution of auxin is induced, which results in auxin accumulation on the lower side of the root. Since auxin is inhibitory to cell elongation in root tissues (Philosoph-Hadas *et al*. 2005), the result is that the root curves towards the gravity vector (Wolverton *et al*. 2002; Swarup and Bennett 2009).

Polar transport of auxin was initially demonstrated through the classical experiments of Went (1935). An agar block with known auxin concentration was placed on the apical surface of a coleoptile cylinder, while one devoid of auxin was placed on the basal surface. After a few hours the greater portion of the auxin was detected in the lower block. Swopping the agar blocks resulted in no auxin being transported through the

coleoptile cylinder, indicating auxin transport in a polar direction through an ordered cellular arrangement. Recognising that cell polarity is a basic requirement for ordered growth and differentiation, Leopold and Hall (1966) proposed a mathematical model for polar auxin transport. It explained that polarity is achieved in auxin transport through the preferential secretion of more auxin from the lower end of the cell compared with the upper end, so as to maintain nett auxin secretion in the basipetal direction.

A more explanatory model of auxin transport was proposed by Rubery and Sheldrake (1974) and Raven (1975), which is based on the chemiosmotic theory. According to those authors, auxin transport is driven by the proton motive force across the plasma membrane. Auxin may enter a cell either through transport via an uptake carrier; or directly in its protonated form, since IAA (pKa = 4.7) is a weak acid and its carboxyl group is more protonated in the acidic conditions (pH = 5.5) of the extracellular matrix. In this state the lipophilic IAA is able to pass through the cell membrane and into the cytoplasm. Upon exposure to the more basic pH in the cytoplasm (pH = 7), IAA loses its proton and becomes charged (hydrophilic), hence trapped within the cytoplasm. Auxin efflux is then facilitated by efflux carriers located on the basal side of the cell. This arrangement gives auxin transport its polar nature (Lomax *et al*. 1995; Leyser 1999). Jacobs and Gilbert (1983), using an immunological approach, verified the basal location of the IAA efflux carrier.

In addition to the passive influx of auxin into cells, Bennet *et al*. (1996) found evidence of carrier-mediated auxin influx, wherein mutations within the *AUX1* gene resulted in auxin-resistant root growth characteristics. Sequence similarity between AUX1 and permeases led to the suggestion that AUX1 is a transport mediator for an amino acid-like signalling molecule. Being structurally similar to the amino acid tryptophan, it was then suggested that auxin (IAA) was the substrate of the AUX1 transport protein. Given the proton-driven nature of plant permeases, and that auxin influx has also been shown to occur through a proton co-transport system (Estelle 1998), Bennett *et al*. (1996) proposed that AUX1 may be an auxin influx protein. Support for this mode of auxin influx was gained with studies by Yamomoto and Yamamoto (1998) involving *aux1* seedlings resistant to auxins that are good transport substrates, such as IAA and 2,4-D.

Putative auxin efflux carriers were isolated by a number of authors at around the same time (Chen *et al*. 1998; Luschnig *et al*. 1998; Müller *et al*. 1998; Utsuno *et al*. 1998). Studies on the *Arabidopsis pin1* mutant, which exhibits greatly reduced auxin transport in the inflorescence axis (Okada *et al*. 1991), have revealed the presence of a basally located auxin efflux carrier (Gälweiler *et al*. 1998). Loss of PIN1 function elicits a growth response analogous to growth in the presence of auxin transport inhibitors. While the PIN1 protein is found in the stem, a second gene family was found in roots that lacked the gravitropism response. Bell and Maher (1990) induced and isolated a number of *Arabidopsis* mutants with altered root graviresponse, called "*Agravitropic*" (*agr*) mutants. While some of these mutants (*agr2* and *agr3*) displayed reduced gravity sensing, the mutant designated *agr1* was totally agravitropic. Studies on these (*agr1*) mutants by Chen *et al*. (1998) showed that the AGR1 protein promoted the efflux of radiolabelled IAA.

Working on *A. thaliana* etiolated seedlings in an attempt to identify genes for ethylene perception, Roman *et al*. (1995) reported the existence of a number of previously unidentified gene complementation groups. Four of these were responsible for ethylene insensitivity, while the fifth group *eir1* (*ethylene insensitive root 1*), defined a class of mutants that were both insensitive to ethylene as well as exhibiting severe agravitropism. Immunolocalisation studies with antibodies against the EIR1 protein revealed the accumulation of the protein on the basal end of cells limited to the plasma membrane of epidermal and cortical cells in the root tip (Müller *et al*. 1998; Leyser 1999). Luschnig *et al*. (1998) then showed *agr1* and *eir1*, as well as other putative auxin efflux carriers such as *Atpin2* (Müller *et al*. 1998) to be alleles of the larger *PIN* gene family. These proteins were revealed to have amino acid sequence similarity to several membrane transport proteins, and their properties were consistent with their role as IAA transport proteins (Luschnig *et al*. 1998; Müller *et al*. 1998). Numerous studies have subsequently supported the role of these carrier proteins, by using mutant *A. thaliana* lines that exhibit loss-of-function of any one or multiple PIN proteins (reviewed in Palme and Gälweiler 1999; Michniewicz *et al*. 2007). The discovery, isolation and characterisation of root-and stem-specific IAA influx and efflux proteins, as well as their localisation within the cell, provided the tools for further research into the

implications of polar auxin transport on gravitropism, as well as root developmental physiology in general.

The physiological signals that are produced upon gravity perception have to be transported to the relevant regions of the root, i.e. its distal and central elongation zones, in order for the appropriate response to be elicited. Polar auxin transport has been shown to be intimately connected with the gravitropic responses observed in these tissues (Chen *et al*. 1999; Muday 2001; Blancaflor and Masson 2003). The asymmetric distribution and localisation patterns of the IAA influx and efflux carriers are consistent with the chemiosmotic theory, and their respective functions of polar auxin transport in the distal and central elongation root zones (Chen *et al*. 1998). Proteins of the efflux PIN family and of the influx AUX1 family are either located basally, or along the upper plasma membrane of cells within the root cap, depending on the orientation of the root with respect to gravity (Chen *et al*. 1999; Moore 2002; Blancaflor and Masson 2003). They are rapidly relocated within the cell when the root is reorientated relative the gravity vector, and an asymmetric auxin gradient is generated so that more auxin is transported to the new bottom edge of the root, where it functions in inhibiting root elongation, thereby allowing downward curvature (Philosoph-Hadas *et al*. 2005; Swarup and Bennett 2009; Vanneste and Friml 2009).

Essentially, the model of polar transport of auxin as the signal and effector of the gravitropic response within the root tip is as follows: auxin is transported acropetally (towards the root tip) via the PIN1 efflux carriers and then via the PIN4 carrier into the quiescent centre. Starch grains within statoliths in the columella region sediment in the direction of gravity, causing a relocation of the PIN3 carrier to the lower side (relative to gravity) of root tip cells (Fig. 1.1). An accumulation of auxin at the basal end then inhibits root elongation in the lower side, causing a directional change in the growing root towards the gravity vector. The auxin gradient is re-established through the PIN7 and PIN2 carriers, which are located in the cortical and epidermal cells. Auxin gradients are then restored through the basipetal delivery back along the root transport (Fig. 1.1) (reviewed by Moore 2002; Perrot-Rechenmann and Napier 2005; Swarup *et al*. 2005; Swarup and Bennett 2009). This model of gravitropism is based on the polar transport

of IAA and, therefore, implicates the IAA-specific protein carries. However, not much is known regarding the influences of other auxin analogues on graviperception, and this area therefore needs further elucidation.

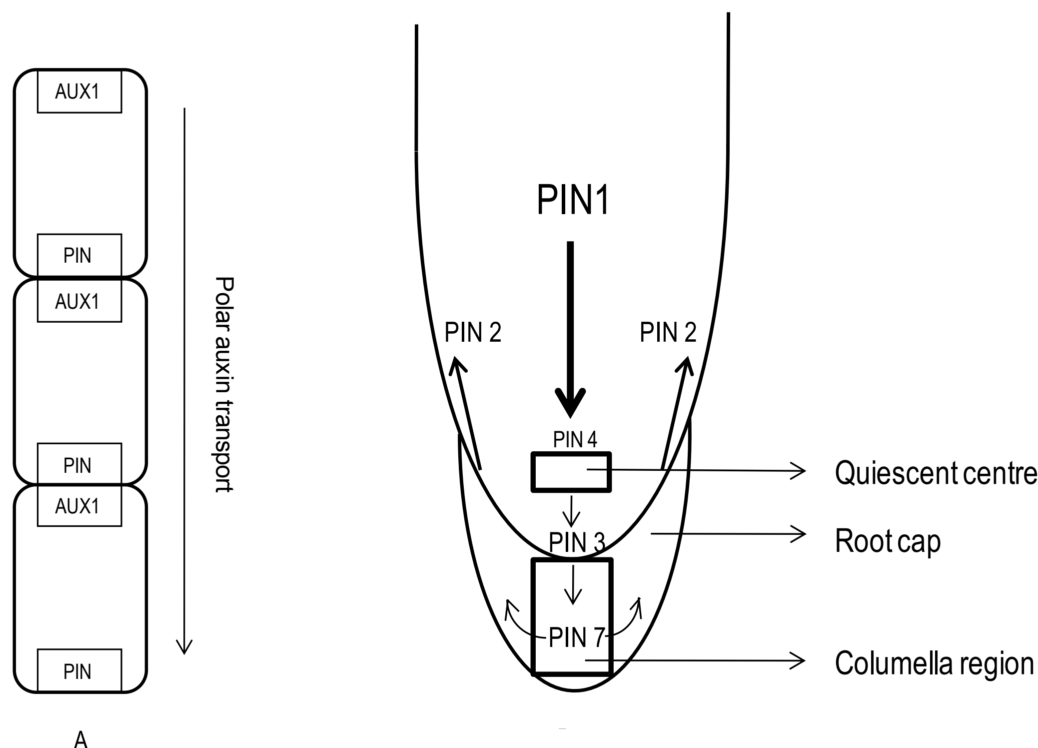

Fig 1.1 Polar auxin transport in the root tip is facilitated mainly through the AUX1 and PIN family of proteins, located on the upper and lower sides of root cells, respectively (A). Auxin is instrumental in effecting a gravitropic response through its polar transport, brought about by the positioning of particularly the PIN3 auxin efflux carrier, which relocates to the lower side of cells upon gravity perception by the sedimentation of amyloplasts within the root cap columella region (B) (Adapted from Michniewicz *et al*. 2007 and Moubayidin *et al*. 2009)

Information regarding carrier-mediated auxin transport materialised from studies using inhibitors of polar auxin transport in intact plants, tissue fragments and cultured cells. For example, it was found for example, that 2,3,5-triiodobenzioc acid (TIBA), a known inhibitor of polar auxin transport, effected an accumulation of labelled IAA in maize coleoptiles in a manner that led to the idea that TIBA inhibited IAA efflux (Hertel and Leopold, 1963). The finding that auxin influx is also carrier-mediated was a result of

research using auxin transport inhibitors. Rubery and Sheldrake (1974) found that uptake of auxin into suspension cells was a saturable process, showing that a protein mediator must be involved in the process.

There are a number of natural and synthetic auxin transport inhibitors that have been identified. The major class of such inhibitors are the phytotropins, which in addition to inhibiting polar auxin transport, also inhibit plant gravitropic and phototropic responses. These are the most widely studied and refer to a class of auxin transport inhibitors that share a common chemical structural theme (Lomax *et al*. 1995). Whatever the chemical class, auxin transport inhibitors inhibit efflux of auxin by non-competitive binding with the transport protein at the site of the catalytic unit of the auxin efflux carrier, but distinct from the auxin binding site (Rubery 1990; Lomax *et al*. 1995). While the bulk of the research has been directed at inhibitors of auxin efflux carriers, inhibitors that target auxin influx carriers have also been identified, such as 1-naphthoxyacetic acid (Parry *et al*. 2001a).

It has been documented that the auxin efflux carrier PIN1 is disrupted by transport inhibitors that interfere with its cycling within the cell (a property that is central to its auxin transport role) and with the membrane trafficking process in general (Geldner *et al*. 2001). The transport inhibitor TIBA has been shown to inhibit membrane trafficking of both the efflux carrier, as well as the influx carrier (AUX1) (Kleine-Vehn *et al*. 2006). Those authors also demonstrated that the AUX1 and PIN1 transport proteins are subjected to membrane trafficking via distinct pathways in *A*. *thaliana*. Interference in auxin signal transduction with ρ-chlorophenoxyisobutyric acid (PCIB) has been shown to regulate Aux/IAA stability and hence root graviperception in *Arabidopsis* (Oono *et al*. 2003). Further, although cytokinins generally inhibit auxin actions in plants (George *et al*. 2008; Geiss *et al*. 2009), their interactions with auxin with respect to root graviperception are unclear. They have been reported to contribute to the regulation of root gravitropism in *Arabidopsis*, particularly during the rapid, early phase of root gravity response (Aloni *et al*. 2004; Aloni *et al*. 2006). However, more recently, cytokinins were shown to influence cell-to-cell auxin transport through the modification of components of auxin transport, in turn influencing auxin efflux and distribution

(Pernisová *et al*. 2009; Růžička *et al*. 2009). The influences of auxin inhibitors and antagonists not only on IAA action, but also other auxin analogues need further elucidation, to clarify the specific roles of these analogues on plant development and physiology, particularly since they are crucial in almost every aspect of plant development and are used interchangeably in micropropagation protocols.

As proteins, influx and efflux carriers bear a degree of specificity (although some overlap has been observed) for the type of auxin that they transport, and this has implications for plant physiological development. Delbarre *et al*. (1996), using tobacco cells, and later Yamamoto and Yamamoto (1998), using the *A. thaliana aux1* mutant, demonstrated that IAA and the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) are substrates for the AUX1 influx carrier. The synthetic auxin 1-naphthaleneacetic acid (NAA) on the other hand, is not transported via the same influx carrier, since it was found to be able to restore gravitropism in the *aux1* mutants (Yamamoto and Yamamoto 1998). NAA has been shown to enter cells through passive diffusion, while its exit from cells is facilitated by efflux carrier proteins (Delbarre *et al*. 1996). Although 2,4-D and IAA share the influx carrier, they are not transported via the same efflux carriers, as demonstrated in *A. thaliana* (Utsuno *et al*. 1998), and in suspension-cultured tobacco cells (Delbarre *et al*. 1996). Work conducted on the Arabidopsis *rib1* mutant, which exhibits resistance to IBA, has shown that IBA is also not transported via the same efflux carriers as does IAA (Poupart and Waddell 2000; Strader and Bartel 2011).

Since different auxins are transported within tissues in different ways (passive and active), and due to the specificity of the protein mediators that allow them to carry out their various functions, it can be tentatively deduced that the auxin requirements for plant growth and development are specific to an auxin type. This has implications for the development of *in vitro* protocols of eucalypts, which empirically select PGRs, based on their apparent organogenic potency rather than their long-term effects on growth and development, or their interactions with other PGRs.

1.5 Aims and objectives of the present study

Vegetative propagation of commercially important eucalypt clones are an important part of tree improvement programmes. *In vitro* organogenesis protocols are not only essential in genetic modification, but also provide a means of propagating valuable and elite clones that would otherwise not be considered for forestry owing to the difficulty in propagating them via mini- or macro-cuttings. For these reasons, such micropropagation protocols need to be optimised in order to allow for quality plantlet generation both *in vitro* and following acclimatisation. The key to organogenesis *in vitro* lies in the appropriate supply of exogenous PGRs and, therefore, any attempt at understanding plant growth and development following *in vitro* regeneration requires an understanding of the roles of the exogenous PGRs in that system.

The final and most important step in any clonal propagation programme is the successful development of roots. Studies have indicated, however, that *in vitro*-produced roots of certain commercially important eucalypts have a root system which, owing to its horizontal and shallow architecture, is more susceptible to uprooting than roots of seedling or macro-propagated plants (Mokotedi *et al*. 2009b). Root development is particularly dependent on auxins, which are implicated in a range of physiological processes such as root cell patterning (Blilou *et al*. 2005), maintenance and zonation of the root meristem (Luijten and Heidstra 2009), and gravitropism (Swarup and Bennett 2009). Since exogenous auxins are used to direct root initiation and development *in vitro*, an understanding of the roles of these exogenous auxins is necessary to realise their control on root development.

The present study was aimed at understanding the specific roles of PGRs during *in vitro* root organogenesis of selected commercially important eucalypt clones. The influence of exogenous auxin type, stability, concentration, and subsequent accumulative effects during the pre-rooting culture stages on *in vitro* and post-acclimatisation root development was investigated. The specific roles of auxin in relation to parameters such *in vitro* vascular differentiation, root tip development and graviperception were studied,

using inhibitors of auxin transport and signal transduction, and antagonists of auxin action.

A good- and two poor-rooting clones were selected in order to understand the interactions between auxins and cytokinins and their relative influences on root development in clones of variable rooting ability. This was aimed at refining micropropagation protocols to allow for root induction not only under conditions of the most potent PGR, but rather the most appropriate PGR. With such information, the present limitations of *in vitro* propagation can be addressed, and the potential of this technology in fundamental research, and in supplying large amounts of clonal material to the forestry industry can be realised. In addition, by understanding the phytohormone needs of eucalypt clones, other vegetative propagation protocols can be optimised to increase yields of both difficult- and easy-to-root clones.

CHAPTER 2: AUXIN STABILITY AND ACCUMULATION DURING *in vitro* SHOOT MORPHOGENESIS INFLUENCES SUBSEQUENT ROOT INDUCTION AND DEVELOPMENT IN *Eucalyptus grandis*

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2.1 Introduction

Eucalyptus spp. and their hybrids are widely propagated and utilised by the forestry industry to help meet the increasing global demand for wood and wood-related products. In many countries, they are preferentially propagated vegetatively as this preserves desired genotypes, allows for relatively accurate site matching of superior clones and confers the advantage of uniformity (in height, tree diameter, wood properties, etc.) (Eldridge *et al*. 1994; de Assis *et al*. 2004), all of which translate to increased economic value from the plantation.

Traditionally, *Eucalyptus* propagation programmes utilise stem cuttings, but this method has limitations as the yield is restricted, the rooting ability amongst clones is variable and tend to decrease as the parent plants age (Eldridge *et al*. 1994; de Assis *et al*. 2004). Hence, alternative methods such as minicuttings, hydroponics and micropropagation are being employed (Denison and Kietzka 1993a; Eldridge *et al*. 1994; de Assis *et al*. 2004). In terms of micropropagation, axillary bud proliferation is generally the preferred choice (reviews by Jones and van Staden 1997; Watt *et al*. 2003; de Assis *et al*. 2004) and, together with some specific mini-cutting techniques (e.g. Schwambach *et al*. 2008), the only viable method for the propagation of difficult-to-root clones (Yasodha *et al*. 2004; Mokotedi *et al*. 2000; George *et al*. 2008).

The reported successes of micropropagation protocols for eucalyptus, including axillary bud proliferation, have centred on achieving the correct balance of plant growth regulators (PGRs) (most notably auxins and cytokinins), in a genotype-specific manner, to achieve maximum *in vitro* shoot proliferation and rooting (reviews by Jones and van Staden 1997; Watt *et al*. 2003; de Assis *et al*. 2004). Further, with respect to rooting, the emphasis has been on root induction and percent rooting, rather than on root quality prior to and post acclimatisation. An exception is the work by Bell *et al*. (1993) which revealed no growth morphological differences between the roots of *E. camaldulensis* obtained from seed and tissue cultured plants after nine months of field growth. In addition, both plant types exhibited sinker roots that were equally capable of penetrating

heavy clay soils, although one clonal line displayed root architecture that was found predominantly in the upper 20 cm of the soil profile. Similarly, Mokotedi *et al*. (2003) found that the propagation method did not significantly affect root hydraulic conductance of nine-month-old micro- and macro-propagated *E. grandis* x *nitens*. However, when micropropagated plants of that hybrid were compared with seed-propagated *E. grandis* and *E. nitens* after sixteen months of field growth, significant differences were found in root architecture, root hydraulic conductance (Mokotedi *et al*. 2009b), growth patterns and in uprooting resistance (Mokotedi *et al*. 2010). In particular, roots of micropropagated plants established just below the soil surface as a few I-beam shaped roots, whereas seedling plants developed numerous T-beam shaped roots, which were significantly more resistant to uprooting than the micropropagated ones (Mokotedi *et al*. 2010). That study employed the standard, routinely-used protocol in our laboratories, adapted for the clone for maximum multiplication, elongation, rooting and acclimation yields, with specific PGRs for each of the *in vitro* stages (Mokotedi *et al*. 2000). The results from that field work may, therefore, be explained by the influences of the supplied phytohormones pre- and post rooting.

