Survival and rooting of selected vegetatively propagated *Eucalyptus* clones in relation to supplied auxin

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As the candidate's supervisors we have approved this dissertation for submission.

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ABSTRACT

Eucalyptus spp. and hybrids dominate the global plantation forestry industry, and vegetative propagation through cuttings is the preferred method for their commercial use. However, the cuttings of some species and hybrids show recalcitrance to rooting. The first aim of this study was to improve percentage rooting of three clones of *E. grandis* x *E. nitens* (Clones 1, 2 and 3) identified by a commercial nursery as having variable rooting abilities. The second was to relate their rooting responses as cuttings to their rooting responses *in vitro*. Minicuttings (3.5 – 4 cm in length) (hereafter referred to as cuttings) were subjected to commercial nursery propagation practices. Initial results revealed that in the absence of exogenous plant growth regulators (PGRs), soft (juvenile, thin diameter) cuttings survived (87 – 95%) and rooted (29 – 32%) significantly better than hard (mature, thick diameter) ones (62 – 71% survival and 2 – 8% rooting). This validated the use of soft cuttings by the nursery and all subsequent studies were conducted with soft cuttings. The other nursery practice of applying the commercial rooting powder Seradix 2 (3 g kg^{-1} indole-3-butyric acid [IBA]) adversely affected the survival and subsequent rooting of cuttings of Clones 1 and 2. Ensuing studies investigated: 1) the effect of mode of IBA application (powder vs. liquid); 2) concentrations of Seradix (0, 0.5, 1, 2 and 3 g kg^{-1} IBA), applied at initial placement of cuttings and two weeks later; and 3) the influence of season on the survival and subsequent rooting of cuttings. Results showed that regardless of the mode of application, IBA significantly reduced percentage survival and rooting in cuttings of Clones 1 and 2. The delayed application of Seradix, two weeks after cuttings were initially set, resulted in a higher percentage survival and rooting than when cuttings were supplied with Seradix at initial placement. Nevertheless, the best survival for Clones 1, 2 and 3 (95%, 99% and 71%, respectively) and rooting (83%, 64% and 47%, respectively) occurred in the absence of Seradix. In addition, the survival and rooting of cuttings were seasonally variable, with particularly low rooting during winter (e.g. for Clone 1, 32%) when compared with summer (e.g. for Clone 1, 83%).

Shoots from all the clones were multiplied *in vitro*, followed by elongation on either of two media (E1= kinetin, α-naphthalene acetic acid [NAA] and IBA; E2 = kinetin and indole-3-acetic acid [IAA]), and then rooting on 0, 0.1 or 1.0 mg l^{-1} IBA. The latter were selected to typify the range of Seradix concentrations used for the cuttings (i.e. no IBA, low and high IBA concentrations). For all

three clones, shoots elongated on E1 or E2 displayed high survival (> 80%) but failed to root without IBA in the rooting medium. For Clones 1, 2 and 3 the best *in vitro* survival (80%, 100% and 100%, respectively) and rooting (40%, 75% and 40%, respectively) occurred when shoots were elongated on E2 and rooted on 0.1 mg l^{-1} IBA. However, 1.0 mg l^{-1} IBA in the rooting medium severely inhibited survival (0 – 50%), irrespective of the clone or the elongation treatment used.

Overall, cuttings demonstrated the best survival and rooting in the absence of exogenous IBA, which suggested that sufficient endogenous auxin was present within the shoots for successful root induction. The application of exogenous IBA may have disrupted the cuttings' endogenous PGR balance resulting in an inhibition of survival and rooting. *In vitro* shoots required a low concentration of IBA (0.1 mg l^{-1}) in order to counteract the antagonistic effect of cytokinins that were supplied during the multiplication and elongation culture stages, and promote rhizogenesis. Essentially, both cuttings and *in vitro* shoots demonstrated adverse survival and rooting responses when subjected to excessively high IBA concentrations.

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Westville Campus and Sunshine Seedlings Services nursery, from March 2011 to September 2012, under the supervision of Professor Paula Watt (UKZN), Dr Oscar Mokotedi (CSIR) and Dr Muhammad Nakhooda (UKZN).