While root induction can potentially be achieved with any auxin type, the mode of action and transport of each auxin within plant tissues differs (Vieten *et al*. 2007), and their specific effects on the physiological development of the plantlet cannot be discounted. For example, indole-3-acetic acid (IAA) is more rapidly taken up by plants and easily conjugated or oxidised to inactive forms (Blakesley 1994; de Klerk *et al.* 1999) than indole-3-butyric acid (IBA), which is more stable and persists for longer in plant tissues (de Klerk *et al*. 1999). These auxin conjugates are then stored within the plant and later hydrolysed to provide free auxin as the plant requires (Blakesley 1994). Furthermore, the differential effects of auxins on root development *in vitro* have been demonstrated in Eucalyptus. For example, based on adventitious rooting studies with *E. globulus* and *E. saligna*, Fogaça and Fett-Neto (2005) concluded that the best rooting response achieved using IBA could possibly be explained by its conversion to IAA, and its higher relative stability over IAA. Those authors further suggested that the more persistent auxins such as NAA inhibit root emergence by remaining in tissues in the free form. Rooting studies on *E. sideroxylon* have also demonstrated a greater callus-

forming tendency of IBA over NAA (Cheng *et al*. 1992), further emphasising the effects of different auxin types and stabilities on tissue development *in vitro*. Since root development progresses in stages that differ in auxin sensitivity, with some developmental stages being inhibited by auxins (de Klerk *et al*. 1999), the choice and longevity of the exogenously-supplied auxins, and their transport with respect to gravistimulation, need to be considered in micropropagation protocols.

The present study tested the hypothesis that *in vitro* root induction and development are adversely affected by exogenous auxin supply above or below a specific concentration range. The type of auxin, its accumulation from previous culture stages, its relative stability and its role in graviperception *in vitro* were considered. Further, it investigated which auxin type successfully effects graviperception *in vitro*, using the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA).

2.2 Materials and Methods

2.2.1 Decontamination and culture initiation

Pure *E. grandis* parent plants with high minicuttings rooting success (clonal material) were obtained from Mondi Business Paper, Hilton, KwaZulu-Natal, from which minicuttings were taken and surface sterilised in 0.02% (w/v) $HgCl_2$ and a drop of Tween® -20 for 10 minutes, followed by 1% (w/v) calcium hypochlorite for 10 minutes and rinsed several times with sterilised distilled water. They were then cut into nodal segments, each with half a leaf intact and placed on 10 ml bud induction medium [MS nutrients (Murashige and Skoog 1962), 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.04 mg l^{-1} (0.21 μM) α-naphthaleneacetic acid (NAA), 0.1 mg l^{-1} (0.44 μM) 6-benzylaminopurine (BAP), 0.05 mg l^{-1} (0.23 μM) kinetin, 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite®, pH 5.6-5.8] in 50 ml culture tubes with snap-on lids for 2 weeks.

2.2.2 Micropropagation protocol

The multiplication and elongation formulations of the standard protocol employed in our laboratories are designated M1 and E1, respectively. Their components were as for bud induction, except for the PGRs. Two multiplication media were used, M1 (standard) contained 0.04 mg l^{-1} (0.21 µM) NAA and M2 lacked auxin. The tested PGRs for the elongation media are given in Table 2.1. Shoots were maintained in 20 ml medium in 100 ml culture bottles (5 shoots per culture bottle) during the multiplication and elongation stages, which were typically 3 and 4 weeks, respectively. Once shoots reached a height of at least 1.5 cm, they were transferred to 10 ml rooting medium in culture tubes, containing ¼ MS nutrients (Murashige and Skoog 1962), 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 15 g l^{-1} sucrose and 4 g l^{-1} Gelrite®. Rooting media were supplemented with auxins at various concentrations and 0.4 mg l^{-1} (0.8 µM) 2,3,5-triiodobenzoic acid (TIBA), as required. Root induction was monitored every 3 to 5 days, and was scored as positive when at least 0.5 cm of the root protruded from the base of the shoot. The mean rooting times were calculated according to the method described by Fett-Neto *et al.* (2001).

All media, including those with the phytohormones, were adjusted to a pH of between 5.6 and 5.8, and decontaminated by autoclaving at 121°C and 1KPa for 20 minutes. Cultures were maintained under a 16-h light (200 µmol m^{-2} s^{-1}) / 8-h dark photoperiod, at 25°C and 23°C, respectively.

2.2.3 Acclimatisation

Rooted shoots were acclimatised in insert trays containing 1:1 peat:perlite mix, supplemented with 1/3 MS nutrients. Shoots were maintained for 2 months in a mist tent and then planted out in 25 litre pots that were kept in a shadehouse at Mondi Business Paper, Hilton, KwaZulu-Natal.

Table 2.1 Plant growth regulators in the tested elongation media

PGR (mg l^{-1}/µM)	Media					
	E1	E2	E3	E4	E5	E6
0.2/0.93 Kinetin	+	-	+	+	+	+
0.3/1.6 NAA	+	-	+	-	-	-
0.05/0.25 IBA	+	-	-	-	+	-
0.37/2.1 IAA	-	-	-	-	-	+

2.2.4 Sample preparation for phytohormone analysis

Analysis of the levels of auxins in shoots were conducted using GC-MS. Samples were prepared as follows: 50 mg of freeze-dried shoots per sample were homogenised and suspended in 500 µl sodium phosphate buffer (pH 7) and incubated for an hour at 4°C. The pH was adjusted to 2.6 with HCl, adsorption of the compounds were facilitated by the addition of Amberlite® XAD-7 (Sigma-Aldrich), and the solution was incubated again at 4°C for another hour. Following 2 washes of 500 µl 1% (v/v) acetic acid and dichloromethane, the samples were dried down and 50 µl of trimethylsilyl-diazomethane was added. The samples were then incubated for 30 minutes at room temperature. Acetic acid (1% v/v) was added to quench the samples, which were then dried down overnight. Samples were ready for GC-MS analysis once heptane was added. Analysis was conducted using the GCT Premier™ benchtop orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer, Waters, USA.

2.2.5 Microscopy

Root tips were prepared for light microscopy by placing them initially in a 2.5% (v/v) gluteraldehyde solution in a 0.1 M phosphate buffer at pH 7.2 for at least 24 hours at 4°C. The primary fixative was removed by several rinses with the phosphate buffer and

the samples were then post-fixed in 0.5% (v/v) osmium tetroxide solution for an hour. Following several rinses in phosphate buffer, the samples were dehydrated through a series of increasing acetone concentrations. The samples were then infiltrated with 50:50 acetone: epoxy resin (Spurr 1969) and left on a shaker for 5 hours before being placed in epoxy resin overnight for further infiltration. Samples were then placed into silicone blocks and the resin was polymerised at 70°C for 8 hours. Sections of 1 µm were collected using the Reichert Ultracut E microtome. These were stained with a 1% (w/v) KI and 1% (w/v) safranin solution. Sections were viewed using the Nikon Biophot® light microscope coupled with the Motic Image Plus 2.0 computer programme.

2.2.6 Statistical analysis

All statistical analyses was carried out using the programme PAST, version 2.01 (Hammer *et al*. 2001). All experiments were repeated at least three times, with sample sizes of at least 3 for phytohormone analysis, and 25 for root induction and development studies.

2.3 Results

The clone of *E. grandis* used in the present investigation was chosen because of its high minicutting rooting success in the production nurseries. To confirm and define the rooting ability of this clone *in vitro*, shoots were produced using the standard *in vitro* protocol conditions (multiplication [M1] and elongation [E1]) used in our laboratory, and then transferred to rooting medium with and without IBA. All shoots rooted in an auxin-free medium and percentage rooting was inversely related to the concentration of IBA supplied (Fig. 2.1). Mean rooting times were recorded as 7.6 days, 11.9 days, and 13.5 days for the auxin-free, 0.1 mg l^{-1} (0.49 µM), and 0.5 mg l^{-1} (2.45m µM) IBA treatments, respectively. Whereas percentage rooting and mean rooting times decreased

with increasing auxin concentration in the rooting medium, the opposite was observed for callus production at the base of the stem, indicating that an excess of exogenous auxin delayed root organogenesis. However, the number of roots per shoot increased significantly with IBA supply, *viz.* 3.16 ± 0.47, 4.36 ± 0.50, and 7.6 ± 0.55 for 0, 0.1 and 0.5 mg l^{-1} IBA, respectively (p = 0.00075), in keeping with the rhizogenic influence of the auxin.

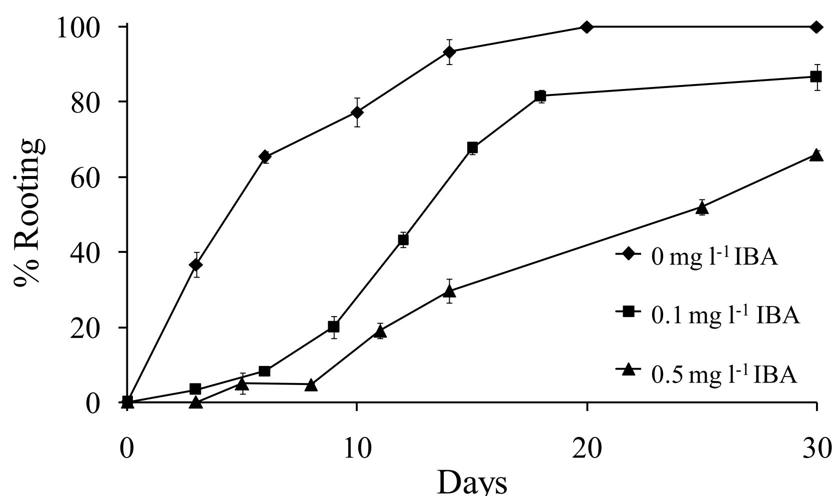

Fig 2.1 The effect of IBA on the percentage rooting of shoots, cultured according to the standard multiplication and elongation protocol. The data were analysed using one way analysis of variance followed by Fisher's least significant difference and found to be significantly different ($P<0.05$). Bars indicate standard deviations of the mean, n = 30

The very high rooting success and relatively rapid root production (100% in 20 days, 7.6 days mean rooting time) of shoots in auxin-free rooting medium (Fig. 2.1) implied that, in this clone of *E. grandis*, root induction was brought about by the action of stored auxins from the previous culture stages (multiplication and elongation). This was, therefore, investigated by culturing shoots on auxin-free multiplication medium, transferring them onto six elongation media (Table 2.1), followed by rooting on auxin-free medium. The overall ability of shoots in an elongation treatment to produce a high

percentage of roots decreased with decreasing levels of auxin exposure in their culture history (Fig. 2.2). Mean rooting times were recorded as E1 = 11 days; E2 = 9.4 days; E3 = 7.7 days; E4 = 4.5 days; E5 = 4.5 days; E6 = 5.4 days. The three elongation media that resulted in the lowest percentage rooting were E4 (no auxin), E5 (0.05 mg l^{-1}/ 0.25 µM IBA) and E6 (0.37 mg l^{-1} /2.1 µM IAA). The 29% rooting success of shoots cultured on E5 can be attributed to the low concentration of IBA in that elongation medium, prior to rooting on auxin-free medium. Even though the elongation medium E6 contained greater levels of auxin (IAA) (0.37 mg l^{-1} /2.1 µM) than the other two auxin-containing elongation media (0.3 mg l^{-1} /1.6 µM NAA in E3 and 0.05 mg l^{-1} /0.25 µM IBA in E5), only 31.3% of shoots from E6 rooted.

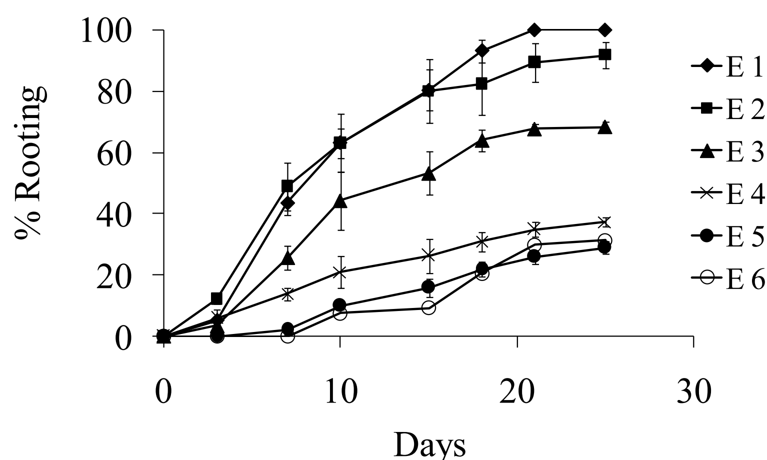

Fig 2.2 Percentage rooting of shoots multiplied on auxin-free medium, elongated on media with different PGRs and subsequently rooted on auxin-free medium. E1 (standard) – E6 as in Table 2.1. Data were subjected to one-way analysis of variance and Fisher's least significant difference. Bars indicate standard errors of the mean, n = 25

Another apparent effect of the elongation media tested was altered graviperception (AG) in roots, defined in this study as root growth greater than 90° away from the gravity vector at any time (Rashotte *et al*. 2000). As listed in Table 2.2, shoots that produced AG roots had significantly reduced levels of IAA compared with those that produced graviperceptive roots.

Further evidence to support the need for free IAA within shoots for graviperception was sought by adding the IAA-specific transport inhibitor TIBA to the rooting medium, together with 0.1 mg l^{-1} IBA, IAA or NAA (0.49 µM, 0.57 µM, and 0.54 µM, respectively). Shoots treated with IBA retained their full rooting ability, while 70.9% of those on IAA were able to induce roots. Only 20.6% of shoots cultured on NAA-containing medium retained their rooting ability as a result of TIBA treatment (Fig. 2.3). Mean rooting times were recorded as 15.8 days, 11.2 days, and 2.6 days for the IBA-, IAA- and NAA-containing TIBA media, respectively. Further, although IBA, and to a lesser extent IAA and NAA, were able to induce roots *in vitro* in the presence of TIBA, IAA availability and its transport within the root seemed necessary to effect root graviperception, since all the shoots produced at least one AG root per shoot. In addition, a qualitative analysis of histological sections of roots grown in the presence of TIBA showed inconsistent starch grain accumulation in the columella cells and in at least 40% of the sections viewed, these starch grains, necessary for gravity sensing, were missing completely (Fig. 2.4).

Table 2.2 Root gravity response observed *in vitro* from shoots exposed to varying levels and types of auxins in the elongation stage and rooted in an auxin-free medium (n = 25). The amount of extractable IAA present in shoots indicates free IAA available just prior to transferring the shoots into rooting media. (Standard deviations of the mean are indicated, significantly different values are denoted by different letters, p<0.05)

Elongation media	Extractable PGR (mg l^{-1})	% shoots with AG roots	IAA (nmol g^{-1} DW)
E1	0.3 NAA, 0.2 kinetin, 0.05 IBA	0	325.7 ± 77.7^{a}
E2	None	70	53.1 ± 46.5^{b}
E3	0.3 NAA, 0.2 kinetin	15	229.5 ± 81.8^{a}
E4	0.2 kinetin	70	45.36 ± 4.24^{c}
E5	0.05 IBA, 0.2 kinetin	60	84.67 ± 10.69^{b}
E6	0.37 IAA, 0.2 kinetin	65	86.7 ± 41.1^{b}

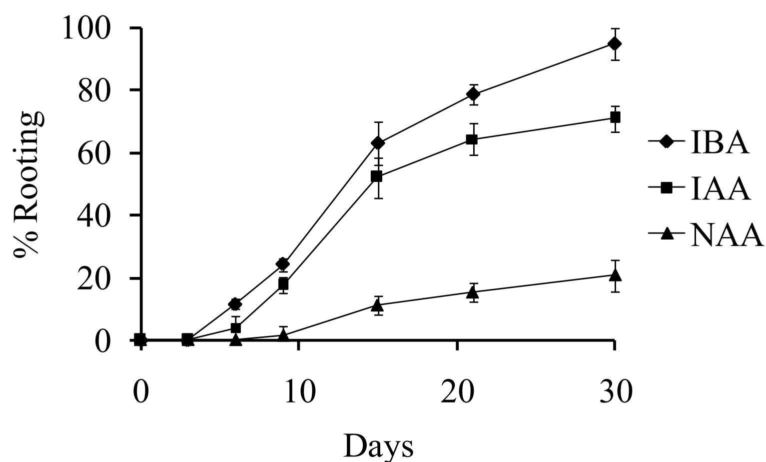

Fig 2.3 Percentage rooting of shoots cultured in rooting media containing 0.1 mg l^{-1} of either IBA, IAA, or NAA, and supplemented with 0.4 mg l^{-1} TIBA. Data were subjected to one way analysis of variance and found to be significantly different (P<0.05). Standard deviations of the mean are indicated, n = 28

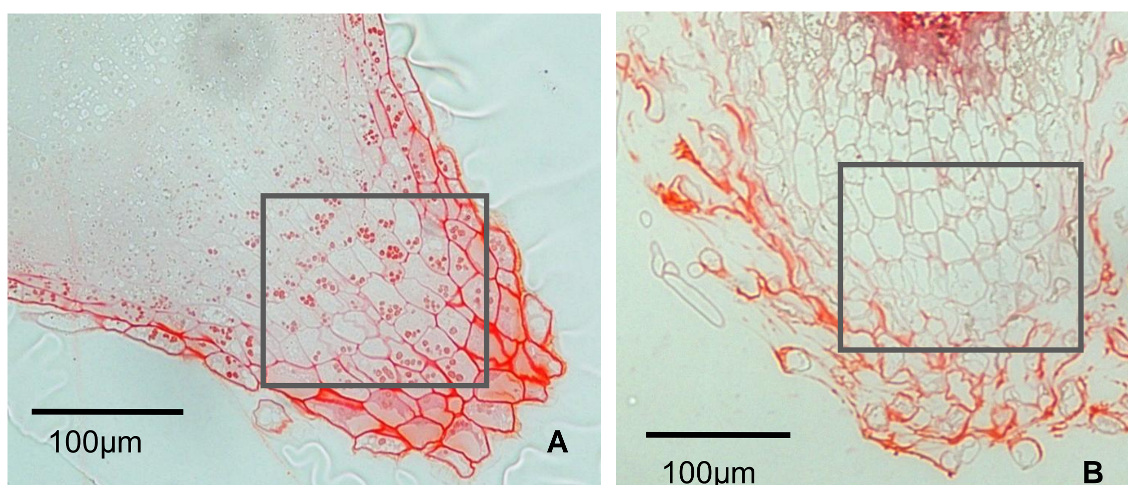

Fig 2.4 Histological sections of root tips from shoots treated with TIBA. Square indicates region of starch grain accumulation; A) present; B) absent

Having established that, in this clone, exogenous auxins accumulate in the shoots during culture, the effects of excess auxin accumulation on rooting was investigated. The persistence of IAA, IBA and NAA in the shoots of this clone was investigated by multiplying and elongating shoots under auxin-free conditions (i.e. multiplication on M2 and elongation on E4), and subsequently rooting them on auxin-containing media (0.1 or 0.5 mg l^{-1}). All shoots produced roots after 21 days regardless of the auxin type (Table 2.3). However, the type of auxin affected the pace of root induction, with the greatest delay in rooting occurring in media containing 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} NAA. For all three tested auxins, mean rooting time was longer in the presence of the higher tested concentration (Table 2.3). The formation of basal callus was observed in all shoots cultured at 0.5 mg l^{-1}, regardless of auxin type, although shoots on IBA- and NAA-media developed larger calli than IAA-treated shoots (results not shown). In addition, increasing the IBA and NAA concentration in the rooting medium, from 0.1 mg l^{-1} to 0.5 mg l^{-1}, resulted in a significant increase in the number of roots produced per shoot and a significant decrease in the mean root length (Table 2.4).