These studies represent original work by the author and have not otherwise been submitted in any form for any degree to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

DECLARATION

I, Natasha Rambaran declare that:

1. The research reported in this thesis, except where otherwise indicated, is my original research.
2. This thesis has not been submitted for any degree or examination at any other university.
3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from those persons.
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2,4-D	2,4-dichlorophenoxyacetic acid
BAP	benzylaminopurine
GA_3	gibberellic acid
GA_4	isomer of gibberellic acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
MS	Murashige and Skoog (1962)
NAA	α-naphthalene acetic acid
PEG	polyethylene glycol
PEMs	proembryogenic masses
PGR	plant growth regulator

1. INTRODUCTION

Eucalyptus, belonging to the family Myrtaceae, is a commercially important genus with a wide variety of uses in the timber, pulp and pharmaceutical industries (Turnbull 1999; Moyo et al. 2011). Although indigenous to Australia and its northern neighbouring islands, *Eucalyptus* plantations now occur in more than 90 countries (Eldridge et al. 1994; Turnbull 1999; da Silva et al. 2010; Rejmánek and Richardson 2011). Due to their fast growth rate, short rotation time and high adaptability to a wide range of environments, *Eucalyptus* species and hybrids have become the most commonly planted hardwood in the world (Campinhos 1999; Boreham and Pallet 2009; Paine et al. 2011).

Eucalyptus plantations were introduced in South Africa in the late nineteenth century to meet the growing timber demands which exceeded the availability from indigenous forests (Wise et al. 2011). The South African forestry industry has since become dependent on the fast growing, low cost wood supplied by various *Eucalyptus* species and hybrids for pulpwood production with smaller amounts used for mining timber and sawlogs (Boreham and Pallett 2009). *Eucalyptus* plantations are grown primarily along the eastern seaboard of South Africa between latitudes 23 °S and 32 °S across a wide range of site conditions (Pallett and Sale 2004). In addition to contributing significantly to the South African economy, the forestry industry provides employment for almost 170 000 people (Godsmark 2010).

Traditionally, eucalypts are propagated by seed. However, plantations established from seeds are characterised by a large variation in growth, form and vigour, traits which are undesirable for commercial forestry (Ahuja 1993; Eldridge et al. 1994). One strategy for improving the productivity of the forestry sector is tree breeding with emphasis on hybridization (Bayley and Blakeway 2002; Titon et al. 2003; Boreham and Pallett 2009). In addition to improved growth characteristics, hybrid combinations of superior genotypes may possess drought, frost, pest and disease tolerance, thereby enabling them to exist on more marginal sites (Denison and Kietzka 1993; Eldridge et al. 1994; Campinhos et al. 1998). Once superior genotypes are produced it is essential that efficient propagation strategies exist to exploit the genetic gain (Watt et al. 1995; Wilson 1998a; Naidu and Jones 2009). Vegetative propagation of superior selected genotypes via macro-, mini- or

microcuttings enables the rapid deployment of 'true-to-type' clones (Hartmann et al. 1997; de Assis et al. 2004; Brondani et al. 2010; Borges et al. 2011).

Since its introduction to South Africa, *Eucalyptus grandis* has been hybridized with *E. camaldulensis, E. urophylla, E. macarthurii, E. nitens* and *E. dunnii* amongst others (Dension and Kietzka 1993; Little et al. 2003). In addition to being cold tolerant, *E. grandis* x *E. nitens* displays good wood qualities ideal for the pulp industry (Dension and Kietzka 1993; Bandyopadhyay et al. 1999). However, *E. grandis* x *E. nitens* hybrids are difficult to propagate vegetatively due to their inability to initiate and develop a good quality root system (Mokotedi et al. 2000; Boreham and Pallett 2009; Naidu and Jones 2009; Mokotedi et al. 2010). Adventitious root formation is an extremely complex process consisting of various phases with each phase having different requirements, specific to a particular species or hybrid (de Klerk et al. 1999; Fogaça and Fett-Neto 2005; Li et al. 2009). The role of auxins as a key determinant in the process of adventitious root formation has been well established (Zaerr and Mapes 1982; Blakesley 1994; Blythe et al. 2007). The discovery that exogenously supplied auxin could potentially induce root formation in cuttings significantly improved plant propagation activities (Blythe et al. 2007; Pop et al. 2011 and references therein). However, as in the case of *E. grandis* x *E. nitens* clones used in this study, the standard nursery practice of supplying cuttings with exogenous auxin did not result in sufficient numbers of rooted cuttings. If desirable *Eucalyptus* genotypes display difficulty rooting as cuttings, *in vitro* techniques may be employed with greater success (Mokotedi et al. 2000; Yasodha et al. 2004; George et al. 2008).

In vitro culture methods for a number of commercially important *Eucalyptus* species and hybrids are well established (le Roux and van Staden 1991a; Watt et al. 2003; Aggarwal et al. 2010; Hung and Trueman 2011) and the various applications include the mass production of selected genotypes, those that have proven difficult to propagate via conventional methods, bulking up of hybrid genotypes and the rejuvenation of physiologically old plant material (Delaporte and Sedgley 2004; Nehra et al. 2005; Brondani et al. 2012a). In addition, *in vitro* tissue culture provides a good system to investigate the relationship between plant growth regulators (PGRs) and their effect on root induction and development under controlled conditions. Consequently, the results derived from the performance of *Eucalyptus* genotypes *in vitro* could possibly be used to improve the propagation efficiency of cuttings.

Hence, the opportunity exists to optimise standard nursery practices by investigating factors which may influence the survival and rooting of cuttings with emphasis on the relationship between supplied auxin and rooting. Consequently, the aims of this study are to: 1) improve the rooting of cuttings of three *E. grandis* x *E. nitens* clones with variable rooting abilities, 2) to investigate their rooting responses *in vitro* and 3) to determine whether these clones displayed similar rooting responses *in vitro* as when propagated via cuttings, thereby providing some elucidation on their PGR requirements.

2. LITERATURE REVIEW

2.1 The history and importance of *Eucalyptus*

As the global demand for forestry products increases and suitable land available for plantations decreases, ensuring the sustainability of the forestry industry has never been more crucial. Indigenous to Australia and the neighbouring islands of New Guinea, Philippines and West Timor, *Eucalyptus* was first described by the French botanist Charles Louis L'Heritier De Brutelle in the late eighteenth century (Eldridge et al. 1994). With more than 800 species, eucalypts are a diverse and versatile genus (Campinhos 1999; da Silva et al. 2010; Rejmánek and Richardson 2011). Initially, they were merely considered botanical curiosities and were not recognised for their potential as commercial forestry species (Turnbull 1999). Within the past few decades they have emerged as the most widely planted hardwood genus in the world, totalling over 20 million hectares (da Silva et al. 2010; Rejmánek and Richardson 2011). As previously mentioned, in addition to being a major source of pulp for high quality paper production, eucalypts also provide timber, firewood, charcoal, essential oils and honey amongst other uses (Turnbull 1999). The fast growth rate, short-rotation time and the ability to adapt to a range of environmental conditions are some of the desirable characteristics which have resulted in *Eucalyptus* species and their hybrids dominating the global forestry industry (Campinhos 1999). Their distribution range extends from the hot, humid tropical lowlands to the cool, temperate highlands (Turnbull 1999).

Major industrial plantations of eucalypts exist in Brazil, China, Spain, India, Portugal and South Africa. Although Brazil dominates the global eucalypt industry (over 4 million hectares), the South African industry (over 500 000 hectares) exemplifies the economic benefits that can occur as a result of well managed plantations (Turnbull 1999; Morris 2008; da Silva et al. 2010). According to the latest available forestry report, eucalypts account for 40.4% of the 1.2 million hectares of afforested land in South Africa (Godsmark 2010). In addition to contributing R 6.7 billion to the economy, the forestry industry provides employment for almost 170 000 people (Godsmark 2010). However, reports of negative impacts on soil water, soil nutrients, soil erosion and biodiversity are some of the controversial issues affecting the proliferation of eucalypt plantations (Turnbull 1999; Liu and Li 2010). Although such concerns may be justified, the competitive advantage of eucalypts as a forestry species cannot be easily dismissed.

2.2 Sexual propagation of *Eucalyptus*

In natural eucalypt forests, propagation occurs from seeds which are then collected and used to establish commercial plantations (McComb and Bennett 1986; Eldridge et al. 1994). Consequently, most of those plantations are characterized by a large variation in growth, form and vigour (Ahuja 1993; Eldridge et al. 1994). Many species of eucalypts, within subgeneric groups, have the ability to hybridise naturally (Griffin et al. 1988). Hybridisation has therefore become an integral part of tree improvement programmes as the combination of desirable genetic characteristics from two species can produce superior eucalypt genotypes which can exist on marginal sites (Denison and Kietzka 1993; Eldridge et al. 1994; Campinhos et al. 1998). As a result of seed heterozygosity, most hybrids experience difficulty producing sufficient quantities of seeds due to irregular flowering and high abortion (Denison and Kietzka 1993; Potts and Dungey 2004). Hence, to meet the increasing demands for forestry products, new approaches to propagate eucalypts rapidly were required.