Table 2.3 Average percentage rooting of shoots cultured in auxin-free multiplication and elongation media, and transferred to rooting media containing IBA, IAA, or NAA at 0.1 mg l^{-1} and 0.5 mg l^{-1}. Standard deviations of the mean are included, n=25

Auxin used	Days	% rooting	
		0.1 mg l^{-1}	0.5 mg l^{-1}
IBA	3	0	0
	7	14.7±4.2	1.7±2.9
	12	95±5	17.7±4.9
	18	100	88.3±11.1
	21	100	98.3±1.6
Mean rooting time (days)		11.6	16.9
IAA	3	0	0
	7	40.7±3.1	10.3±5.5
	12	91.7±7.6	65.7±11
	18	96.7±2.9	82.3±15.8
	21	100	98.7±1.3
Mean rooting time (days)		10.6	13.8
NAA	3	0	0
	7	7.7±3.8	3.3±5.7
	12	55±5.6	4.2±7.4
	18	93.7±5.1	92±7.2
	21	100	98±2
Mean rooting time (days)		14.5	15.1

Following one-month acclimatisation, 100% plantlet survival was recorded, and new root growth was observed in all (100%) shoots, regardless of the auxin type or concentration used *in vitro* for rooting. Calli that had developed *in vitro*, degraded during acclimatisation. However, differences in the new root architecture were apparent in that the new roots were thicker than those roots that developed *in vitro*. Shoots rooted with 0.5 mg l^{-1} auxin (regardless of type) developed new roots that tended towards I-beam architecture, while those shoots rooted with 0.1 mg l^{-1} auxin produced new roots that tended towards T-beam architecture. This developmental response was more apparent in the shoots treated with IBA and NAA *in vitro*, compared with IAA treatment for root induction *in vitro* (Fig. 2.5).

Table 2.4 Mean rooting time, root number and root length produced from shoots cultured on auxin-free multiplication and elongation media, and transferred to rooting media with IBA, IAA or NAA, n = 25 (Standard deviations of the mean are included for root length, significantly different values are denoted by different letters, p<0.05)

Auxin (mg l^{-1})	Mean rooting time (days)	Mean root number	Mean root length (cm)
0.1 IBA	11.6	$6±1.1^a$	$6.76±0.25^d$
0.5 IBA	16.9	$9±0.7^b$	$1.90±0.25^e$
0.1 IAA	10.6	$4±0.3^c$	$7.63±0.32^d$
0.5 IAA	13.8	$4±0.3^c$	$7.86±0.25^d$
0.1 NAA	14.5	$5±0.6^a$	$7.86±0.85^d$
0.5 NAA	15.1	$7±0.5^a$	$4.70±0.45^f$

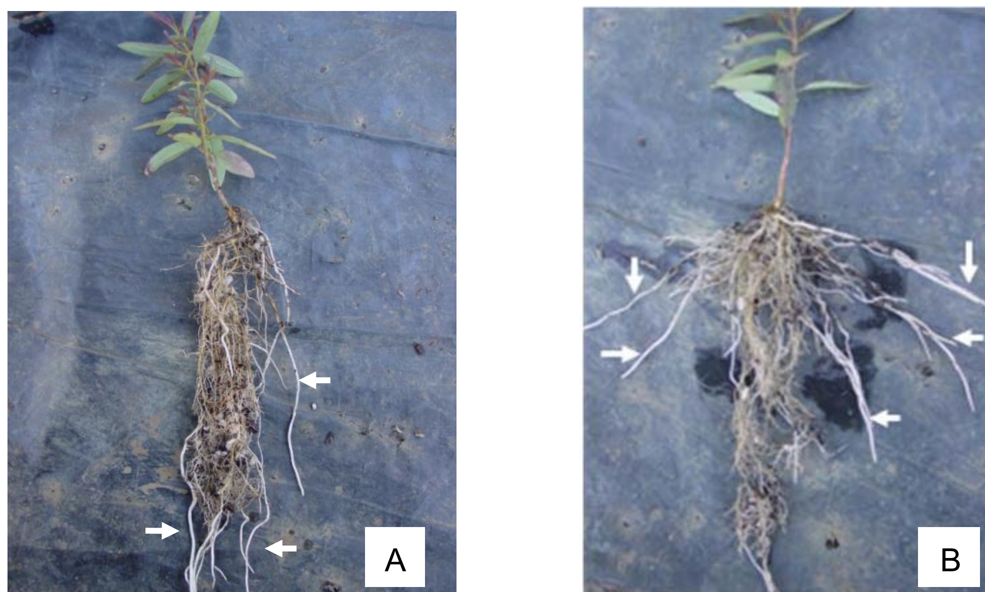

Fig 2.5 New root growth observed in shoots following one-month acclimatization; A) *in vitro* root induction with 0.1 mg l^{-1} auxin (IAA, IBA or NAA); B) *in vitro* root induction with 0.5 mg l^{-1} auxin (IAA, IBA or NAA). The new roots that developed during acclimatisation are indicated with white arrows. During acclimatisation, shoots that were rooted *in vitro* with 0.5 mg l^{-1} auxin developed roots that tended to grow at a smaller angle to the soil surface (tending more towards I-beam) compared with the 0.1 mg l^{-1} auxin treatment (T-beam)

2.4 Discussion

The physiological effect of each auxin on plant tissue development, with respect to growth and morphogenesis, differs in terms of uptake, conjugation, transport and metabolism within plant tissues (Blakesley 1994; George *et al*. 2008). A number of studies and reviews have highlighted the relatively higher stability of IBA compared with IAA in media and in plant tissues (Nordström *et al*. 1991; Epstein and Ludwig-Müller 1993; Ludwig-Müller 2000), and this property has been exploited in *in vitro* protocols that require adventitious root formation. The two natural auxins IAA and IBA

are rapidly taken up by plant tissues *in vitro* to form conjugate products with sugars and amino acids, which then serve as storage for free auxin when it is required (Woodward and Bartel 2005). Although IBA can act as an independent auxin (Ludwig-Müller 2000), its conversion to IAA via β-oxidation is often cited as the pathway through which it acts (Epstein and Ludwig-Müller 1993; Woodward and Bartel 2005; George *et al*. 2008). Further, some authors have suggested that IBA conjugates serve as a better source of free IAA than IAA conjugates (Wiesman *et al*. 1989), based on its relative stability to oxidation. Conjugates of IAA are often subjected to irreversible deactivation through oxidation (Epstein and Ludwig-Müller 1993), although in general conjugation is reversible for all auxins (de Klerk *et al.* 1999). The synthetic auxin NAA has also been reported to form conjugates (Goren and Bukovac 1973; Smulders *et al*. 1990; Centeno *et al*. 1999) and their hydrolysis sustains levels of NAA over relatively long periods in culture (Centeno *et al*. 1999). These metabolic properties of auxins result in IAA being the least stable, compared with IBA and NAA, whether in conjugated or free form (de Klerk *et al*. 1999).

In the tested clone, endogenously produced auxins, together with the auxins that were added during the multiplication and elongation stages (at the concentrations of the standard protocol) were sufficient at inducing adventitious roots, rendering exogenous auxin supply in the rooting medium unnecessary (Fig. 2.1). Since the addition of IBA to the rooting medium was unnecessary for this clone, the excess exogenous auxin resulted in basal callus formation which delayed root emergence.

The influence of auxin accumulation from the pre-rooting culture steps on root induction was demonstrated with the removal of the auxins from the multiplication and elongation stages (Fig. 2.2). The total percentage rooting decreased with decreasing auxin exposure in the culture history, and was coupled with the loss in ability of some roots to perceive gravity (Table 2.2). The low total percentage rooting observed from shoots cultured on elongation medium 6 (E6), in which IAA was added at a higher concentration than NAA and IBA in elongation media 3 (E3) and 5 (E5) respectively, is consistent with the low stability of IAA in plant tissues (de Klerk *et al.* 1999; Ludwig-Müller 2000). Even though the auxins supplied in the present study were not equimolar,

the observed rooting responses still reflected established knowledge on the stabilities and rhizogenic efficiencies of the tested auxins *in vitro* and in plant tissues (reviewed by George *et al*. 2008).

The need for auxin (in particular IAA) availability to influence graviperception was shown in that shoots cultured without any auxins during multiplication and elongation showed the highest percentage of AG roots (Table 2.2). This was supported by GC-MS analysis, which showed that shoots that produced AG roots had less free IAA compared with those that produced graviperceptive roots *in vitro*. These results indicate that even though other auxins (IBA and NAA in the present study) may be used in the culture history, *in vitro* shoots require sufficient levels of free IAA in order to form graviperceptive roots.

Given that auxin transport is central to auxin action, this property serves as a useful tool in elucidating the function of different auxins in plant responses. Auxins need to be transported in either a basipetal or acropetal direction, depending on the root cell type, to effect gravitropism (Chen *et al*. 1999). While starch grain sedimentation within the statoliths, in root columella cells, is known to be the means of root graviperception, the effecter of the gravitropic signal is auxin transport (Evans 1991; Chen *et al*. 1999; Kiss 2000; Morita and Tasaka 2004). A number of studies have acknowledged that the transport of IAA within root tip cells results in gravitropic bending (Palme and Gälweiler 1999). IAA and IBA transport has been shown to be protein-mediated, although these natural auxins do not share their influx and efflux transport proteins (Poupart and Waddell 2000). IAA influx into cells is mediated by the AUX1 protein family (Bennett *et al*. 1996; Yamamoto and Yamamoto 1998), while efflux is facilitated by proteins of the PIN family (Chen *et al*. 1999; Palme and Gälweiler 1999). The synthetic auxin NAA has been shown to enter cells through passive diffusion, while its exit from cells is protein-mediated (Delbarre *et al*. 1996).

The results obtained in this study with the addition of the IAA-specific transport inhibitor TIBA to the rooting medium, together with IBA, IAA, or NAA (Fig. 2.3), illustrated the interconversion between IBA and IAA in the tested clone. Uptake of the auxins supplied in the medium would have been through the cut surface via the xylem,

together with water and nutrients. Since it has been shown (Ludwig-Müller 2000) in several cuttings systems that IBA is transported better acropetally than IAA, it is possible that IBA was either taken up and converted to IAA, or that IBA was directly involved in root induction. The latter would result in the significantly highest percentage rooting from IBA-treated shoots, compared with the IAA and NAA treatments. Even though IAA efflux was inhibited by TIBA, 70.9% of shoots treated with IAA for root induction retained the ability to induce roots. This could be attributed to the fact that TIBA treatment may actually have led to the cellular accumulation of IAA through the inhibition of IAA efflux, resulting in root induction, or the *in vitro* conversion of IAA to IBA for root induction. Such a conversion has been previously reported in roots, coleoptiles and leaves of maize (Ludwig-Müller and Epstein 1991). Although the conversion of NAA to IBA has not been reported, it would explain the lowest percentage rooting by NAA-treated shoots (not discounting the influence of endogenous auxins), compared with those supplied with IBA and IAA, all in the presence of TIBA. Since NAA relies largely on PIN-mediated efflux from cells (Yamamoto and Yamamoto 1998; de Klerk *et al*. 1999), its influence on root induction was reduced in the presence of TIBA (Fig. 2.3) While it cannot be conclusively deduced that IBA acted independently of IAA for root induction in the tested clone, a direct correlation does exist between IAA availability and root graviperception in this clone (Table 2.2).

Once roots had developed *in vitro* in the presence of TIBA, many displayed AG behaviour. This suggests that in this case the IBA translocation within root tissues was insufficient in effecting graviperception, and that uninhibited auxin transport through the IAA efflux transporter was necessary. Such auxin transport is also necessary for the accumulation of starch grains in the root tip, since histological sections revealed that TIBA-treated shoots developed roots with inconsistent statolith presence, and in some cases statoliths were missing completely (Fig. 2.4). Stange (1985) also reported on the inhibition by TIBA of starch accumulation in meristematic tissues of *Riella helicophylla* by TIBA.

While IBA remains the most commonly-used auxin for adventitious root induction (in both micropropagation and minicuttings protocols), *in vitro* root induction in the clone under study was also achieved with IAA and NAA (Table 2.3). However, it appears that root development following induction was influenced by the stabilities of these three auxins in the shoots. Studies on root development indicated that plant tissues display varying sensitivities and responses to auxins, even being inhibited by them at some phases of root development (reviewed by de Klerk *et al*. 1999). Apple microcuttings were unaffected by auxins and cytokinins during the initial dedifferentiation phase, but became sensitive at 72 to 96 hours, when adventitious roots were induced, after which the auxin became inhibitory to root development (de Klerk *et al*. 1999).

The investigation into the influence of excess auxin supply during *in vitro* root induction on callus and root development, both *in vitro* and during acclimatisation, revealed that shoots treated with IAA (the least stable auxin) were the quickest to induce roots (Table 2.3) and produced longer roots (Table 2.4) at the highest tested concentration. Shorter mean rooting times correlated with greater root elongation over the investigation period. Similar observations were recorded by Fogaça and Fett-Neto (2005) with *E. globulus* and *E. saligna*, following root induction with IBA, IAA and NAA, with some discrepancies attributable to endogenous auxin effects. These responses emphasise the inhibitory effect that stable and persistent auxins exert during the phases following root induction (de Klerk *et al*. 1999). Hence, the relative stabilities of auxins in plant tissues invariably have implications for root development following induction *in vitro*. This suggests the necessity to use a less stable auxin (e.g. IAA) for root induction *in vitro*, or alternatively a pulse auxin treatment in the rooting stage.

In conclusion, the results imply that the properties of the auxins used in micropropagation programmes need to be considered in terms of the explant's endogenous and exogenous phytohormone requirements and varying sensitivities to these during the stages of root induction and development. The administered PGRs should also provide for the physiological requirements of the developing roots, such as graviperception. The most commonly encountered natural auxin, IAA, appears to fulfil these requirements. Nevertheless, preliminary studies on certain poor-rooting

Eucalyptus in our laboratory have shown that the in vitro percentage root induction is approximately 20% with 0.1 mg l-1 IAA and increases to approximately 80% under the same concentration of IBA, indicating that IBA may still be necessary for root induction in difficult-to-root clones, due to its more potent rhizogenic action compared with IAA. Presently, the effects of the different auxins and their accumulation on root induction, root characteristics, and in the early stages of acclimatisation in such „poor-rooters' are being investigated, along with the effects of cytokinin on auxin action.

CHAPTER 3: THE CHOICE OF AUXIN ANALOGUE FOR ROOT INDUCTION *in vitro* INFLUENCES POST-INDUCTION DEVELOPMENT IN *Eucalyptus grandis*

3.1 Introduction

The commercial and economic importance of the world's *Eucalyptus* plantations is well known and documented (Eldridge *et al*. 1994; Turnbull 1999; Watt *et al*. 2003). Amongst the wide range of their products the most important include paper and pulp, charcoal, and timber for furniture and construction (Eldridge *et al*. 1994; Turnbull 1999; Watt *et al*. 2003). The increasing demand for these commodities and the associated favourable growth characteristics of members of the genus has led to a concomitant dominance of eucalypt plantations worldwide (Merkle and Nairn 2005). This has been achieved through breeding programmes, the use of hybrids and prudent nursery and clonal practices. Selected superior eucalypt clonal lines, both pure and hybrid, are perpetuated through vegetative propagation in order to preserve desired genotypes and traits (Denison and Kietzka 1993a; Denison and Kietzka 1993b; Watt *et al*. 2003). This allows for increased true-to-type plantlet yield, more efficient site-matching and uniformity in the plantations (Eldridge *et al*. 1994).

While propagation through macrocuttings has proven successful for a number of eucalypt clones (Eldridge *et al*. 1994), mini- or micro- cuttings confer even greater advantage in terms of speed of rooting, root quality and an improvement in rooting potential, coupled with decreased production costs (Eldridge *et al*. 1994; de Assis *et al*. 2004). In conjunction with these approaches, micropropagation through *in vitro* practices provides increased plantlet multiplication rates (Le Roux and van Staden 1991), and may be the only practical means of propagating certain difficult-to-root clones (Mokotedi *et al*. 2000; Yasodha *et al*. 2004; George *et al*. 2008). As a result, there are numerous published *in vitro* protocols for the propagation and maintenance of superior selected eucalypt genotypes (Le Roux and van Staden 1991; Jones and van Staden 1997; Watt *et al*. 2003).

Fundamental to all vegetative propagation programmes is the attainment of fully functional plants. In this regard root ontogeny is often an area of research focus and this is particularly true for the eucalypts of commercial importance (de Assis *et al*. 2004).

As previously discussed (Nakhooda *et al*. 2011) (Chapter 2), the aim of the rooting stage of these propagation programmes and their specific micropropagation protocols has been to achieve a high percentage rooting (Jones and van Staden 1997; Trindade and Pais 1997; de Assis *et al*. 2004; Mankessi *et al*. 2009; Nourissier and Monteuuis 2008). However, with few exceptions (Bell *et al*. 1993; Mokotedi *et al*. 2010), reports have not documented root growth, quality and morphology of *in vitro*-produced roots post-acclimatisation, or compared these traits with those of seed-, macro or mini-cuttings- propagated eucalypt clones. The study by Mokotedi *et al*. (2010) showed that after 16 months acclimatization, micropropagated plants displayed a relatively weaker root system than macro- and seed- propagated eucalypt clones, due to a shallow horizontal root architecture. Most eucalypt micropropagation protocols prescribe the use of one or more of the auxin analogues to induce roots *in vitro* (reviewed by Jones and van Staden 1997). However, preliminary findings by Nakhooda *et al*. (2011) (Chapter 2) indicated that the choice of auxin analogue [IAA (indole-3-acetic acid) or IBA (indole-3-butyric acid)] and concentration used for the *in vitro* multiplication and elongation stages of the micropropagation protocol influenced both *in vitro* root gravity perception and post acclimatisation root architecture, and that IAA was integral to those processes. That study specifically investigated the influence of auxin analogues supplied during the multiplication and elongation stages on subsequent root development. In the present study, the influence of the auxin analogues IAA and IBA on root development was investigated when these analogues were added to the rooting medium, the final stage of every micropropagation protocol.

Linked to any study of root development is an understanding of auxin transport and action. The major form of natural auxin found in plants, IAA, has been shown to be transported in a basipetal direction, through diffusion (Delbarre *et al*. 1996; Kramer and Bennett 2006), or predominantly through membrane-bound transport proteins of the AUX 1 (Bennett *et al*. 1996; Parry *et al*. 2001b), PGP (Terasaka *et al*. 2005; Mravec *et al*. 2008) and PIN families (Gälweiler *et al*. 1998), providing influx and efflux of auxin in a polar manner, primarily through the phloem (George *et al*. 2008; Tromas and Perrot-Rechenmann 2010). Specifically in roots, auxin is laterally distributed in the root cap, as the primary signal in gravitropic bending (Chen *et al*. 1999; Friml 2003; Swarup

and Bennet 2009). Studies conducted using *Arabidopsis* have implicated auxin and auxin transport in numerous root physiological processes such as regulation and maintenance of root meristem and zonation (Luijten and Heidstra 2009), root cell patterning (Blilou *et al*. 2005) and, along with cytokinins (Campilho *et al*. 2009), auxins influence vascular development (Mattsson *et al*. 1999; Ye 2002).

Much of the understanding of auxin transport and action has developed through studies utilising auxin inhibitors and antagonists (Geldner *et al*. 2001; Oono *et al*. 2003; de Rybel *et al*. 2009; Kuderová and Hejátko 2009). These inhibit auxin action in various ways. For example, 2,3,5-triiodobenzoic acid (TIBA) blocks polar auxin by competing for auxin binding sites (Geldner *et al*. 2001) and ρ-chlorophenoxyisobutyric acid (PCIB) inhibits auxin signal transduction by impairing the auxin signalling pathway (Oono *et al*. 2003). Cytokinins are also known to work antagonistically with auxins in many root developmental processes (Brault and Maldiney 1999; George *et al*. 2008).

While the use of an auxin for root induction in eucalypt micropropagation protocols is often encountered in the literature (reviewed by Jones and van Staden 1997), the choice of analogue needs further investigation. This is achievable through the use of auxin inhibitors and antagonists, which can serve to confirm the specific roles of each auxin analogue during root development. With such information, *in vitro* protocols can be refined, potentially at each stage, to produce maximum plantlet yield without compromising plantlet quality, from the *in vitro* stages through to post-acclimatisation. The present contribution is a continuation of our studies on eucalypt root ontogeny *in vitro* (Nakhooda *et al*. 2011) (Chapter 2). The role of auxins on root induction, graviperception, cell patterning, vascular differentiation, and root tip development were investigated, both at the root induction stage and 3 weeks after root induction.

3.2 Materials and Methods

3.2.1 Decontamination and culture initiation

One year old potted plants of a pure *Eucalyptus grandis* clone were obtained from Mondi Business Paper, Hilton, KwaZulu-Natal. Minicuttings from these plants were decontaminated and placed onto bud induction medium, as described previously (Nakhooda *et al*. 2011) (Chapter 2).