2.3 Vegetative propagation of *Eucalyptus* via cuttings

Asexual (vegetative) propagation produces individuals genetically identical to the parent plant (i.e. clones). Using vegetative propagation, superior genotypes selectively bred through tree improvement programmes or identified from natural populations and then screened for traits of interest, can be rapidly propagated on a commercial scale (Hartmann et al. 1997). Conventional vegetative propagation techniques include cuttings, grafting and air layering (Eldridge et al. 1994). As the latter two are expensive, labour intensive and not commercially viable, cuttings are the preferred method for conventional vegetative propagation of eucalypts (Cresswell and de Fossard 1974; McComb and Bennett 1986). With the rapid development of biotechnology in recent years, innovative techniques to propagate elite trees vegetatively have become available. Amongst them, *in vitro* tissue culture exploits the property of totipotency, which is the inherent ability of every plant cell exposed to the correct stimuli and environmental conditions, to potentially regenerate into a complete plant (Kleinschmit et al. 1993; Hartmann et al. 1997). As discussed later in Section 2.4, in cases where desirable *Eucalyptus* genotypes display an inability to root as cuttings, *in vitro* techniques may be employed with greater success (Yasodha et al. 1997).

Clonal forestry presents several advantages over seed propagated plantations, the most significant being the uniformity of trees produced (Zobel 1993). Trees displaying uniformity in terms of growth characteristics and wood properties enable cost effective harvesting and enhanced product quality (MacRae and Cotterill 1997). *Eucalyptus* species and hybrids exhibit a strong Genotype x

Environment interaction which enables matching clones to sites previously considered marginal (Denison and Kietzka 1993; de Assis et al. 2004). Since its inception, large scale clonal programs have contributed significantly to increasing eucalypt biomass production worldwide. The achievements made by the clonal forestry programs in Brazil and the Peoples' Republic of Congo with various eucalypt species have become legendary with many countries developing clonal programs based on those successes (Ritchie 1994). In South Africa, Mondi Business Paper modelled their clonal programme on the Aracruz operation but with modified procedures, specific to the local context (Denison and Kietzka 1993). A major benefit of clonal forestry is the potential for site-specific management which is especially important in South Africa due to the diverse range of climates in which eucalypt plantations occur (Morris 2008). In the cooler temperate regions, *E. grandis* x *E. nitens* dominate as the superior growth rate of *E. grandis* is combined with the cold tolerance of *E. nitens* (Morris 2008). In subtropical regions, *E. grandis* has been combined with the superior disease tolerance of either *E. urophylla* or *E. camaldulensis* (Denison and Kietzka 1993; Morris 2008). In low rainfall regions *E. dunnii* and *E. smithii* are grown as they are capable of high drought tolerance (Morris 2008).

Cuttings are a section of stem, root or leaf from a parent plant that are placed under environmental conditions conducive to the development of roots and shoots, thus producing new, independent plants which are genetically identical to the parent plant (Hartmann et al. 1997; George et al. 2008). The propagation of eucalypts from rooted cuttings (macrocuttings) reached a commercial scale in the 1980s (de Assis et al. 2004). Following that approach, trees displaying desirable characteristics are identified within field plantations. These trees are then felled and the resprouting coppice used as rooted cuttings (macrocuttings) (Eldridge et al. 1994). This practice requires reserving large areas of plantations and good quality trees annually, solely for the purpose of obtaining material for cuttings. In the competitive Forestry Industry this wastage of resources is not ideal. Consequently, this system was refined by the introduction of clonal hedges which serve the purpose of parent plants by providing a constant source of cutting material (de Assis et al. 2004). Clonal hedges or gardens are traditionally initiated by rooting stem cuttings and are commonly employed by the forestry industry to provide macrocuttings, which are usually 8 – 10 cm long with basal diameters between 2 – 5 mm (Stape et al. 2001). However, vegetative propagation via macrocuttings is limited to only a small number of *Eucalyptus* species and hybrids. This is mainly because macrocuttings display variable rooting abilities, which is often attributed to the gradual decrease of rooting potential with the aging of parent plants (MacRae and Cotterill 1997, Wilson 1998a). Consequently,

it became necessary to investigate alternative methods such as *in vitro* cultures systems to support clonal forestry programmes (Cunha et al. 2009b).

One of the major advantages associated with *in vitro* culture systems is the ability to administer and replenish nutrients and carbohydrates to rejuvenate mature plants (Hartmann et al. 1997). These rejuvenated plants are then acclimatised and used to establish micro-clonal hedges (refer to Section 2.3.1). Cuttings obtained from parent plants propagated via micropropagation are referred to as "microcuttings" and are usually 2 – 3 cm long with a basal diameter of 0.4 – 1 mm (Stape et al. 2001; de Assis et al. 2004). Since rooting potential reaches a maximum at highest juvenility stage, microcuttings obtained from rejuvenated parent plants demonstrate greater rooting percentages than macrocuttings (de Assis et al. 2004). The recognition of the relationship between juvenility and rooting was the reason for the commercial introduction of minicuttings. Like microcuttings, minicuttings are usually 2 – 3 cm long with a basal diameter of 0.4 – 1 mm but are obtained from mini-clonal hedges (see Section 2.3.1) which are essentially smaller, more juvenile clonal hedges (Stape et al. 2001).

As cuttings are relatively fragile, a hospitable, supportive environment and intensive management regime are essential to avoid their mortality (Wilson 1998a). Factors influencing survival and rooting of cuttings include the genetic predisposition of the species or hybrids to form roots, the physiological status and age of the parent plant, the application of 'rooting hormones' and the climatic conditions of the propagation environment (Leakey et al. 1992; Hartmann et al. 1997). The combination of endogenous and exogenous factors contributes significantly to either the success or failure of a propagule to initiate roots.

2.3.1 Parent Plants

Although the traditional clonal hedge system enables the mass production of propagules, being outdoors means they are highly affected by the weather, particularly difficult to control and consequently, they require intensive labour and management (de Assis et al. 2004). The introduction of micro- and minicuttings necessitated the use of micro- and mini-clonal hedges to serve as parent plants. Micro- and mini-clonal hedges, being significantly smaller than clonal hedges, enabled super-intensive management, which resulted in technical and economic benefits in addition to improved rooting in cuttings (Saya et al. 2008). In addition, they alleviated problems associated with outdoor clonal hedges such as poor nutritional status, reduced photosynthetic rates

and leaf diseases (de Assis et al. 2004). The newer micro- and mini-clonal hedge systems include indoor hydroponics. Sand beds with drip irrigation or intermittent flooding are currently the two most widely used hydroponic systems in Brazil since their major advantage is the maintenance of an optimal nutritional status of the parent plants, which is a key contributor to promoting rooting in cuttings (de Assis et al. 2004; de Assis 2011).

All plants progress through a series of developmental stages known as phase changes (Taiz and Zeiger 2004). Plant development is characterised by three phases comprising of a juvenile, transitional and adult (mature) phase (Hartmann et al. 1997). The developmental process from juvenile to maturity is known as ontogenetic aging and is distinctly different from chronological or physiological aging (Scianna 1998). In woody plants, maturation is associated with decreased growth rates, a greater tendency for plagiotropism, changes in reproductive competence and differences in stem and foliage characteristics when compared to their more juvenile counterparts (Ritchie 1994; Leakey 2004). In addition to the morphological changes associated with maturation, there are several physiological, anatomical and biochemical changes accompanying the process (Hartmann et al. 1997). The diminishing rooting ability of cuttings from mature parent plants has been well established (Leakey et al. 1992; Maile and Nieuwenhuis 1996; de Assis et al. 2004; Mankessi et al. 2011). Possible explanations for this include the accumulation of rooting inhibitors, the reduced endogenous concentration of rooting hormone promoters and the decreased sensitivity of tissues to exogenous applications of rooting hormones (Greenwood and Hutchinson 1993; Hartmann et al. 1997). Hence, to ensure high rooting percentages, parent plants need to be maintained in the juvenile phase. As discussed above, although the loss of juvenility in parent plants can cause a severe decline in rooting ability of the cuttings, there are numerous factors responsible for variation in rooting ability and these all need to be investigated before any conclusions are drawn.