3.2.2 Micropropagation protocol

After bud induction, explants were cultured for two weeks on multiplication medium, followed by four weeks on elongation medium. Multiplication medium was composed of MS nutrients (Murashige and Skoog 1962), 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.04 mg l^{-1} (0.21 μM) α-naphthalene acetic acid (NAA), 0.1 mg l^{-1} (0.44 μM) 6-benzylaminopurine (BAP), 0.05 mg l^{-1} (0.23 μM) 6-furfurylaminopurine (FAP/kinetin), 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite®. Elongation medium contained MS nutrients, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.3 mg l^{-1} NAA, 0.1 mg l^{-1} indole-3-butyric acid (IBA), 0.2 mg l^{-1} kinetin, 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite®. Both stages were conducted using 20 ml of media in 100 ml culture bottles.

Elongated shoots (approximately 1.5 cm) were then individually transferred to 10 ml rooting medium in 40 ml culture tubes. This medium comprised ¼ MS nutrients, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 15 g l^{-1} sucrose and 4 g l^{-1} Gelrite®. The auxin analogues IAA and IBA, the auxin inhibitors TIBA (0.8 μM/0.4 mg l^{-1}) and PCIB (10.7 mg l^{-1}/50 μM) and the auxin antagonist kinetin (1 mg l^{-1}/4.6 μM) were added to the media, where indicated. Shoots were recorded to have rooted when at least 0.5 cm of the root protruded from the base of the shoot. These studies were conducted using Magenta® plant culture boxes, to provide space for observations of graviperception of the developing roots *in vitro*. All media, together with the phytohormones and auxin inhibitors, were adjusted to pH 5.6 to 5.8, before decontamination through autoclaving

at 121ºC and 1 kPa for 20 minutes. All cultures were maintained under 16-hour light (200 µmol.m^{-2}.s^{-1})/8-hour dark photoperiod, at 25 and 23ºC, respectively.

3.2.3 Microscopy

Shoot-root junction, root sections taken midway between the root tip and the shoot, and root tips were prepared for light microscopy and histological analysis by initially placing them into 2.5% (v/v) gluteraldehyde solution prepared in a 0.1 M phosphate buffer, at pH 7.2, for at least 24 hours at 4ºC. Following primary fixation, samples were rinsed several times in the phosphate buffer to remove all traces of fixative. Samples were then dehydrated using a series of acetone solutions of increasing concentrations. Dehydrated samples were subsequently infiltrated with 50:50 acetone:epoxy resin (Spurr 1969), left on a shaker for 5 hours, and then placed in full epoxy resin overnight to allow for further resin infiltration. This was followed by resin embedding in silicon blocks and polymerisation at 70ºC for 8 hours. Sample sectioning of 1 µm was conducted using the Riechert Ultra-cut E microtome, followed by staining using a 1% (v/v) safranin solution and a 1% (w/v) KI solution for visualisation of starch grains in root tips. Section viewing and measurement analysis (mean root diameter) was achieved using the Nikon Biophot® light microscope coupled with the Motic Image Plus 2.0 computer programme.

3.2.4 Statistical analysis

All statistical analyses were carried out using PAST, version 2.01 (Hammer *et al*. 2001). The experiments were repeated at least 3 times, each with a minimum sample size of 30. Measurements of root length were conducted *ex vitro* using a tape measure.

3.3 Results

The specific roles of auxin on root induction and post-induction development were investigated, using an *E. grandis* clone, known to be „easy-to-root' *in vitro* and as minicuttings (Nakhooda *et al*. 2011) (Chapter 2). As demonstrated (Table 3.1), initial experiments with exogenous auxins showed that neither IAA nor IBA in the rooting medium had any significant effect on the percentage rooting of shoots of the tested clone compared with shoots cultured on auxin-free medium, with shoots rooting to over 85% (100% in the absence of exogenous auxin). Since this clone relies mainly on endogenous auxin for root induction (Nakhooda *et al*. 2011) (Chapter 2), the effects of the inhibitors and antagonist on root development can be related to either the endogenous auxin (in the absence of an exogenous analogue), or to the supplied exogenous auxin.

3.3.1 Auxin inhibitor and antagonist exposure at root induction

The addition of the auxin inhibitors and antagonist to the rooting medium had varying effects on root production (Table 3.1). Kinetin significantly decreased percentage rooting and mean root number (except for the IAA-containing rooting medium), and resulted in the formation of basal callus. In the IBA-containing rooting medium, kinetin also significantly reduced the mean root diameter and induced the largest observed basal callus formation. The inhibitor of auxin signal transduction PCIB (Oono *et al*. 2003) almost completely inhibited root production, regardless of exogenous auxin supply. When rooting occurred, only one root per shoot was produced with the significantly smallest mean root diameters recorded. The inhibitor of auxin transport TIBA (Geldner *et al*. 2001) resulted in a significant reduction in percentage rooting in the auxin-free and IAA-containing rooting media compared with the control. However, no significant difference in percentage rooting was recorded for the shoots on the + IBA + TIBA treatment compared with those on + IBA – TIBA medium (Table 3.1). The largest basal callus formation was again recorded in the IBA-containing rooting medium with TIBA.

In addition, the + IBA + TIBA combination resulted in the largest mean root diameter obtained from all the tested rooting treatments.

Given that root induction and zonation is dependent on auxin transport (Luijten and Heidstra 2009), cross sections of the shoot-root junctions were taken to determine the origins of adventitious roots. This approach was also used to establish if the auxin antagonist treatments influenced the cellular origins of the adventitious roots *in vitro*. The results revealed that, for all treatments, at the time of root induction, a fully-developed shoot vascular cambium was absent (Fig. 3.1). Instead, there was only a procambial region with primary phloem and primary xylem vessels scattered throughout the procambium. Adventitious roots appeared to originate from the procambium region (Fig. 3.1). This root developmental morphology was consistent across all rooting treatments (with or without IBA or IAA) and irrespective of auxin inhibitor or antagonist presence (Fig. 3.1).

Table 3.1 Rooting parameters (± SD) across all the investigated auxin treatments after 30 days for the tested *E. grandis* clone in vitro. Shoots were rooted on media containing auxin inhibitors (PCIB, TIBA), auxin antagonist (kinetin), together with either of the indicated auxin analogues (IAA or IBA), or in an auxin-free environment. Callus was quantified as less than 2 mm; between 2 mm and 5 mm; and greater than 5 mm, as indicated by +, ++ and +++, respectively

Auxin treatment (mg l^{-1})	Antagonist Treatment	% Rooting	Mean root number	Mean root diameter (µm)	Callus
0	Control	100 ± 3.8^{a}	5 ± 0.8^{a}	794.5 ± 54.3^{a}	-
	Kinetin	73.4 ± 8.2^{b}	3 ± 1.0^{b}	832.7 ± 43.1^{a}	+
	PCIB	2.1 ± 0.9^{c}	1 ± 0.6^{c}	524.6 ± 78.3^{b}	-
	TIBA	62.1 ± 5.6^{b}	6 ± 2.1^{a}	1187.5 ± 213.6^{c}	++
0.1 IAA	Control	94.8 ± 7.3^{a}	5 ± 1.6^{a}	835.6 ± 44.8^{ad}	-
	Kinetin	69.6 ± 9.7^{bd}	4 ± 1.4^{ab}	875.9 ± 62.6^{a}	+
	PCIB	2.7 ± 1.9^{c}	1 ± 0.8^{c}	575 ± 96.2^{b}	-
	TIBA	72.1 ± 6.2^{b}	6 ± 2.4^{ad}	1216.3 ± 143.7^{c}	++
0.1 IBA	Control	85.7 ± 12.9^{ae}	7 ± 1.2^{ad}	929.7 ± 82.7^{d}	+
	Kinetin	52.7 ± 11^{d}	4 ± 1.4^{b}	859 ± 74.1^{a}	+++
	PCIB	4.3 ± 3.1^{c}	1 ± 0.4^{c}	632.7 ± 85.9^{b}	-
	TIBA	79.7 ± 3.5^{e}	8 ± 1.4^{d}	1305.4 ± 56.3^{e}	++ to +++

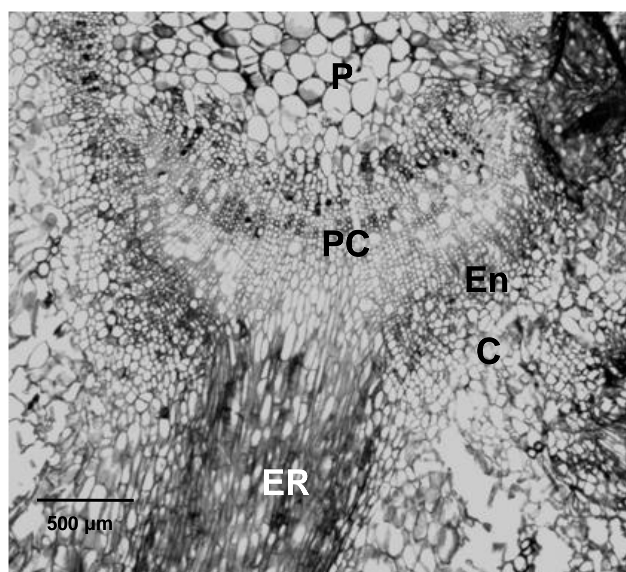

Fig. 3.1 Stem section showing emerging adventitious root (ER). New root had formed from the procambium (PC). The stem endodermis (En) is visible, as is the cortex (C) and pith (P). At this stage, only primary xylem and primary phloem were present

3.3.2 Auxin inhibitor and antagonist exposure post root induction

In this investigation, aimed at determining the effects of auxin antagonists on *in vitro* root development post-induction, shoots were rooted using the standard rooting medium, supplemented with 0.1 mg l^{-1} IBA. This auxin was added to the rooting medium to complement endogenous IAA, the presence of which was confirmed by Nakhooda *et al*. (2011). Ensuring the presence of both auxin analogues would allow for further investigation into the specific roles of each of these natural auxins in root development in the tested clone. Three days after root emergence, rooted shoots were placed onto a rooting medium containing either 1 mg l^{-1} kinetin, 10.7 mg l^{-1} PCIB or 0.4 mg l^{-1} TIBA, each with 0.1 mg l^{-1} IBA. After 3 weeks *in vitro*, compared with the control treatment (no auxin inhibitor or antagonist) (Fig. 3.2A), the kinetin-treatment had no significant impact on root elongation, but did significantly reduce the mean root diameter of the elongating root. It also produced roots that displayed altered gravity (AG) perception (Fig. 3.2B; Table 3.2), which was defined as root growth with greater than 90º deviation from the gravity vector (Rashotte *et al*. 2000; Nakhooda *et al*. 2011).

Exposing shoots to PCIB (which inhibits auxin signal transduction) resulted in a significant increase in root elongation (Fig. 3.2C), coupled with a significant decrease in mean root diameter (see later), compared with the control (Table 3.2). However, PCIB treatment did not affect the ability of the elongating root to respond to the gravity vector (Table 3.2). On the other hand, exposing shoots to TIBA (which inhibits IAA transport) significantly retarded root elongation, while increasing the mean root diameter, compared with the control (Fig. 3.2A and D; Table 3.2). In addition, the TIBA treatment resulted in roots which did not appear to respond to the gravity vector (Fig. 3.2D). Cross-sections of the shoot-root junction after 3 weeks in each of the modified rooting media revealed no change in the root-shoot junction morphology compared with that already described (Fig. 3.1).

Fig. 3.2 Morphology of roots of the tested *E. grandis* clone following various auxin antagonist treatments post-induction. Three days after normal root induction and emergence, shoots were transferred to A) control rooting medium, where no antagonist was added; B) rooting medium containing 1 mg l^{-1} kinetin; C) 10.7 mg l^{-1} PCIB; and D) and 0.4 mg l^{-1} TIBA. Images were recorded after 3 weeks

Table 3.2 The mean root diameter, change in root length (±SD) and gravitropic responses after 3 weeks in culture. Shoots were exposed to the indicated auxin inhibitors and antagonist 3 days after root induction and emergence in the presence of 0.1 mg l^{-1} IBA. G = gravitropic; AG = altered graviperception

Treatment	Mean root diameter (µm)*	Δ Root length (mm)	Gravitropic response
Control	552.8 ± 4.7[a]	2.8 ± 0.9[a]	G
Kinetin	278.6 ± 70.7[b]	3.7 ± 0.4[a]	AG
PCIB	129.2 ± 62.2[b]	12.2 ± 2.4[b]	G
TIBA	833.4 ± 64.5[c]	0.8 ± 0.2[c]	AG

*At 3 days after root induction, mean root diameter was recorded as 489 ± 32µm

Histological analysis of roots that developed in the presence of the tested auxin inhibitors or antagonist revealed a marked change in the vascular organisational integrity of TIBA-treated roots. Kinetin- and PCIB- treatments did result in altered graviperception and a significant increase in root elongation, respectively, coupled with a loss in cortical organisation (similar to that of Fig. 3.3B), compared with the control. The TIBA-treatment, on the other hand, led to an increase in vascular bundle area, in which vessel organisation and tissue patterning was lost (Fig. 3.3B). Additionally, root cortex organisation was lost (Fig 3.3), compared with the normally developing roots *in vitro* (Fig. 3.3A).

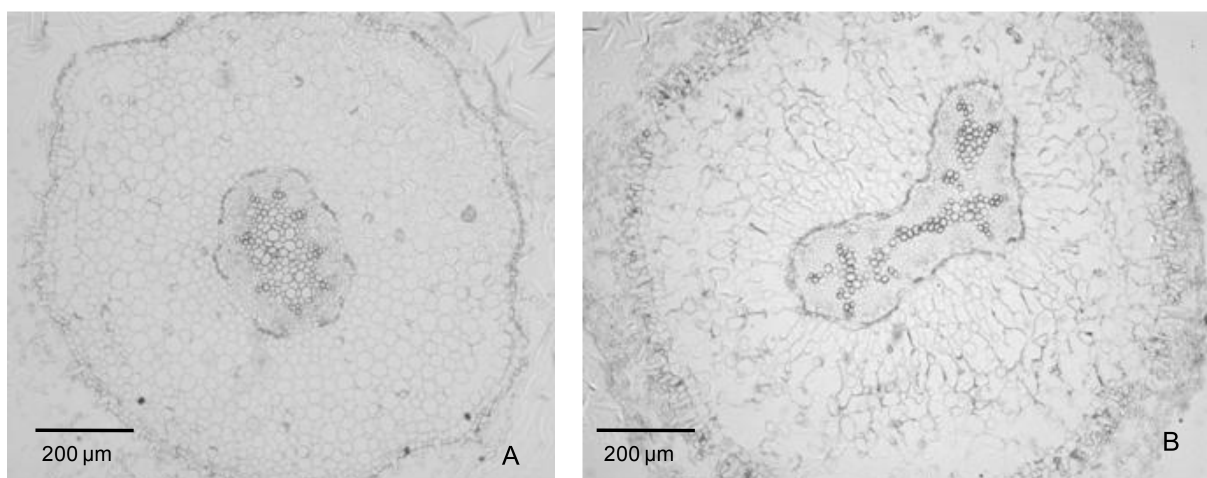

Fig. 3.3 Cross-sections of roots, taken midway between the root tip and the shoot, showing: A) morphology of a normal (control) root produced from shoots not exposed to any auxin antagonist; and B) typical root development from shoots treated with 0.4 mg l^{-1} TIBA five days after normal root induction. Restricting IAA transport resulted in an alteration in vascular patterning

The loss of cortical organisation in the roots of auxin inhibitor- and antagonist- treated shoots was also apparent in longitudinal sections of their root tips (Fig. 3.4). Under the influence of post induction supply of kinetin, the root tips developed a characteristic curvature away from the gravity vector, with no discernible organisation in the root meristematic region and columella cells (Fig. 3.4A). Above the meristematic region, the cells of the cortex lacked discernible structure. Post-induction treatment of shoots with PCIB or TIBA resulted in the collapse of the columella region, coupled with the collapse of cortical integrity. Only the quiescent centre and root meristematic regions of roots maintained tissue/cellular integrity in the presence of TIBA or PCIB (Fig. 3.4B). As a result, the root meristematic area just above the root cap appeared bulbous compared with the rest of the elongating root. Even though starch grains were visible in the collapsed columella region of TIBA-treated roots (Fig. 3.4B), these roots remained unresponsive to the gravity vector (Table 3.2) as a result of IAA transport inhibition. Of

note, PCIB-treated roots remained graviresponsive (Table 3.2), despite having similar root tip morphology to TIBA-treated roots (Fig. 3.4B). Treating shoots with TIBA was previously shown to result in a loss of starch-grain accumulation within the root cap columella region (Nakhooda *et al*. 2011).

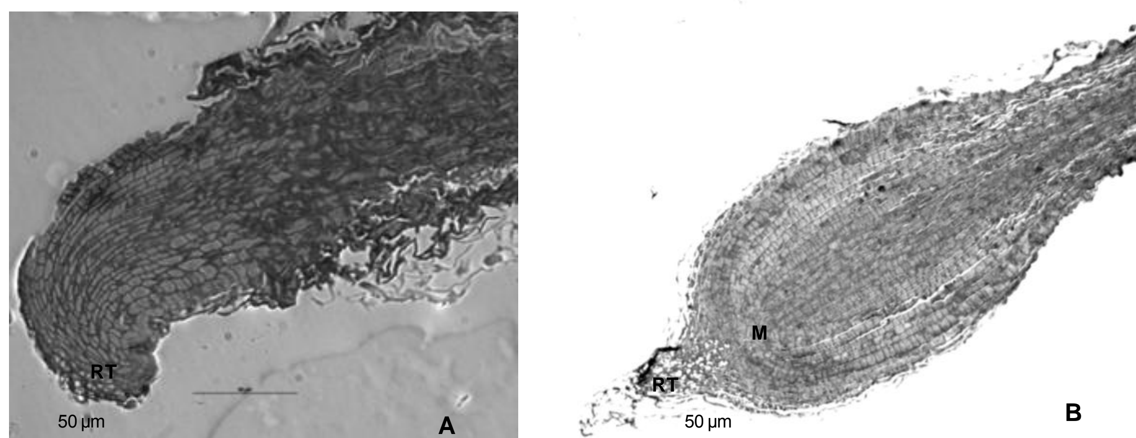

Fig. 3.4 Typical root development when shoots were rooted on normal root induction media supplemented with 0.1 mg l^{-1} IBA, and then transferred, after 3 days, to rooting medium containing the auxin antagonists A) kinetin and B) PCIB or TIBA, *in vitro*. RT = root tip; M = meristematic region

3.4 Discussion

It is well established that auxins are integral to root induction and development (Hartmann *et al*. 1997; George *et al*. 2008). Recent research in our laboratory has shown that at least in some *Eucalyptus* clones, the choice of auxin analogue supplied in the pre-rooting micropropagation stages is critical, in that the natural auxin IAA was necessary for root functioning processes such as graviperception. The auxin analogues NAA (α-naphthalene acetic acid) and IBA could not act as substitutes to IAA (Nakhooda *et al*. 2011) (Chapter 2). These auxin requirements, at least for some

eucalypt clones, could potentially explain the horizontal root architecture (post-acclimatisation) reported by Mokotedi *et al*. (2010), in which IBA was used for rooting eucalypts *in vitro*. While the study by Nakhooda *et al*. (2011) focussed on *in vitro* root development effected by auxin supply during the multiplication and elongation stages prior to rooting, the present investigations focused on the supply of auxin at the rooting stage. Both PCIB and TIBA, and the cytokinin antagonist kinetin, significantly reduced the percentage rooting of the tested clone, regardless of the auxin analogue (IAA or IBA) used for root induction, although only PCIB addition completely inhibited rooting (Table 3.1). Of the two tested auxin analogues, exogenous IBA generally resulted in the greatest basal callus formation. Being a more potent rhizogenic auxin than IAA (Nordström *et al*. 1991; Epstein and Ludwig-Müller 1993; Ludwig-Müller 2000; George *et al*. 2008), IBA also resulted in significantly larger mean root diameters, when supplied in the absence of kinetin or in the presence of TIBA, the inhibitor of IAA efflux (Christie and Leopold 1965; Geldner *et al*. 2001). Since auxin stimulates cell growth (George *et al*. 2008) and retards root elongation (Woodward and Bartel 2005), these observations indicate that the exogenous IBA was converted to IAA in situ, and at least in the tested clone, may serve as a source of IAA, as previous authors have noted in other plant species (Woodward and Bartel 2005).