The nutritional status of the parent plants is another key factor determining the ability of the cuttings to form roots (Cunha et al. 2009b). The nutritional status of a parent plant influences the amount of carbohydrates, auxins and metabolic compounds, which are all necessary for successful root development in cuttings (Hartmann et al. 1997). Mineral nutrients have specific functions in plant metabolism which include functioning as constituents of organic structures, as activators of enzymatic reactions or as charge carriers and osmoregulators (Li et al. 2009). Macro- and micro-nutrients involved in the metabolic processes associated with differentiation and formation of the

root system are considered essential for the development of good quality roots (Taiz and Zeiger 2004). Generally, parent plants should be well nourished with macronutrients such as phosphorus, potassium, calcium, magnesium and should be moderately deficient in nitrogen to promote rooting in cuttings (Hartmann et al. 1997). However, Rosa et al. (2009) established a direct relationship between increased nitrogen concentration and the number of potential cuttings produced by *E. dunnii* parent plants. Since nitrogen is either directly or indirectly involved in all metabolic pathways, an increased nitrogen concentration should promote greater photosynthetic activity which enables a higher biomass production (Taiz and Zeiger 2004). Cunha et al. (2009b) established that endogenous nitrogen content is negatively correlated with the rooting of *E. urophylla* cuttings and positively correlated with the rooting of *E. grandis* cuttings.

The supply of a high concentration of calcium (300 mg l^{-1}) during the induction phase increased the root number in *E. globulus* shoots (Schwambach et al. 2005) which, according to Bellamine et al. (1998), could be due to the involvement of calcium in cell division and the transport of auxin. However, Cunha et al. (2009c) determined that endogenous calcium concentrations of 700 mg l^{-1} affected rooting negatively in a number of *E. grandis, E. urophylla* and *E. grandis* x *E. urophylla* clones. Supplied concentrations of both phosphorus and potassium < 760 mg l^{-1} reduced the root length of *E. globulus* cuttings (Schwambach et al. 2005). In contrast, cuttings from an *E. grandis* hybrid displayed increased rooting with increasing supply of phosphorus (Cunha et al. 2009c).

The literature reveals conflicting results with regards to the optimal range of the various nutrients supplied to achieve rooting of *Eucalyptus* cuttings (Dell and Wilson 1985; Arezki et al. 2001, Schwambach et al. 2005; Trueman et al. 2012). As the optimum concentration of the various mineral nutrients required for adventitious root formation in cuttings varies with the species or clone, age and management practice, constant nutritional monitoring of the parent plants is required (Cunha et al. 2009b; Neto et al. 2011). It is imperative to develop a nutrient regime capable of providing all the essential nutrients to the parent plants for subsequent production of cuttings capable of rhizogenesis (Cliffe 2006; Neto et al. 2011).

The light and temperature conditions to which the parent plants are exposed can be manipulated to ensure maximum rooting of cuttings are achieved (Rosa et al. 2009). Light and temperature can influence the uptake and metabolism of nutrients and hormones, while light intensity significantly influences the concentration of endogenous phenolic substances. These, in turn, depending on the

species involved, can act as either rooting promoters or inhibitors (Hartmann et al. 1997; de Assis et al. 2004). Generally, reducing irradiance can result in anatomical changes such as decreased lignification and cell differentiation which promote the initiation of roots (Hartmann et al. 1997). However, a recent study determined that increasing radiation had a positive effect on the rooting of a variety of eucalypt species and hybrids (Cunha et al. 2009a).

The quality and quantity of light available to the parent plant directly affects photosynthetic activity whose products are essential for the development of roots (Taiz and Zeiger 2004). The carbohydrate content of the parent plants must be sufficient to supply the cuttings with the energy required to initiate roots (Veierskov 1988). In addition to carbohydrates being the energy source for plant tissues, they also have an important regulatory role in rooting (Corrêa et al. 2005). For example, glucose is an important signalling molecule for abscisic acid and ethylene which in turn directly influence auxin, the primary rooting PGR (León and Sheen 2003).

The temperature to which parent plants are subjected can potentially affect the quality and quantity of cuttings produced, as well as the subsequent root formation of cuttings (Trueman et al. 2012). High temperatures (28 – 33°C) increased the quantity of cuttings produced from *E. citriodora* and *E. dunnii* parent plants. According to Cliffe (2006) a number of Brazilian and Uruguayan nurseries producing eucalypt cuttings negated the use of rooting hormones by optimising environmental conditions and employing intensive parent plant management. In this way, rooting percentages of 85 – 95% were often achieved.