The basal stem morphology from which the roots developed was similar in all the tested treatments. Histological analysis revealed that a vascular cambium had not developed at the time at which *in vitro* shoots were placed onto rooting medium in the current study. Adventitious roots had developed from the meristematic procambium (Fig. 3.1). According to the general model of adventitious root formation from stem cuttings of woody plants, roots arise from secondary phloem, but may also originate from the vascular cambium and phloem (Hartmann *et al*. 1997). In addition, Ye (2002) stated that in woody plants, the vascular tissues develop from either meristematic procambium or vascular cambium. Of particular interest to the present work is a study by Baltierra *et al*. (2004) using *E. globulus*, which showed that adventitious roots *in vitro* originated from either old vascular tissue or from newly-formed xylem. In our laboratory, roots from minicuttings of *E. grandis* x *nitens* have been found to originate from developed shoot xylem arches, a feature not prominent in micropropagated shoots at the time of

rooting (Fig. 3.1). Considering this, it is suggested that, when applying the present *in vitro* protocol, the time at which elongated shoots are placed onto root induction medium may contribute to the differences in subsequent root architecture compared with macro- and seed- propagated eucalypts following acclimatisation, as reported by Mokotedi *et al*. (2010).

The addition of auxin inhibitors or antagonist post root induction, revealed the specific need for IAA to produce the known root development and physiological responses (Hartmann *et al*. 1997) in the tested eucalypt clone. The presence of either the auxin antagonist kinetin or the IAA-specific transport inhibitor TIBA, resulted in the loss of root gravity perception, a response not observed in PCIB-treated shoots. In addition, the mean root diameters of kinetin and PCIB treatments were significantly reduced compared with the control, but a significant increase in this parameter resulted from TIBA-treatment (Table 3.2). These results indicate that following root induction *in vitro*, the loss in gravity perception and hence horizontal root architecture (Mokotedi *et al*. 2010; Nakhooda *et al*. 2011) may be due to a disruption in IAA efflux and not to a loss in auxin signal transduction. A disruption in auxin efflux, induced either through auxin transport inhibition or through auxin regulation via a cytokinin (Pernisová *et al*. 2009; Su and Zhang 2011), is suggested to have resulted in a redistribution and/or accumulation of auxin within the root cells. This in turn interfered with gravitropism and root cap development, events that rely on regulated auxin transport and specific distribution with respect to auxin maxima and minima concentrations (Muday 2001; Moore 2002; Pernisová *et al*. 2009). Furthermore, studies using *Arabidopsis* have implicated the PIN family of proteins, responsible for auxin efflux, as the determinant in root growth and patterning (Blilou *et al*. 2005). The rooting response observed in the presence of TIBA (Table 3.2) indicated that IAA efflux is a requirement in root development, and that IAA cannot be successfully replaced by its analogue IBA in the tested eucalypt clone.

Disturbances in auxin transport and action also resulted in changes in root vascular patterning (Fig. 3.3), which was most prominent with the TIBA treatments. Under these conditions, a qualitative increase in the vascular bundle area was observed, coupled with

a loss in vascular patterning compared with the control (Fig. 3.3). The continuity of the IAA signal appeared necessary for the maintenance of vascular patterning and IBA could not replace IAA in this regard. These results are similar to those observed in *Arabidopsis*, in which conditions of reduced auxin transport resulted in increased vascular tissue development that were less ordered than those of normal auxin transport and perception (Berleth *et al*. 2000).

Accompanying the alterations in root development, graviperception and vascular patterning brought about by the disruption of auxin flow, changes in root tip development were also noted. The presence of kinetin resulted in a distinct curvature of the root cap away from the gravity vector, while that of PCIB or TIBA resulted in the collapse of the root cap structure, with little interference to the root meristematic region (Fig. 3.4). This maintenance of the quiescent centre and meristematic region, despite interruptions in auxin transport, is in keeping with the requirements of these regions, in that the quiescent state is linked to high levels of auxin through accumulation via auxin transport (Kerk and Feldman 1995; Kerk *et al*. 2000). The collapse of the root cap (Fig. 3.4B) may explain the observed loss in graviperception under conditions of auxin efflux interruption through kinetin (Pernisová *et al*. 2009; Su and Zhang 2011) or TIBA action (Christie and Leopold 1965; Geldner *et al*. 2001). Even though starch grains were visible in the root cap in a number of root tip sections (e.g. Fig. 3.4B), the asymmetric redistribution of auxin is the ultimate gravity response effecter (Muday 2001; Moore 2002). A similar collapse was not observed in kinetin-treated roots, but the possible interference of auxin efflux by cytokinin action did result in a loss in graviperception (Fig. 3.4A). Exogenous cytokinin has been shown to induce bending towards the application site in *Arabidopsis*, thus supporting the inhibitory role that cytokinins play in root gravitropism (Aloni *et al*. 2004), as supported by the present findings.

These results highlight some key root developmental aspects and requirements for *in vitro* rooting of *E. grandis* shoots. The conversion of exogenous IBA to IAA was established, in that specifically inhibiting IAA transport without inhibiting IBA transport, impeded several root developmental events which were not affected in the control. Adventitious root induction *in vitro* was found to form from shoot meristematic

procambium in all treatments, regardless of the presence of auxin inhibitors or antagonist. Of the inhibitors or antagonist treatments imposed following normal root induction, only a disruption in auxin efflux, i.e. a disruption in the asymmetric distribution of auxin in the root, was found to alter gravity perception. This brought about morphological changes in the root cap and alterations in vascular patterning. These critical root developmental events rely on polar transport of IAA (Chen *et al*. 1999; Muday 2001; Moore 2002; Ye *et al*. 2002).

As previously mentioned, IBA is the auxin most widely used in commercial vegetative propagation practices, including eucalypt culture (Hartmann *et al*. 1997; de Klerk *et al*. 1999; de Assis *et al*. 2004; George *et al*. 2008). It is chosen on the basis of its rhizogenic efficacy which results from its higher stability in plant tissues (George *et al*. 2008). This, in turn, also makes IBA the preferred choice for clones that display difficulty in rooting, and do not respond well to IAA application (Epstein and Ludwig-Müller 1993; Ludwig-Müller *et al*. 2005). However, a previous (Nakhooda *et al*. 2011) (Chapter 2) and the present study, show that the most potent auxin may not necessarily be the most suitable auxin in terms of root development and quality, and that IAA cannot always be substituted for in certain root developmental responses. Ongoing work in our laboratory, has shown that at least in some poor-rooting eucalypt clones, IAA can have equal rhizogenic ability to IBA, provided that cytokinin exposure in the pre-rooting culture history is reduced (see Chapter 4). It may therefore be possible to refine eucalypt micropropagation protocols to utilise exogenous IAA for both easy- and difficult-to-root clones, thereby ensuring the quality of the developed roots (e.g. gravitropism and vascular development). Such traits are particularly important for commercially important trees such as eucalypts, in ensuring healthy and productive forests.

CHAPTER 4: THE PROPERTIES AND INTERACTION OF AUXINS AND CYTOKININS INFLUENCE THE ROOTABILITY OF *Eucalyptus* CLONES *in vitro*

4.1 Introduction

Eucalyptus forestry programmes have proven invaluable in their contribution to meeting timber demands worldwide. Profitable plantations have been established in more than 70 countries, and the products of these establishments continue to serve the wood, paper, pulp, and charcoal industries, among others (Eldridge *et al*. 1994; Turnbull 1999; Watt *et al*. 2003). In order to remain competitive, forestry programmes need to seek and maintain superior genotypes that confer traits of interest (e.g. preferred timber properties), allow for genotype-to-site matching, and other strategies to increase production yields. In the pursuit of this, superior eucalypt hybrids have found favour, and their selection programmes often seek to combine stress tolerance with superior wood characteristics in a sustainable, cost effective manner that meets industrial requirements (Watt *et al*. 2003; de Assis *et al*. 2004).

The traditional eucalypt propagatory method, i.e. via seedlings, is not often possible for hybrids (Denison and Kietzka 1993b), consequently, vegetative propagation, e.g. through macro-, mini- or micro-cuttings is often the only option, and it has the benefit of ensuring the maintenance and continuity of the value-added traits (Denison and Kietzka 1993a; Denison and Kietzka 1993b). Even for pure species, vegetative propagation has many advantages over seedling propagation, most notably the increase in yield and the maintenance and conservation of superior genotypes (Eldridge *et al*. 1994). However, propagation through macro- and mini-cuttings has its limitations in that the rooting ability amongst clones is variable and is known to decrease with the age of the parent plants (Eldridge *et al*. 1994; de Assis *et al*. 2004). Micropropagation potentially addresses such shortcomings by providing a highly controlled environment that yields high shoot multiplication rates (Le Roux and van Staden 1991), improved potential, speed and quality of rooting (de Assis *et al*. 2004), and is an efficient and often the only viable method of propagating difficult-to-root clones (Mokotedi *et al*. 2000; Watt *et al*. 2003; Yasodha *et al*. 2004).

A number of studies have investigated and developed micropropagation protocols for the commercially important pure and hybrid eucalypt clones (Le Roux and van Staden 1991; Jones and van Staden 1997; Watt *et al*. 2003). These are based on the empirical manipulation of key plant growth regulators - notably auxins and cytokinins - to achieve the desired morphogenesis in each of the *in vitro* culture stages. In general, the initial stages of bud induction from minicuttings (taken from the parent plant) and subsequent shoot multiplication are achieved using either a single cytokinin type or a combination of cytokinins to encourage shoot proliferation. Shoot elongation is then stimulated by a combination of auxins and cytokinins and lastly rooting is accomplished using one or more types of auxins at various concentrations, depending on the clone in question (Jones and van Staden 1997; George *et al*. 2008). Since each of the established protocols empirically addressed the micropropagation needs of a specific clone, their interclonal application often leads to large variations in propagation and rooting success.

As core to the success of a micropropagation protocol is the ability of the shoots to produce roots (de Assis *et al*. 2004), there is a large body of published reports on the rooting efficiencies of eucalypts in response to the various auxin types and concentrations, on an empirical and clone-specific basis (Jones and van Staden 1997; Watt *et al*. 2003). However, little attention has been paid to the antagonistic effects of the auxin and cytokinin types on morphogenesis, in particular the inhibitory effect that persistent cytokinins may exert on root induction of eucalypt shoots *in vitro*.

Both auxins and cytokinins are recognised as the key signalling molecules in plant development (Moubayidin *et al*. 2009). It is accepted that elevated cytokinin content favours shoot development, elevated auxin content favours root development, whilst equal concentrations of both results in callus formation (George *et al*. 2008). While this general model dictates the use of these phytohormones during the various stages in *in vitro* protocols, the properties of the various cytokinins and auxins used should also be considered. Indole-3-acetic acid (IAA) is the most frequently encountered natural auxin, and is known to be more easily oxidated than the other natural auxin indole-3-butyric acid (IBA), or the synthetic auxin α-naphthaleneacetic acid (NAA) (de Klerk *et al*.

1999; George *et al*. 2008). Similarly, the properties of the various cytokinins differ, with the synthetic cytokinin kinetin (6-furfurylaminopurine (FAP)) been shown to persist longer (George *et al*. 2008) than the natural cytokinin compounds such as *trans*-zeatin, which is rapidly degraded by the enzyme cytokinin-oxidase (Mok and Mok 2001; Haberer and Kieber 2002; George *et al*. 2008). An enzyme other than cytokinin oxidase is thought to be involved in kinetin degradation in some plant species (Forsyth and van Staden 1987). Given that the most commonly-used auxins and cytokinins in eucalypt culture vary in their stabilities, their interaction at each of the culture stages, and subsequent effect on rooting, need further elucidation.

In the present study, the apparent inability of two eucalypt clones to root „with ease', *in vitro*, was investigated in relation to the perception and conjugation of the most commonly-used auxins in eucalypt culture. The tested hypothesis was that the inhibition of root induction in some difficult-to-root clones is due to cytokinin persistence from the pre-rooting culture stages, resulting in a supra-optimal cytokinin:auxin ratio in shoots prior to rooting. The aim is to optimise *in vitro* protocols for eucalypt culture, to maximise yields of both easy- and difficult-to-propagate clones.

4.2 Materials and Methods

4.2.1 Decontamination and culture initiation

The eucalypt clones used in the present study were a pure *Eucalyptus grandis* (TAG31) and an *E. grandis* x *E. nitens* (GN155). A further *E. grandis* x *nitens* natural hybrid (NH58) was later used to confirm observations. All clones were obtained from Mondi Business Paper, Hilton, KwaZulu-Natal. They were chosen on the basis of their rooting performance as mini-cuttings in the Mondi nursery, i.e. as examples of a „good rooter' (easy-to-root) (TAG31) and of two „poor rooters' (difficult-to-root clones) (GN155 and NH58). Cuttings of the parent plants were surface decontaminated in 0.02% (w/v)

$HgCl_2$ with a drop of Tween® -20 for 10 minutes, followed by 1% (w/v) calcium hypochlorite for 10 minutes. After several rinses in sterilised distilled water, they were cut into nodal segments, each with a half-leaf, and placed on bud induction medium, containing MS nutrients (Murashige and Skoog 1962), 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.04 mg l^{-1} (0.21 µM) NAA, 0.1 mg l^{-1} (0.44 µM) 6-benzylaminopurine (BAP), 0.05 mg l^{-1} (0.23 µM) kinetin, 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite® for 2 weeks.

4.2.2 Micropropagation protocol

The multiplication and elongation media were as for bud induction, except that elongation media contained different combinations of plant growth regulators (Table 4.1; E1 represents the standard elongation medium used for eucalypt micropropagation in our laboratory). Shoots were maintained in culture bottles on 20 ml of medium during the multiplication and elongation stages, which typically lasted 3 and 4 weeks, respectively. Upon reaching a height of at least 1.5 cm, shoots were transferred to 10 ml of rooting medium (¼ MS nutrients, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 15 g l^{-1} sucrose and 4 g l^{-1} Gelrite®), in culture tubes. Manipulations of the rooting media (where indicated) included the addition of the auxins IAA, IBA, or NAA at 0.1 mg l^{-1} (0.57 µM, 0.49 µM, and 0.54 µM, respectively) or at 0.5 mg l^{-1} (2.85 µM, 2.46 µM, and 2.7 µM, respectively). These compounds were purposely supplied as mg l^{-1} to mimic reported protocols. Two studies were undertaken with clone 2 (poor rooter), where the following were added to the rooting medium: 50 µM of the inhibitor of auxin signal transduction ρ-chlorophenoxyisobutyric acid (PCIB) (to test its auxin-responsive ability) and 2 mM of the auxin conjugation inhibitor 1,6-dihydroxyacetophenone (DHAP) (to test the effects of auxin conjugation on root induction).

All media were adjusted to a pH of 5.6 - 5.8 prior to autoclaving at 121°C and 1KPa for 20 minutes, with phytohormones added as per suppliers' instructions. Maintenance of cultures was under a 16-h light (200 µmol m^{-2} s^{-1})/ 8-h dark photoperiod at 25°C and

23°C, respectively. Mean rooting times were evaluated according to the method reported by Fett-Neto *et al*. (2001).

Table 4.1 Concentration and composition of plant growth regulators (PGRs) in the eight different elongation media (E1-8) used in this study. N = absent; Y = present

PGR (mg l^{-1})/(µM)	Media							
	E1	E2	E3	E4	E5	E6	E7	E8
0.2 /0.93Kinetin	Y	Y	Y	Y	N	Y	N	N
0.3 /1.6 NAA	Y	Y	N	N	Y	N	N	N
0.05 /0.25 IBA	Y	N	Y	N	Y	N	N	N
0.37 /2.1 IAA	N	N	N	Y	N	N	N	Y
0.2/0.91 *trans*-zeatin	N	N	N	N	N	N	N	Y

4.2.3 Sample preparation for phytohormone analysis

Concentrations of auxins and cytokinins within whole shoots, just prior to rooting, were evaluated using gas chromatography-mass spectrometry (GC-MS). A preliminary study indicated no significant differences in phytohormones along the 1.5 cm long *in vitro* shoots (results not shown). Freeze-dried shoots were homogenised and suspended in 500 µl sodium phosphate buffer (pH 7) and incubated for 1 hour at 4°C. The pH was then reduced with HCl to 2.6, and compound absorption was carried out, facilitated by the addition of Amberlite® XAD-7 (Sigma-Aldrich). The solution was incubated for a further hour at 4°C, followed by two washes of 500 µl of 1% (v/v) acetic acid and dichloromethane. Samples were then dried down, 50 µl of 2 M trimethylsilyl-diazomethane was added, and samples were incubated for 30 minutes at room temperature. Samples were then quenched with the addition of acetic acid (1% v/v) and dried down overnight, followed by heptanes addition and sample analysis, using the

GCT Premier™ benchtop orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer, Waters, USA.

4.2.4 Statistical analysis

All statistical analyses were carried out using PAST, version 2.01 (Hammer *et al*. 2001). Experiments were repeated at least 3 times, with sample sizes of at least 25 for rooting studies, and at least 3 for phytohormone analysis.

4.3 Results

The *in vitro* rooting responses of the tested clones recorded in this study (Fig. 4.1) reflected their reported behaviour as minicuttings in the nursery (Wallis, *pers. comm.*[1]). After exposure to standard multiplication and elongation conditions (E1-Table 4.1), and 30 days in rooting medium containing 0.1 mg l^{-1} IBA, the percent rooting of the clones were 85% for TAG31 (good rooter), 45% for GN155 (poor rooter) and 41% for NH58 (poor rooter) (Fig. 4.1), with mean rooting times of 12, 5.9 and 4.6 days, respectively. The high *in vitro* rooting efficiency of TAG31 has been established previously (Nakhooda *et al*. 2011) (Chapter 2) and, together with NH58, were used in some studies for comparative purposes against GN155, which was the focus of subsequent investigations.

To further characterise the poor-rooting GN155, its rooting response in the presence of different auxin analogues was assessed. The response of its shoots to 0.1 mg l^{-1} and 0.5 mg l^{-1} IBA, IAA and NAA, following elongation on the standard medium (E1), indicated that none of the tested auxins yielded greater than 50% rooting (Fig. 4.2) Mean rooting times for each auxin analogue (0.1 mg l^{-1} and 0.5 mg l^{-1}) were recorded as IBA = 5.9 and 7.6 days; IAA = 3.9 and 8.9 days; and NAA = 6.1 and 6.4 days. The 0.1 mg l^{-1} IAA treatment resulted in the least efficient root production (30%) (Fig. 4.2). Furthermore, upon these treatments, basal callus was observed in all the shoots, with

[1] Jackie Wallis, Mondi Business Paper, Hilton, KwaZulu-Natal, South Africa

callus production being greatest at 0.5 mg l^{-1}, regardless of the type of auxin used (Fig. 4.3). At this concentration, IBA and NAA resulted in larger basal callus formation than IAA. Callus formation was also observed in places other than the cut end of the shoot but no root formation was observed from them (Fig. 4.3).

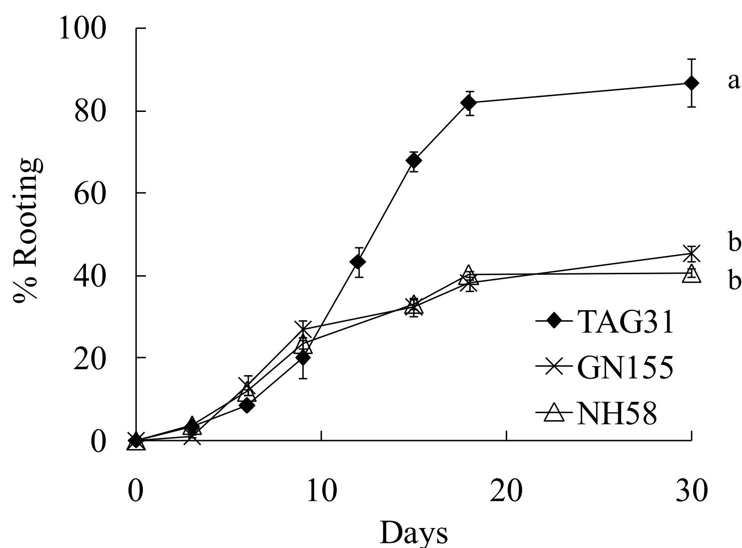

Fig. 4.1 Percentage rooting of shoots of TAG31, GN155 and NH58, over 30 days on rooting medium containing 0.1 mg l^{-1} IBA. Shoots were produced on standard multiplication and elongation media. The values are the mean ± SE (n=30), different letters denote significant differences as determined by one way analysis of variance ($P<0.05$)

The extent to which the shoots of GN155 were able to perceive exogenous auxin was then tested by including the inhibitor of auxin signal transduction, PCIB (Oono *et al.* 2003) in the rooting medium, which was also supplemented with 0.5 mg l^{-1} IBA, IAA or NAA (Table 4.2). In the presence of 50 µM PCIB, none of the auxin treatments were able to induce root production significantly by day 30 (Table 4.2).

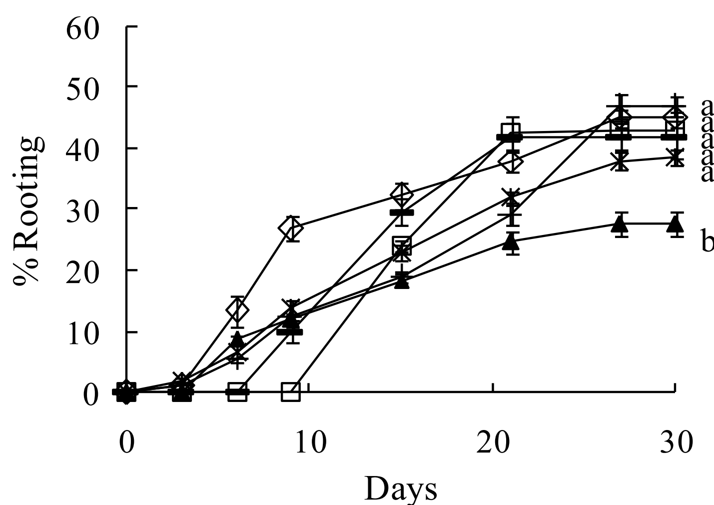

Fig. 4.2 Percentage rooting of GN155 shoots, over 30 days on rooting medium containing 0.1 mg l^{-1} or 0.5 mg l^{-1} IBA (◊, □), IAA (▲, |) or NAA (X, ▬). Shoots were produced on standard multiplication and elongation media. The values are the mean ± SE (n=30). The data were analysed using one way analysis of variance followed by Fisher's least significant difference. Different letters indicate significant differences ($P<0.05$)

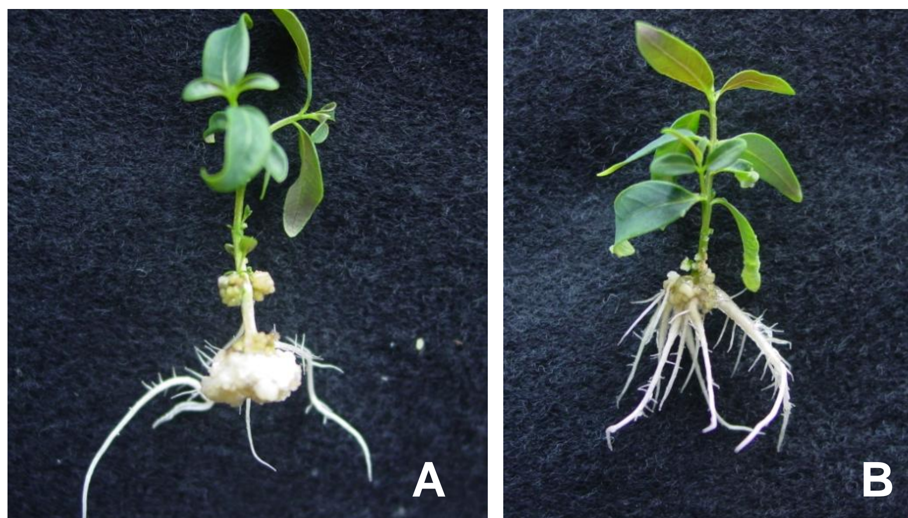

Fig. 4.3 Typical basal callus formation in response to A) 0.5 mg l^{-1} IBA or NAA, or B) 0.5 mg l^{-1} IAA

Table 4.2 Percentage rooting of GN155 shoots cultured on rooting media containing 0.5 mg l^{-1} indole 3-acetic acid (IAA), indole 3-butyric acid (IBA) or 3-napthalene acetic acid (NAA), together with the auxin signal transduction inhibitor p-chlorophenoxyisobutyric acid (PCIB, 50 µM). The values are the mean ± SE (n=30). The data were analysed using one way analysis of variance followed by Fisher's least significant difference. All values were found to be statistically similar (P>0.05 for all statistical comparisons)

	% Rooting		
Time (days)	IAA	IBA	NAA
5	0	0	0
15	6.3±5.5	0	0
30	6.3±5.5	6±5.7	5.3±5.5

In order to test the hypothesis that rooting efficiency is dependent on a clone's ability to hydrolyse auxin conjugates (van der Krieken *et al*. 1992; Epstein and Ludwig-Müller 1993; Epstein *et al*. 1993; George *et al*. 2008), the rooting ability of GN155 was assessed by including the auxin conjugation inhibitor DHAP, together with 0.1 mg l^{-1} IBA, in the rooting medium (Fig. 4.4). By the end of the 30 day culture period, no significant increase in rootability was observed, with rooting still below 55% in the presence of 2 mM DHAP (Fig. 4.4). Mean rooting time for the 0.1 mg l^{-1} IBA (without DHAP) was 5.9 days, 6.5 days for the 0.1 mg l^{-1} + 1 mM DHAP, and 7.7 days for the 0.1 mg l^{-1} + 2 mM DHAP treatments.

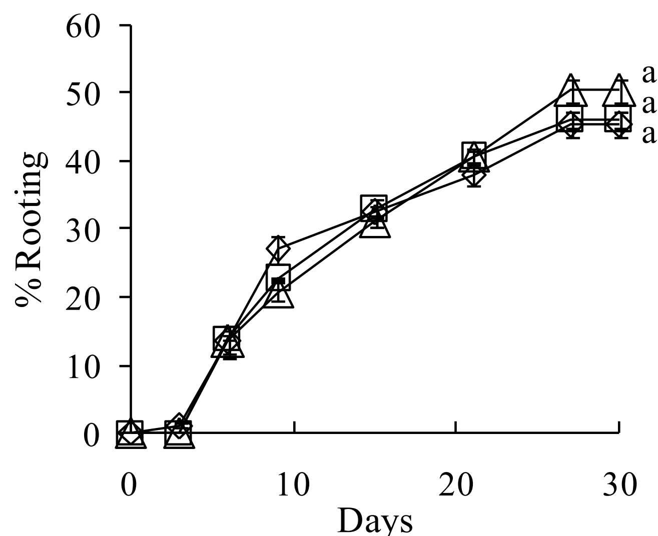

Fig. 4.4 Percentage rooting of GN155 shoots, over 30 days on rooting medium containing 0.1 mg l^{-1} IBA alone (◊), and 0.1 mg l^{-1} IBA with either 1mM (□) or 2 mM 1,6-dihydroxyacetophenone (DHAP) (Δ). Shoots were produced on standard multiplication and elongation media. The values are the mean ± SE (n=30). The data were analysed using one way analysis of variance followed by Fisher's least significant difference, and found to be statistically similar, as indicated by common letters ($P>0.05$)

Having established that GN155 was able to perceive exogenous auxin (Table 4.2) and inhibition of exogenous auxin conjugation did not markedly increase its rooting efficiency (Fig. 4.4), the effect of plant growth regulators (PGRs) supplied during the elongation stage on rooting was tested. Shoots of TAG31 (good rooter) and GN155 (poor rooter) were transferred from multiplication medium onto seven elongation media (E1 to E7), each with different combinations of auxin and cytokinin analogues (variation on the standard elongation medium, E1) (Table 4.1). Following these treatments, the shoots were transferred to rooting medium lacking auxin. The results show that the levels and combinations of the PGRs used during shoot elongation significantly influenced the rooting ability of the shoots of both tested clones (Table 4.3). TAG31 rooted best (100%) when elongated on the standard medium (E1, containing kinetin, NAA and IBA), on the medium lacking kinetin (E5, containing

NAA and IBA) and on the medium devoid of PGRs (E7) (Table 4.3). However, its percentage rooting was significantly inhibited when kinetin was supplied alone (E6), or in conjunction with the unstable auxin IAA (E4), or with a low concentration of the stable auxin IBA (E3) (Table 4.3). While a similar trend in rooting ability in response to the auxins and cytokinin present in the elongation media was observed for shoots of GN155, it was apparent that this clone was more dependent on exogenous auxin to counteract the inhibitory effects of kinetin on root production than TAG31 (Table 4.3). In the presence of kinetin, root production was inhibited in relation to the concentration and stability of the auxins used in the elongation stage. This was indicated by the percentage rooting of GN155 shoots following elongation on media E2, E3 and E4 (Table 4.3). While no significant difference in rooting was observed between E1 and E2 (containing the relatively stable, but synthetic NAA), a significant increase in rooting resulted following elongation on media containing kinetin and either a low concentration of the relatively stable IBA (E3), or a higher concentration of the less stable IAA (E4) (Table 4.3). The percentage rooting of GN155 shoots elongated with kinetin alone was not significantly different from that obtained following elongation on E3 or E4, eluding to the possible effects of endogenous auxins on rootability (Table 4.3, see later for further discussion).

As the rooting results of GN155 from the E1 and E7 treatments show, the absence of exogenous phytohormones from the elongation stage did not significantly affect root production (Table 4.3). However, rooting was significantly enhanced (80.3%) by removing the kinetin while retaining the auxins NAA and IBA (E5) during shoot elongation, prior to rooting on an auxin-free rooting medium. The rooting ability of this clone was restored (100% rooting) with the addition of 0.1 mg l^{-1} IAA, IBA or NAA to the rooting medium (Fig. 4.5), with mean rooting times of 12.3, 14.9 and 15.2 days for the IAA, IBA and NAA treatments, respectively.

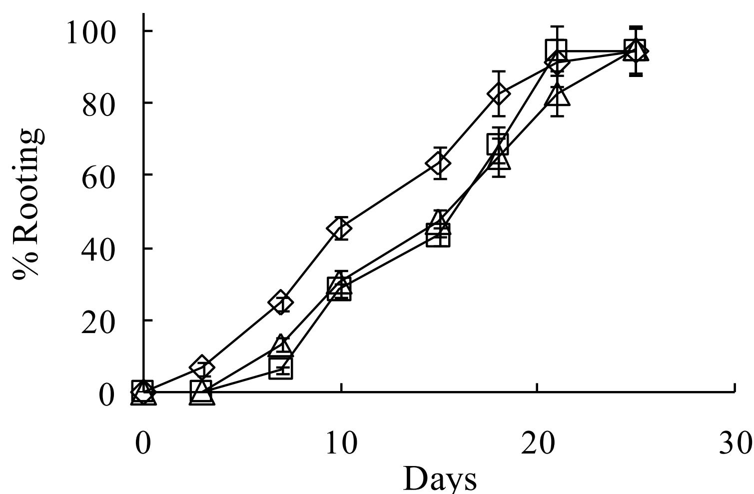

Fig. 4.5 Percentage rooting of shoots of GN155, elongated on E7 (Table 4.1) and transferred to rooting media containing 0.1 mg l^{-1} IAA (◊), IBA (□) or NAA (Δ). The values are the mean ± SE (n=30). The data were analysed using one way analysis of variance followed by Fisher's least significant difference and found to be statistically similar (P>0.05)

The inability of GN155 shoots to produce roots, when subjected to the standard protocol (E1), and the restoration of rootability by removing kinetin from the elongation medium suggested that exogenous cytokinin during the pre-rooting culture stages inhibited root production, either directly or through the alteration of endogenous phytohormone levels in the shoots. To test this, the endogenous levels of kinetin, IAA, IBA and NAA of shoots of TAG31 and GN155, cultured on selected elongation media (E1, E5, E6 and E7) were determined using GC-MS (Table 4.3). As expected, shoots produced on media containing no phytohormones (E7) had the lowest levels of these compounds and those produced on media containing kinetin (E1 and E6) had the highest synthetic cytokinin content, irrespective of the clone identity. The addition of the auxin analogues IBA and NAA (E1 and E6) resulted in an increase in endogenous IAA content to levels comparable or in excess of those of the auxin analogues themselves (Table 4.3). After elongation on E1, the ratio of kinetin to auxin was four times higher in GN155 than in TAG31 (2.6 and 0.58, respectively). However, when shoots of GN155 were elongated

on E5 (excluding kinetin, but containing NAA and IBA), kinetin:auxin was reduced to 0.39 and rooting increased to 80% (Table 4.3). A strong relationship between the kinetin:auxin ratio and percent rooting (R^2 = 0.943) of shoots of both tested clones following elongation on selected media, indicated the inhibitory effect of high kinetin:auxin on root induction (Fig. 4.6).

Table 4.3 Percentage rooting of shoots of TAG31 and GN155 after 30 days, following elongation on different media (E1-E7), and rooted in the absence of exogenous auxins. E1 = standard protocol (Table 4.1). The values are the mean ± SE (n=30). The data were analysed using one way analysis of variance followed by Fisher's least significant difference and values that do not share letters are significantly different ($P<0.05$) from each other

Elongation media	PGR (mg l^{-1})	% rooting	
		TAG31	GN155
E1	0.3 NAA, 0.2 kinetin, 0.05 IBA	100[a]	10±2[d]
E2	0.3 NAA, 0.2 kinetin	68.3±2.9[b]	6±5.3[d]
E3	0.05 IBA, 0.2 kinetin	29±3.6[c]	18±2[e]
E4	0.37 IAA, 0.2 kinetin	31.3±5.5[c]	19±2.6[e]
E5	0.3 NAA, 0.05 IBA	95±8.6[a]	80.3±4.5[f]
E6	0.2 kinetin	37.3±2.5[c]	21.3±4.2[e]
E7	None	91.7±7.6[a]	9.3±1.1[d]

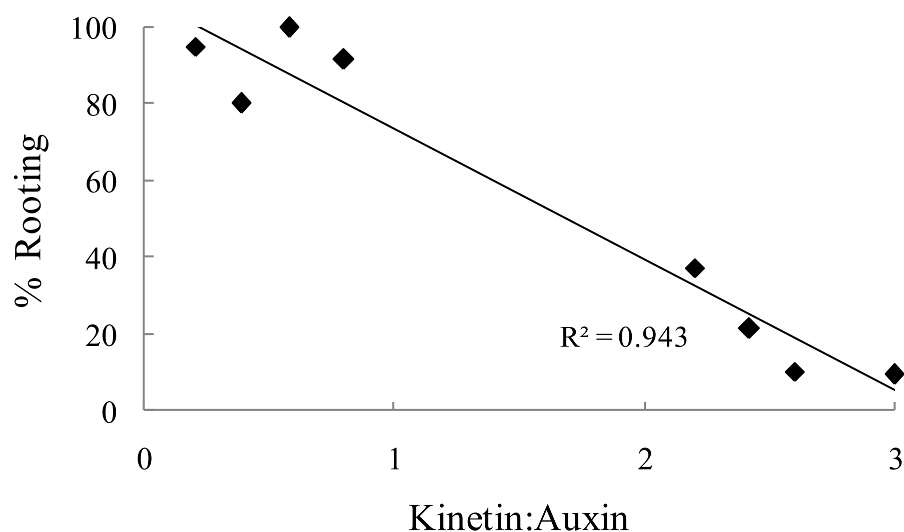

Fig. 4.6 Linear regression of rootability (percentage rooting) to endogenous cytokinin: auxin ratios during the elongation culture stage

Since kinetin appeared to accumulate to higher amounts in the shoots of GN155 than in TAG31 and subsequently inhibited rooting, an additional elongation medium was tested (E8). It contained *trans*-zeatin, a cytokinin less stable than kinetin (George *et al*. 2008), and was selected in an attempt to reduce the inhibitory effect of cytokinin accumulation on root induction. This *trans*-zeatin-containing medium (E8) was comparable to E4, except that kinetin was replaced with 0.91 µM *trans*-zeatin (Table 4.1). Following elongation, shoots were transferred to rooting media containing 0.1 mg l^{-1} IAA. Under these conditions, percentage rooting was significantly higher in the *trans*-zeatin (E8) than in kinetin-containing (E4) medium, albeit lower than on the medium devoid of cytokinins (E5) (Fig. 4.7). Mean rooting times for shoots on these treatments (Fig. 4.7) were 12.3, 8.11 and 2.9 days for cytokinin-free, *trans*-zeatin and kinetin-containing media, respectively. The observed inhibitory effect of kinetin and *trans*-zeatin supply during shoot elongation on subsequent rooting was then tested with NH58, another poor rooting clone (Fig. 4.1). Following elongation on E4 (kinetin-containing), E5 (cytokinin-free), and E8 (*trans*-zeatin-containing) (Fig. 4.8), it became apparent that the rooting ability of the two tested poor-rooters could be significantly improved by

omitting or modifying the cytokinin type used in the elongation medium. Recorded mean rooting times were 11.2, 7.9 and 4.9 days for the cytokinin-free, *trans*-zeatin and kinetin treatments, respectively.

Table 4.4 Average concentrations (± standard error of the mean) of extractable IAA, IBA, NAA and kinetin in elongated shoots of the tested clones, prior to rooting in an auxin-free medium, following each elongation treatment. Refer to Table 3 for % rooting for each treatment. The ratio of kinetin: auxin is indicated

Clone	Elongation medium	Extractable PGR (µmol/g DW)				Total cytokinin/total auxin
		IAA	IBA	NAA	Kinetin	
1	E1	0.33±0.08	0.33±0.02	0.15±0.04	0.47±0.2	0.58
	E5	0.13±0.015	0.02±0.007	0.22±0.13	0.12±0.03	0.2
	E6	0.12±0.1	0.04±0.03	0.07±0.03	0.51±0.2	2.2
	E7	0.08±0.04	0.02±0.01	0.014±0.01	0.09±0.07	0.8
2	E1	0.2±0.05	0.05±0.02	0.14±0.09	1.01±0.2	2.6
	E5	0.18±0.06	0.03±0.008	0.63±0.3	0.33±0.04	0.39
	E6	0.17±0.08	0.09±0.06	0.02±0.013	0.68±0.35	2.42
	E7	0.06±0.04	0.02±0.004	0.007	0.28±0.16	3

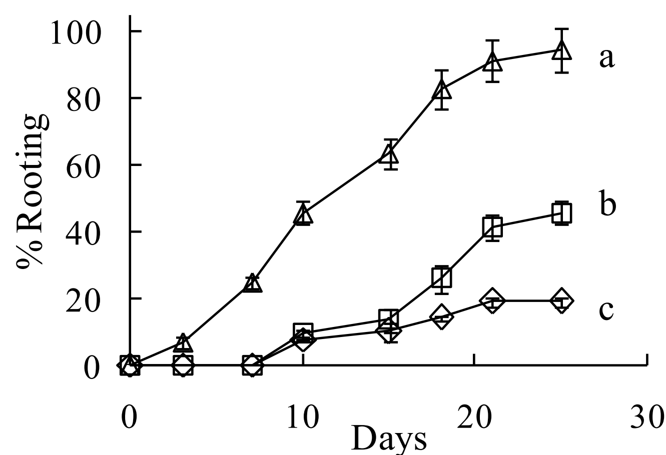

Fig. 4.7 Percentage rooting of shoots of GN155, elongated on either E4 (kinetin-containing medium) (◊), E7 (no cytokinins) (Δ), or E8 (*trans*-zeatin-containing medium) (□), followed by rooting on medium containing 0.1 mg l^{-1} IAA. The values are the mean ± SE (n=30). The data were analysed using one way analysis of variance followed by Fisher's least significant difference. Different letters denote significant differences ($P<0.05$)

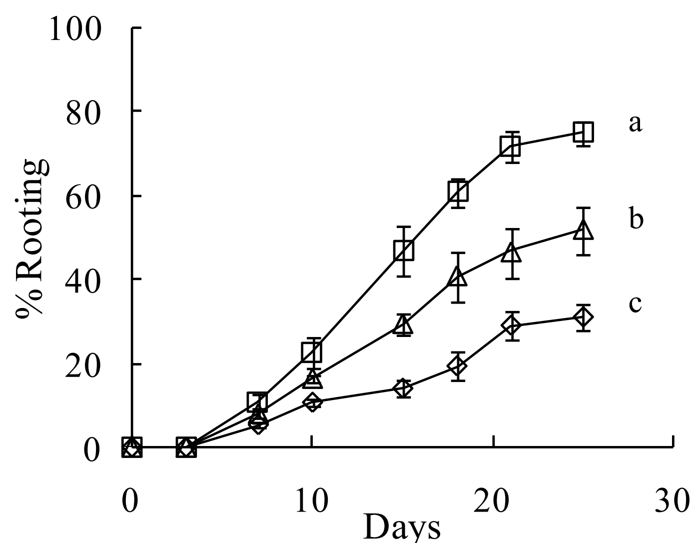

Fig. 4.8 Percentage rooting of shoots of NH58, elongated on either E4 (kinetin-containing medium) (◊), E7 (no cytokinins) (□), or E8 (*trans*-zeatin-containing medium) (Δ), followed by rooting on media containing 0.1 mg l^{-1} IAA. The values are the mean ± SE (n=30). The data were analysed using one way analysis of variance followed by Fisher's least significant difference. Different letters denote significant differences (P<0.05)

4.4 Discussion

Successful root induction is a critical step in vegetative propagation programmes, and different species and genotypes within a species are known to vary greatly in this regard (Eldridge *et al*. 1994; Jones and van Staden 1997; George *et al*. 2008). Such variation has been attributed to a range of biotic and abiotic factors (Geiss *et al*. 2009), most notably the availability of PGRs, particularly auxins and cytokinins (George *et al*. 2008).

The present study was aimed at elucidating the effects of auxins and cytokinins on root induction in three eucalypt clones of varying rooting ability (Fig. 4.1), using an *in vitro* approach. It was initially found that the popular auxins employed in *Eucalyptus* sp.

micropropagation, i.e. IBA, IAA and NAA, did not increase the rooting ability of GN155 (poor-rooter) (Fig. 4.2), none of which were able to produce over 50% rooted shoots, even at 0.5 mg l^{-1}. Predictably, given that IAA is the least stable of the tested auxins (George *et al*. 2008), treatment of GN155 shoots with 0.1 mg l^{-1} IAA resulted in the lowest rooting potential. While callus production was visible at the base of all shoots, those treated with 0.5 mg l^{-1} IAA developed the least basal callus, in keeping with the higher stabilities of IBA and NAA in plant tissues (George *et al*. 2008). The lack of any significant rooting response from shoots of GN155 in the presence of PCIB, a known inhibitor of auxin signal transduction (Oono *et al*. 2003), indicated that it was able to perceive the exogenous auxin (Table 4.2). This, together with shoot basal callus formation in the presence of exogenous auxin in the rooting stage (without PCIB), suggested that in this poor-rooter (GN155), the supplied auxin was directed towards callus formation, rather than rhizogenesis.

Studies into auxin metabolism have shown that auxins are rapidly taken up by cells either through influx carrier proteins or through passive diffusion (reviewed by Leyser 1999; Muday and de Long 2001). Further, they are rapidly conjugated or oxidated to inactive forms through enzymatic action within the cell (de Klerk *et al*. 1999; George *et al*. 2008), which results in only a small portion of the supplied auxin occurring in the free form (de Klerk *et al*. 1999). This has led to the suggestion that a clone's ability to produce roots depends on the ease and timing at which it can hydrolyse these conjugated auxins to free auxin forms (van der Krieken *et al*. 1992; Epstein and Ludwig-Müller 1993; Epstein *et al*. 1993; George *et al*. 2008). In support of this, work conducted by Epstein *et al*. (1993) on cuttings of sweet cherry, showed that an easy-to-root cultivar metabolised IBA conjugates slower than a difficult-to-root cultivar, leaving free IBA available for a longer period within the shoots. Such conjugates have been suggested to serve as a sustainable source of auxin (Wiesman *et al*. 1989). The addition of the auxin conjugation inhibitor DHAP significantly improved the percentage rooting in difficult-to-root cuttings in that study (Epstein *et al*. 1993). However, such a response was not obtained in the present investigation with the addition of 2 mM DHAP. This did not significantly increase the rootability of GN155 shoots, indicating that this clone's poor rooting ability could not be attributed solely to its inability to hydrolyse auxin

conjugates (Fig. 4.4). Auxin metabolism is not autonomous, and a complex interaction exists between auxin and a number of other plant growth regulators. Of interest to the present study, was the interaction between auxins and cytokinins as they are the main PGRs used in *in vitro* regeneration protocols (George *et al*. 2008). The general model of organogenesis states that a high auxin to low cytokinin ratio favours root formation, while a high cytokinin to low auxin ratio favours shoot proliferation (Skoog and Miller 1957; George *et al*. 2008), a principle applied in micropropagation protocols. Both auxins and cytokinins are essential in the regulation of the cell cycle (Mok and Mok 1994) and they have been shown to work antagonistically within root tissues (Brault and Maldiney 1999; George *et al*. 2008; Kuderová and Hejátko 2009). Cytokinins have also been found to modulate auxin-induced organogenic processes through the regulation of auxin efflux (Pernisová *et al*. 2009). Apart from research based on establishing effective (largely clone-specific) *in vitro* protocols (e.g. Le Roux and van Staden 1991; Jones and van Staden 1997; Gomes and Canhoto 2003; Arya *et al*. 2009), relatively few studies have documented the interaction between auxins and cytokinins on the rootability through micro- or macro-propagation of commercially important eucalypt clones, or the reasons for poor rooting percentages achieved with many of these clones.

On the hypothesis that the plant growth regulators used during the pre-rooting culture stages affected the tested clones' rootability, both clones were elongated on a range of media (Table 4.1) and subsequently transferred to rooting medium without exogenous auxins. Compared with GN155 (poor rooter), shoots of TAG31 (good rooter) displayed a greater ease of rooting, relative to the exogenous auxin type and concentration supplied in the elongation treatments (Table 4.3). The more stable auxins, IBA and NAA, in elongation yielded higher rooting percentages for clone 1 than the elongation medium with IAA (except in E5, in which the IBA concentration was minimal). Shoots of GN155 on the other hand, were not able to achieve more than 22% rooting when elongated on media containing kinetin, either with or without auxins. Eliminating kinetin from the elongation medium (E7) of GN155 did, however, result in at least 80% rooting in auxin-free rooting medium and 100% rooting in media containing 0.1 mg l^{-1} auxin (either as IAA, IBA or NAA) (Fig. 4.5). This indicates that the poor rootability of some eucalypt clones may be due to excess cytokinin supplied and stored during the

pre-rooting culture stages. This proposal was further supported by GC-MS analysis of IAA, IBA, NAA and kinetin in the shoots of both tested clones, following elongation on selected media (Table 4.4), but prior to rooting. From these results, it was deduced that the exogenous kinetin in the elongation stage heavily influenced the cytokinin:auxin ratio, thereby inhibiting root induction in GN155 (Fig. 4.6).

Cytokinins themselves have complex metabolic pathways which include conjugation and degradation reactions (Mok and Mok 1994; van Staden and Crouch 1996; Haberer and Kieber 2002; George *et al*. 2008). Natural cytokinins (such as *trans*-zeatin and isopentenyladenine) are degraded by the naturally-occuring enzyme cytokinin oxidase (Mok and Mok 2001; Haberer and Kieber 2002; George *et al*. 2008). The rapid enzymatic breakdown of some natural cytokinins has been regarded as the reason for their ineffectiveness in many culture protocols. In this context, it has been suggested that cytokinins that are not substrates of the cytokinin oxidase enzyme (such as kinetin), may last longer in plant tissues (George *et al*. 2008). In the present study, the synthetic cytokinin kinetin would therefore have likely persisted within shoots of the two poor-rooting clones. Since cytokinins generally delay or even inhibit root formation (Brault and Maldiney 1999; George *et al*. 2008; Kuderová and Hejátko 2009), the percentage rooting of GN155 and NH58, following elongation with either kinetin or *trans*-zeatin (Figs. 4.7 and 4.8), reflected the relative persistence of these cytokinins in shoots. These results suggest that a relationship exists between cytokinin depletion and root formation in the tested poor-rooting eucalypt clones, regardless of genotype. The use of *trans*-zeatin instead of kinetin during elongation did significantly increase the rooting ability of GN155 (Fig. 4.7) and NH58 (Fig. 4.8), while complete cytokinin omission during elongation resulted in even higher rooting percentages in both clones (GN155 and NH58) (Figs. 4.7 and 4.8).

Collectively, the data indicate that in the development of micropropagation protocols for specific clones, the complex interactions that exist between the two main phytohormone groups - auxins and cytokinins - and, in particular, their stabilities and metabolic requirements within plant tissues need to be considered in order to achieve the objective of each culture stage. Improving eucalypt micropropagation protocols to

increase the yield of difficult-to-propagate clones (such as in the present study), is an essential tool in tree improvement programmes, which are highly beneficial to the forestry industry (de Assis *et al*. 2004)

It is possible that the present observations have implications for vegetative propagation of eucalypts through minicuttings under nursery conditions, in that altering the endogenous cytokinin:auxin ratios of minicuttings through treatments of parent plants (e.g. exogenous PGRs may potentially result in greater rooting abilities of known poor-rooting clones). This in turn would greatly enhance yield rates in forestry programmes.

FINAL REMARKS

5.1 Background

As previously discussed, vegetative propagation is a valuable tool in the management practices and propagation of commercially-important forestry species, such as Eucalyptus. Not only does it allow for the preservation of superior selected genotypes, but it also eliminates the erratic and/or poor yields of plants characteristic of propagation that relies on seed production. In addition, vegetative propagation often represents the only viable means of perpetuating valuable hybrid genotypes. Since the 1950s, eucalypts have and continue to be widely vegetatively propagated, mainly through macro- or mini- cuttings. Efforts to increase the productivity of plantations have led to the development of several methods to supply superior material to the industry, such as clonal hedges and hydroponics (Denison and Kietzka 1993a; de Assis *et al.* 2004). These methods were aimed at maintaining desired genotypes while providing a sufficient amount of superior planting material for commercial forest deployment. One of the drivers behind these developments was the need to increase the rooting potential of cuttings from elite genotypes. Initially, as it was recognised that rooting ability decreased as the parent material aged (de Assis *et al*. 2004), i.e. juvenile material rooted better than older material, efforts were directed at maintaining juvenility. However, despite significant advances made towards increasing the rooting ability of commercially important pure and hybrid eucalypts, the difficulty of producing adventitious roots from cuttings of many clones has persisted (Eldridge *et al.* 1994; de Assis *et al.* 2004; López *et al*. 2010). As a result, many potentially valuable clones have been excluded from commercial forestry (Eldridge *et al.* 1994; de Assis *et al.* 2004; Saya *et al*. 2008).

An important advancement in the understanding of adventitious rooting was achieved using *in vitro* technology (Trindade and Pais 1997; Gomes and Canhoto 2003; Nourissier and Monteuuis 2008). The process of micropropagation was found to restore juvenility of old material, resulting in microcuttings (sourced from micropropagated plants) rooting more easily than macro- or mini- cuttings (de Assis *et al*. 2004). In

addition to restoring juvenility, micropropagation offered benefits such as high shoot multiplication rates, the potential to root valuable clones that could not be readily rooted as cuttings, and provided a means of plantlet regeneration following genetic modification. However, due to the lack of knowledge regarding their long-term field performance, micropropagated plants have largely been excluded from commercial forestry deployment. As discussed, only a few reports have documented field trials of micropropagated eucalypts (e.g. Bell *et al*. 1993; Watt *et al.* 1995; Mokotedi *et al.* 2010). Those of Bell *et al.* (1993) and Mokotedi *et al.* (2010) investigated various parameters regarding the above- and below- ground performance of micropropagated plants compared with plants from seed- or cuttings- propagation. Whereas in both of those studies, little or no significant differences were reported for above-ground physiology, that by Mokotedi *et al*. (2010) found differences at 16 months in the field. These included micropropagated plants exhibiting a root system that was significantly less resistant to vertical uprooting than roots of seedlings or cuttings. Given the fast growth rates and heights of productive eucalypts forests, trees with reduced anchorage are of particular concern in this industry.

While several factors govern the formation of adventitious roots *in vitro*, one of the key determinants is the exogenous application of PGRs (Geiss *et al.* 2009). Since various genotypes respond differently to exogenous PGR application (Fogaça and Fett-Neto 2005; Nourissier and Monteuuis 2008; Mankessi *et al.* 2009), a range of exogenous PGRs and their concentrations have been empirically prescribed for root induction *in vitro* (Table 1.2). Attempts at promoting adventitious rooting in difficult-to-root genotypes have frequently led to the use of a range of auxin analogues with varying potencies (Table 1.2). These analogues – natural or synthetic – are often unique in their perception, transport, metabolism, or interaction with other endogenous and exogenous growth regulators (de Klerk *et al*. 1999; de Rybel *et al*. 2009; Tromas and Perrot-Rechenmann 2010). These differences could potentially result in dissimilar regulation of growth and development, in keeping with the specific perception and transport routes of the various PGRs, as reports suggest (Mok and Mok 2001; Moubayidin *et al.* 2009; Petrášek and Friml 2009). In addition to such variations in PGR action, shoot responses

to exogenously applied PGRs are often genotypic (Fogaça and Fett-Neto 2005; Nourissier and Monteuuis 2008; Mankessi *et al.* 2009).

In order to understand the roles of PGRs on the rooting ability of various eucalypt genotypes, the approach taken in the present study was to use eucalypt genotypes of differing rooting abilities and incorporate inhibitors and antagonists of auxin transport and action to investigate the specific roles of auxin analogues on *in vitro* eucalypt root induction and development. The selected clones - a good-rooting *E. grandis* (TAG31) and two poor-rooting hybrids - *E. grandis* x *nitens* (GN155) and *E. grandis* x *nitens* natural hybrid (NH58) – were utilised to develop an understanding of the interactions of auxins and cytokinins in root development, relative to the genotypic responses to exogenously applied PGRs.

5.2 PGR choice for *in vitro* root induction and development

5.2.1 The need for IAA

The forestry industry has a range of clones with differing morphogenic abilities at its disposal. Considering the varied responses that these eucalypt genotypes display to PGRs *in vitro*, variations in the stabilities and modes of action of cytokinin and auxin analogues are useful when empirically selecting an analogue for shoot and root production. As often mentioned in this document, when devising a protocol for *in vitro* organogenesis, the worker's tendency has been to select a cytokinin or an auxin that results in the best shoot or root production, assessed in terms of number or appearance (e.g. Warrag *et al.* 1990; Nourissier and Monteuuis 2008; Mankessi *et al.* 2009). The range of cytokinin and auxin analogues and the concentrations that have been prescribed for the micropropagation of the various commercially-important clones is testament to this (Table 1.2). However, while phytohormone stability or „potency' may be desirable for a specific *in vitro* stage of morphogenesis, the results of the studies presented here

indicate that the persistence of a stable phytohormone may hamper morphogenesis in subsequent stages, where its presence may be inhibitory. A further consideration is that both good- and poor- rooting clones are affected to different degrees by this hormone carryover effect, illustrating genotypic effects to PGR application and action. For example, in the present study, the tested poor-rooters (GN155 and NH58), were more susceptible to the inhibitory effects of kinetin persistence from the pre-rooting culture stages on root induction than TAG31 (good-rooter).

The data collected using the good-rooting clone TAG31, suggested that the auxins supplied during the multiplication and elongation stages persisted into the rooting stage (Figs. 2.1 and 2.2). This was supported by the fact that this clone rooted best in auxin-free rooting medium. The supply of exogenous auxin (IBA, IAA or NAA) at the rooting stage possibly resulted in a supra-optimal auxin environment, leading to a reduction in rooting ability (Fig. 2.1). As a consequence of reducing the auxin exposure of shoots from the pre-rooting culture stages, however, a sub-optimal auxin environment was created, which resulted in a reduction in percentage rooting, with a concomitant reduction in root graviperception (Fig. 2.2; Table 2.2). Roots that displayed reduced gravitropism were shown, histologically, to have inconsistent or no starch grain accumulation is root cap columella cells compared with normal graviperceptive roots *in vitro* (Fig. 2.4). This indicated that eucalypt shoots require auxins within a specific concentration range in order to induce roots and ensure the correct physiological properties of these roots, such as gravitropism, are met. In addition, a direct relationship was recognised between shoot IAA concentration and root graviperception (Table 2.2). These findings not only supported the need for auxins in adventitious rooting and in gravitropism, but also suggested that the specific properties of IAA (transport and action) are critical in root development.

The typical stages of adventitious root development are cellular dedifferentiation, induction and initiation, development of root initials into root primordia and, finally, root emergence (Hartmann *et al*. 1997; Kevers *et al*. 1997; de Klerk *et al*. 1999; Geiss *et al*. 2009). *In vitro* studies using apple shoots have indicated that although cells are responsive to and require auxins during the dedifferentiation and the induction to

initiation stages, auxins become inhibitory following root primordia development (de Klerk *et al*. 1995; de Klerk *et al*. 1999). If auxins are inhibitory following the development of root primordia, then maintaining a supra-optimal auxin environment *in vitro* hampers root development and emergence, resulting in a reduction in percentage rooting, as was observed with the good-rooting clone TAG31 (Fig. 2.1). The rationale for successful *in vitro* rooting should, therefore, be to maintain an optimum auxin environment, which is also dynamic and in keeping with the changing auxin sensitivities of the developing adventitious roots. One way to achieve this is through the use of auxins and cytokinins that degrade as they would in the natural formation of adventitious roots, following their respective actions. This would eliminate the possibility of their persistence into subsequent stages of morphogenesis where they may be inhibitory. For example, an auxin should be freely available within plant tissues for the duration of the dedifferentiation, induction and initiation stages of adventitious rooting, and then rapidly „disappear' (through conjugation or oxidation, as reported for most auxins) (Centeno *et al*. 1999; Woodward and Bartel 2005), before the subsequent stages of rooting. The differing stabilities of the auxin analogues and their concentrations used in micropropagation protocols are compliant with this requirement, as shown in the present work (Chapter 2).

Studies undertaken to investigate the influence of the stabilities of IAA, IBA or NAA on *in vitro* rooting of TAG31 shoots (Chapter 2), revealed that root induction and post-acclimatisation development were dependent on the respective analogue's stability. While all of those tested analogues have been reported to be inactivated through conjugate formation (Centeno *et al*. 1999; Woodward and Bartel 2005), IAA is relatively more unstable compared with IBA and NAA, due to its rapid oxidative degradation (de Klerk *et al*. 1999; Woodward and Bartel 2005). For these reasons, in TAG31, following the induction of adventitious roots, IBA and NAA were likely to have persisted longer in plant tissues compared with IAA, resulting in a supra-optimal and thus inhibitory auxin environment with regard to root development, following the initiation of adventitious roots in TAG 31. Hence, it is proposed that when IBA and NAA were supplied at a relatively high concentration in the rooting medium (0.5 mg l^{-1}), their respective stabilities allowed for their persistence into the acclimatisation stage.

This also resulted in the formation of new roots with architecture tending towards I-beam (Fig. 2.5). These findings may explain the results reported by Mokotedi *et al*. (2010), in which micropropagated eucalypts, rooted *in vitro* using IBA, developed I-beam shaped roots in the field, with compromised anchorage as a result of significantly reduced uprooting resistance. In view of the results presented here, a less stable auxin such as IAA is suggested to be more suitable for the long-term development of quality roots.

Many aspects of the current understanding of auxin transport and action have been gained through the use of auxin inhibitors and antagonists (Geldner *et al*. 2001; de Rybel *et al*. 2009). Adopting a similar approach in this study allowed for the specific roles of IAA, IBA and NAA on *in vitro* root morphogenesis to be investigated. It was found that, whereas all three analogues were able to induce roots in TAG31 successfully, in the presence of the IAA-specific transport inhibitor TIBA in the rooting medium, subsequent root development was impaired (Figs. 2.3; 3.2D, 3.3B and 3.4B). These findings supported the model that describes the polar transport of IAA through the AUX1 and PIN protein families of influx and efflux transporters as the effector of root graviperception (Swarup and Bennett 2009). Despite other auxin analogues (IBA and NAA) being suitable for root induction, their action could not replace that of IAA during root development in the tested clones. Furthermore, when shoots of TAG31 were transferred to medium containing TIBA or PCIB (inhibitors of IAA efflux and auxin signal transduction, respectively) after root induction, alterations in root vascular patterning and root tip development were recorded. These results not only confirmed the role of auxins in vascular patterning and root tip maintenance (Berleth *et al.* 2000; Ye 2002), but also emphasised that IAA was required in the investigated root development parameters, by virtue of its specific polar transport. Bearing in mind that IAA availability and transport are essential for root development, one questions whether the increased rooting potential of shoots achieved by many workers through the use of more stable auxin analogues (e.g. Gomes and Canhoto 2003; Nourissier and Monteuuis 2008; Mankessi *et al*. 2009) justifies their use in eucalypt micropropagation. This is raised, given that the quality of roots induced using stable analogues, and potentially of the quality of the whole plant may be compromised. This choice of auxin should, therefore,

be considered in view of the close relationship between plant productivity and root architecture (Lynch 1995). Even though analogues such as IBA and NAA may outperform IAA in certain root developmental responses, such as the induction of adventitious roots, IAA is required in virtually all root physiological processes. This being the case, why and how do IAA analogues (e.g. IBA and NAA) function in certain root physiological responses (e.g. root induction), and not others (e.g. gravitropism or the maintenance of root vasculature)? The mechanisms of auxin signal perception, transduction and transport explain the basis behind these variations in the actions of the auxin analogues.

As discussed earlier, auxin is perceived by receptors of the Transport Inhibitor Response/Auxin Signalling F-box (TIR1/AFB) family of proteins, which are subunits of a ubiquitin ligase complex (SCF^{TIR1}) responsible for the degradation of Aux/IAA auxin transcription repressors (Tromas and Perrot-Rechenmann 2010; Simon and Petrášek 2011). This receptor has been shown to recognise IAA and at least two other synthetic auxin analogues - NAA and 2,4-D (Dharmasiri *et al*. 2005; Kepinski and Leyser 2005). However, to-date, no evidence suggests that IBA is included among its recognition molecules. Recent reports have documented a dual-specificity phosphatase-like protein, IBA Response 5 (IBR5), which promotes and regulates auxin response gene expression through a pathway distinct from the TIR/AFB-mediated repressor degradation in *Arabidopsis* (Strader *et al.* 2008). The fact that *Arabidopsis ibr5* mutants are resistant to the actions of IBA, supports this (Monroe-Augustus *et al*. 2003). Those findings suggest that alternate pathways of auxin signalling exist, with IBA perception and signal transduction being different to that described for IAA, which is core to the system controlling auxin gene expression and action (Tromas and Perrot-Rechenmann 2010). The implication is that when exogenous IBA is supplied *in vitro*, it is either converted to IAA or is perceived as IBA, in which case it acts independently of IAA. Both of these pathways have been reported in plants (Tromas and Perrot-Rechenmann 2010; Simon and Petrášek 2011), as well as instances where exogenous IBA interacts with endogenous IAA to elicit the required auxin response (Ludwig-Müller *et al*. 2005).

In the present study, IBA was able to induce roots in the presence of the IAA-specific transport inhibitor TIBA when IAA was unable to do so, indicating that IBA can act independently at least for root induction in eucalypts. However, root development following induction relied on IAA, since the presence of TIBA resulted in agravitropism, alterations in vascular patterning and changes in root tip development (Figs. 3.3B and 3.4B). These results suggest that the IBA analogue can only transiently replace the actions of IAA, possibly by virtue of its alternate perception and signalling pathway. The findings presented in this study also highlighted the importance of auxin transport via the AUX1 and PIN influx and efflux transporters, characteristic of IAA.

Although auxin influx and efflux transporters maintain a degree of specificity by virtue of being proteins, some cross-functioning within these transport proteins has been reported. AUX1, the protein responsible for IAA influx, also facilitates influx of the synthetic auxin analogue 2,4-D, but not the influx of NAA or IBA (Delbarre *et al.* 1996; Yamamoto and Yamamoto 1998; Tromas and Perrot-Rechenmann 2010). Similarly, the PIN proteins responsible for IAA efflux also facilitates NAA efflux, but not the efflux of 2,4-D or IBA (Delbarre *et al.* 1996; Utsuno *et al.* 1998). This implies that 2,4-D and NAA are incapable of sustaining plant responses that require auxin polar transport through the AUX1 and PIN transporters (e.g. gravitropism). As the IBA analogue does not share either of the influx or efflux transport proteins with IAA (Poupart and Waddell 2000; Strader and Bartel 2011), and even though it is transported in a polar manner (Poupart and Waddell 2000; Rashotte *et al.* 2003), it did not replace IAA polar transport in the tested clones.

It appears that the central principle in root morphogenesis, with respect to the auxins, is creating and maintaining an auxin concentration gradient and maintaining auxin homeostasis through the biosynthesis, conjugation, transport, degradation, distribution and even interconversion of auxin analogues (Petrášek and Friml 2009; Simon and Petrášek 2011). As the most likely auxin implicated in root morphogenesis is IAA, other auxin analogues have been suggested to contribute to the creation of IAA concentration gradients within root tissues and to the overall maintenance of IAA homeostasis (Petrášek and Friml 2009; Simon and Petrášek 2011). These analogues may, therefore,

participate in specific parts of the complete auxin response (i.e. localised response), but are limited in their action compared with IAA. This is manifested in detectable but not sustained auxin effects. For example, IBA is an endogenous auxin and may act independently from IAA in exerting certain auxin responses (Ludwig-Müller 2000; Ludwig-Müller 2007; Simon and Petrášek 2011). However, given that no IAA-independent biosynthetic pathway has yet been identified for IBA, and the endogenous levels of IBA depend on those of IAA (Simon and Petrášek 2011), it is more likely that IBA serves as a source of IAA. Storing auxins as IBA ensures auxin availability, since IBA conjugates are a more stable storage form of auxins from which IAA can be synthesised via peroxisomal β-oxidation enzymes (Epstein and Ludwig-Müller 1993; Zolman *et al*. 2008; Strader and Bartel 2011). It is suggested, therefore, that analogues of IAA contribute to the maintenance of IAA concentration gradients and homeostasis, which are reported to be the critical factors determining a number of aspects of plant growth and morphogenesis (Kieffer *et al* 2010; Tromas and Perrot-Rechenmann 2010; Simon and Petrášek 2011).

5.2.2 Choosing a cytokinin: a case for natural analogues

Exogenous cytokinins are necessary during *in vitro* organogenesis to promote shoot multiplication (George *et al*. 2008), a key feature of micropropagation. As with the auxins for root production, the choice of cytokinin is based on its shoot-producing efficiency. This is often also clone-specific for eucalypts and consequently, a number of analogues and their concentrations have been reported in micropropagation protocols (Table 1.2).

Impaired auxin response and the resulting difficulty in rooting has been suggested to be, among others, 1) a consequence of defective auxin signal transduction (Oono *et al*. 2003; Li *et al.* 2009); 2) a failure to hydrolyse auxin conjugates to free auxins when necessary (Epstein *et al.* 1993); or 3) due to the presence of auxin antagonists such as cytokinins (Brault and Maldiney 1997). In this study, investigations using GN155 (poor-rooter) revealed that the apparent lack of response to exogenous auxins with

respect to root formation was not due to the inabilities of those clones to transduce the exogenous auxin signal (through the use of PCIB), or to hydrolyse conjugated auxins to free forms (through the use of DHAP). As known antagonists of auxin action (Brault and Maldiney 1999; Moubayidin *et al.* 2009), cytokinins have been reported to impair auxin responses (Pernisová *et al*. 2009). As with auxins, cytokinin analogues can vary in stability, which is dependent on their respective degradation pathways (Mok and Mok 2001, Haberer and Kieber 2002). Those cytokinins that are not targets for the enzyme cytokinin-oxidase (e.g. the synthetic cytokinin kinetin), which rapidly inactivates compounds such as *trans*-zeatin, have been suggested to persist longer in plant tissues than targets of the enzyme (George *et al*. 2008). Having observed the inhibitory effects of persistent auxins on root development (Fig. 2.5), an hypothesis linking persistent cytokinins from the pre-rooting culture stages with a reduction in rooting ability was tested. Gas-chromatography-mass spectrometry (GC-MS) analysis revealed an inverse relationship between shoot kinetin:auxin and rooting ability (Fig. 4.6). This implied that the rooting ability of at least two poor-rooting eucalypts was dependent on kinetin absence from shoots. Even though kinetin was not supplied during *in vitro* rooting, the nature of its metabolism resulted in its persistence, from the multiplication and elongation stages, into the rooting stage. In support of this, was the observation of the significant increase in the rooting ability of the shoots of GN155 and NH58 (both poor-rooters) resulted, when using the less-stable *trans*-zeatin in the pre-rooting culture stages. Under those conditions, together with 0.1mg l^{-1} IAA in the rooting medium of GN155 and NH58, rooting ability of shoots was recorded as 100% (Fig. 4.5).

Since rooting ability is dependent on cytokinin absence from shoots, the use of a stable cytokinin for shoot multiplication may present a long-term disadvantage for root morphogenesis *in vitro*. While the micropropagation of eucalypts via cytokinin-free culture has been previously described for certain clones (Trueman and Richardson 2007), the majority of commercially-important eucalypts require exogenous cytokinin/s for shoot proliferation (e.g. Gomes and Canhoto 2003; de Andrade *et al*. 2006; Aggarwal *et al*. 2010). Being a synthetic cytokinin, kinetin is often favoured for shoot multiplication over its less-stable analogues, since it is not as easily degraded by natural enzymes (George *et al.* 2008). However, if adventitious rooting is the ultimate aim of

vegetative propagation, then any antagonist effect to the rooting process should be eliminated. In the present study, the metabolism of the natural cytokinin *trans*-zeatin, a target for degradation by cytokinin oxidase, was shown to be in synergy with the aims of *in vitro* propagation. While it may not be as stable as kinetin, its metabolism allows for a reduction in antagonism with the auxins, necessary in the latter rooting stages of micropropagation. This has been shown to be especially useful for the two tested poor-rooting eucalypt clones (Figs. 4.7 and 4.8).

5.3 Auxin-cytokinin interactions in the regulation of *in vitro* root development

As antagonists in plant development, auxins and cytokinins regulate each other to direct various aspects of root development, such as root induction, vascular differentiation, maintenance of the root meristem and gravitropism (Aloni *et al*. 2006), all of which were investigated in the present study. Significant interaction occurs between auxins and cytokinins in the regulation of plant development and reports have also suggested that cytokinins may inhibit enzymes that conjugate free IAA (Gaspar et al. 2003; Moubayidin et al. 2009). These interactions direct root architecture and ensure that appropriate and timely developmental events occur, in relation to environmental cues and the needs of the plant (Aloni *et al*. 2006). For example, root tips have been shown to possess the highest concentration of free cytokinins, owing to the expression of *IPT* genes (involved in cytokinin biosynthesis, see Chapter 1) in the root cap (Aloni *et al*. 2006). However, the root tip is also responsible for gravity perception, a process in which polar transport of IAA is inseparable (Chen *et al.* 1999; Philosoph-Hadas *et al.* 2005; Swarup and Bennett 2009). In effecting the gravity response, both cytokinins and auxins are redistributed in the root tip with respect to the gravity vector, working to inhibit growth on the lower root side and promote growth on the upper root side so as to result in the root bending towards the gravity vector (Aloni 2004; Aloni *et al*. 2006). It has also been suggested that the primary signal of gravitropism, i.e. signalling the start

of a gravitropic response, are the cytokinins in the root cap (Aloni *et al*. 2004). This is then followed by polar IAA transport, responsible for effecting and sustaining gravitropism (Swarup and Bennett 2009).

Both auxins and cytokinins are also responsible for *in vitro* vascular development. In support of published reports (Ye 2002; Aloni 2004), polar transport of IAA was shown in this study to be necessary for the induction and differentiation of vascular tissues (Chapter 3). Although cytokinins do not induce vascularisation (Aloni *et al.* 2006), they have been reported to promote vascular differentiation in roots and shoots, in the presence of IAA (Aloni 1995; Aloni *et al*. 2006). The maintenance of the IAA concentration gradients through polar transport is an oft-encountered feature in root development. As discussed earlier, IAA analogues are suggested to contribute to the pool of free IAA and its subsequent homeostasis (Simon and Petrášek 2011). The implication is that for cytokinin-auxin interaction to take place during *in vitro* root development, the auxins and cytokinins chosen for micropropagation protocols should have properties that enable interaction. The abilities of these key phytohormones to be activated or inactivated by oxidation, conjugated or degraded by enzymes at the appropriate points in their respective responses, are critical in their control. A potential imbalance in this auxin-cytokinin homeostatic control of root development may result when more stable analogues are introduced into the cytokinin and auxin pools, as is routinely practiced during micropropagation practices. This may explain the aberrant root development (compared with seedlings) observed in field-trials from micropropagated plants and from some macrocuttings, both rooted using IBA (Mokotedi *et al*. 2010). In order to address this, vegetative propagation protocols for eucalypts, particularly those of micropropagation, need to be modified to accommodate auxins and cytokinins that are capable of interaction and reciprocal regulation.

5.4 Conclusion and future prospects

A suite of *Eucalyptus* genotypes, matched to an array of sites and environmental conditions, are in commercial forestry use worldwide. Within this suite of economically-viable genotypes, there exists a range of responses to the vegetative propagation methods used, with some genotypes more resistant to vegetative propagation than others. Hence, a need often exists to modify propagation protocols to improve plantlet yields. In the commercial environment, foresters have addressed such modifications empirically, often on a clone-specific basis. Frequently, more stable growth regulators are administered where such organogenesis is required, e.g. IBA for adventitious root induction, whether for cuttings in industry or *in vitro* in the preparation of parent material for microcuttings. However, the results of the present work suggest that at least *in vitro*, the most stable growth regulators may not be the best options for root morphogenesis, when considering their respective properties and interactions with various endogenous and exogenous factors.

The roles of several exogenous factors on *in vitro* root development have been previously investigated by a number of authors, with the aim of improving the rootability of various commercially important *Eucalyptus* species. For example, the *in vitro* adventitious rooting potential of shoots has been shown to be dependent on sucrose concentration in *E. sideroxylon* (Cheng *et al*. 1992), on temperature in *E. saligna* and *E. globulus* (Corrêa and Fett-Neto 2004), on several minerals in *E.globulus* (Schwambach *et al*. 2005), and on various auxin types, phenolic compounds and light intensities in *E. saligna* and *E. globulus* (Fogaça and Fett-Neto 2005). The majority of such studies have reported on the effects of exogenous factors on adventitious rooting, but have fallen short of adequately considering the interactions of such exogenous factors on endogenous ones, e.g. PGRs.

In the present investigation, the relationship between endogenous and exogenous auxins and cytokinins in adventitious root development - the critical point in any vegetative propagation programme - was established for one good- and two poor- rooting eucalypt

clones *in vitro*. However, there still exists a need to extend these findings to include a greater number of eucalypt genotypes that exhibit different responses with regard to PGRs. Several authors have reported on genotypic responses to PGRs in the vegetative propagation methods used, and in many cases certain genotypes exhibit recalcitrance to a particular protocol where other genotypes respond well (e.g. Fogaça and Fett-Neto 2005; Nourissier and Monteuuis 2008; Mankessi *et al.* 2009). In the present study, it was found that for a genotype that can easily produce adventitious roots *in vitro*, by virtue of its higher endogenous auxin:cytokinin ratio than a poor-rooting clone, exogenous auxin presents an obstacle to root induction (Fig. 2.1). Similarly, in the tested poor-rooting genotypes, where the endogenous cytokinin:auxin ratio is higher than in a good-rooting genotype, the persistence of exogenous cytokinins from the multiplication and elongation stages *in vitro*, inhibited root induction (Figs. 4.7 and 4.8). In addition, the presented findings that established that the poor-rooting genotypes were able to produce 100% rooting, when the specific PGR requirements for root induction were considered, also suggest that this may be the case for other poor-rooting genotypes. This indicates that many eucalypt genotypes may not necessarily be poor-rooters, but have been exposed to the incorrect PGR treatments with regards to their respective genotypic requirements for root induction *in vitro*. The implication of this is that increased emphasis should be placed on adapting and modifying vegetative propagation protocols to be more sensitive to genotypic requirements. Although this has been attempted empirically, such modifications must be guided by investigations into the actions of, and interactions between PGRs with respect to root induction for several genotypes. In terms of root development following induction *in vitro*, future investigations will need to focus on the association between *in vitro*-supplied PGRs and nutrients, and their influence in shaping root architecture, since a relationship does exist between nutrient availability and root patterning (López-Bucio *et al*. 2003), and auxin distribution patterns and concentration gradients within roots and lateral root formation (Laskowski *et al.* 2008).

From the results of the present study, it was suggested that in both good- and poor-rooting eucalypts, exogenous auxin analogues may contribute to the establishment and maintenance of IAA concentration gradients and IAA homeostasis. As it was also found

that the interactions between IAA and the cytokinin pathway are critical in the regulation of root development, vegetative propagation protocols should aim for the least interference between these and other PGR interactive pathways (Jaillais and Chory 2010). As previously mentioned, however, many clones do not respond to IAA in the rooting stage, which necessitates the use of more stable auxin analogues such as IBA for adventitious rooting. Hence, future studies will need to investigate methods of vegetative propagation wherein an increased responsiveness of shoots to IAA is achieved. Owing to the high level of control afforded by the *in vitro* system, studies conducted in this manner are valuable in identifying the factors that can lead to increased IAA responsiveness or on the influence of PGR interactions and properties on adventitious rooting.

The results obtained may indicate that *in vitro* responses could be used as a predictor of the *ex vitro* rooting requirements of eucalypt cuttings. For example, based on the current observations of the inhibitory effects of stable auxins on root induction and development *in vitro*, preliminary studies in our laboratory have indicated that a possible supra-optimal environment, through the use of IBA-containing rooting enhancers, also inhibits adventitious rooting of eucalypt cuttings in some clones (unpublished). However, more stable exogenous auxins such as IBA continue to be prescribed for root induction of eucalypt cuttings, particularly in poor-rooting clones (Goulart *et al*. 2008; Trueman and Richardson 2008; Borges *et al*. 2011). Hence, future investigations that consider the actions of, and interactions between PGRs will be invaluable in improving plantlet yields for a number of commercially-important species. Such investigations should consider the link between the treatments and environmental conditions that parent material are maintained under, and the endogenous PGR levels in the parent material and in the cuttings sourced from them.

Studies based on the adventitious rooting ability of cuttings with respect to parent plants have primarily focussed on increasing the rooting ability of cuttings through the maintenance of juvenility of the parent material and ensuring that parent material is nutritionally balanced (López *et al*. 2010). This is achieved through intensely-managed programmes in industry (de Assis *et al*. 2004). Some factors that are considered in the

maintenance of optimum conditions for parent plants are planting density, genetic level (i.e. first or second generation material) and level of silvicultural intensity (Pallett and Sale 2004). With regard to sourcing the best cuttings for adventitious rooting ability and plant survival, the approach taken is often empirical. For example, factors such as cutting length (Naidu and Jones 2009), cutting type and genotype (Mankessi *et al.* 2010), and various growth regulators (Goulart *et al.* 2008) have all been reported to influence the adventitious rooting ability of cuttings. While such studies have been useful in addressing adventitious rooting and plant survival on a clone-specific basis, a deeper understanding of the relationship between environmental conditions, treatment of parent material, distribution of endogenous PGRs within parent material, and genotypic and topophysic effects is lacking.

The distribution pattern of PGRs, as a function of the environmental conditions and treatment of parent plants, is an area that should receive substantial attention. Topophysic effects, related to the number and position of nodes of the explants, have been reported to influence morphogenesis in eucalypts (Hung and Trueman 2011). Hence, establishing the distribution gradients of PGRs in shoots, with respect to nodes, apical buds, and the base of the shoot will allow for cuttings to be sourced from regions of suitable auxin content for adventitious rooting. This in turn will provide a more reliable source of information than empirical means, towards improving yields of cuttings. Recently, auxin distribution patterns within *in vitro* shoots of *E. globulus* were investigated, through the distribution patterns of the IAA influx and efflux transporters (Fett-Neto *et al.* 2011). Those authors found that auxin efflux via the PIN carrier was necessary to create an auxin concentration gradient and induce adventitious roots.

The possibility of incorporating cytokinin inhibitors in the rooting stage to eliminate cytokinin inhibition of auxin transport and action also requires consideration. While several compounds with anti-cytokinin activity have been reported in the literature (George *et al.* 2008), the various modes of cytokinin biosynthesis and their complex metabolic pathways (Mok and Mok 2001) will require that these compounds are rigorously tested to identify those that specifically participate in adventitious rooting. This will minimise further interferences between the PGR interactive pathways in other

aspects of plant development, apart from rooting. This future area of research will be helped in no small part by ongoing developments towards our understanding of signal transduction pathways and PGR interactions in plant physiology.

Significant advancements are being made in the area of *Eucalyptus* genomics. With the sequencing of the *E. grandis* genome (Mizrachi *et al*. 2010; Myburg *et al*. 2011), it has become possible, through careful manipulations of environmental conditions, to identify and isolate genes that are expressed in response to PGRs and the environment in which plants are maintained. Such investigations will reveal the factors that influence and regulate adventitious rooting gene expression. In turn, future studies in this field will provide an understanding of the relationship between endogenous and exogenous PGRs, the genes that are up-regulated or down-regulated in response to their perception, and the basis behind the various genotypic responses to PGRs.

In addition to fundamental research, the common objective of the present study and future work in the area of plant physiology and biotechnology is to improve genotypes, enhance methods of propagating them, and elevate yields of not only *Eucalyptus*, but also other commercially-important forestry species. This is necessary for the forestry industry to secure a sustainable, high-yielding and reliable source of planting material to meet the growing global demands for timber, while minimising the pressures on natural forests and its associated biodiversity.

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APPENDIX 1: SAMPLE OF GC-DERIVED CHROMATOGRAM