2.3.2 Selection of cuttings

Cuttings taken sequentially down a stem have variable rooting abilities due to chronological, morphological and physiological differences at different positions of the stem (topophysis) (Leakey et al. 1992). Consequently, intra-clonal differences in both growth and rooting potential are related to the effects of topophysis since different parts of the stem can differ with regards to their water, carbohydrate, nutrient and hormone concentrations (Leakey et al. 1992; de Assis et al. 2004). Reuveni et al. (1990) established that thick, non-lignified *E. camaldulensis* macrocuttings rooted better than thin macrocuttings and those authors recommended the use of cuttings with a diameter greater than 3 mm. Maile and Nieuwenhuis (1996) determined that hard, woody cuttings of *E. nitens* had a greater rooting percentage than soft ones and attributed that to thicker cuttings having greater carbohydrate reserves, which are required to promote rooting. However, Wilson (1993)

reported that although the survival of *E. globulus* macrocuttings improved with increasing cutting length and woodiness of the stem, the rooting ability decreased slightly. In a later study, Wilson (1999) determined that as the diameter size of *E. globulus* macrocuttings increased, both their survival and rooting decreased. However, this trend was not observed in *E. grandis* macrocuttings (Wilson 1994a), leading that author to later postulate that stem volume, rather than cutting length and diameter, was directly related to rooting potential (Wilson 1994b). Naidu and Jones (2009) determined that the length of *E. grandis* x *E. urophylla* cuttings obtained from conventional hedge banks significantly affected rooting percentage and growth, and recommended that for optimal operational production should be between 8 – 10 cm. Figueiredo et al. (2011) established that longer, thicker cuttings of *E. grandis* x *E. urophylla* displayed higher initial growth but the differences tended to disappear over time.

Since auxin is synthesized in regions of active growth and young leaves, apical cuttings may contain more endogenous auxin available for root induction, thereby enabling apical cuttings to survive and root better than axillary cuttings (Blakesley et al. 1991; Blythe et al. 2007, Borges et al. 2011). A recent study by Borges et al. (2011) showed that apical *E. grandis* x *E. globulus* minicuttings were superior to non-apical cuttings with regards to survival (80% compared with 40%) and rooting (90% compared with 65%) abilities.

2.3.3 The role of auxins in adventitious root formation

The success of vegetative propagation via cuttings is determined by adventitious root induction and development. Root formation is a complex process consisting of three physiological phases, each having different requirements (de Klerk et al. 1999; Li et al. 2009). The induction phase of rooting describes the initial period of molecular and biochemical events preceding any morphological changes in the cutting (Li et al. 2009). During the initiation phase, cell division and root primordial organisation occur while the expression phase is characterised by the emergence and growth of the roots (Li et al. 2009). Each phase needs to progress optimally to facilitate the development of a good quality root system (Bellamine et al. 1998).

It is well established that auxins are a key determinant in the process of adventitious root formation (Zaerr and Mapes 1982; Blythe et al. 2007). In addition, auxins are responsible for a wide array of developmental responses and processes such as embryo patterning, vascular differentiation, root and shoot patterning and tropisms (Alabadí et al. 2009). Endogenous auxin is synthesized in shoot

apices and young leaves and then transported to other tissues to coordinate growth and facilitate responses to environmental stimuli (Pop et al. 2011). Although indole-3-acetic acid (IAA) is generally the most abundant endogenous auxin in plants, indole-3-butyric acid (IBA) and naphthalene acetic acid (NAA) are even more effective than IAA in promoting rooting (Blazich 1988; Li et al. 2009; Pop et al. 2011). This could be related to greater stability, longer persistence in tissues, as well as differences in metabolism and transport (Blazich 1988; Li et al. 2009; Pop et al. 2011).

The interdependent physiological stages of adventitious root formation are associated with changes in endogenous auxin concentrations (Blakesley et al. 1991). The discovery that exogenously applied auxin can potentially induce root formation in cuttings improved plant propagation activities in many species including eucalypts (Blythe et al. 2007). Exogenously applied auxins enter through the base of the cutting and are rapidly taken up by pH trapping and by influx carriers (Rubery and Sheldrake 1973). Upon entering the system auxins are converted either via oxidation or conjugation (Woodward and Bartel 2005). While NAA does not undergo oxidation, IAA and IBA may be inactivated irreversibly by oxidation (Woodward and Bartel 2005).

The supply of exogenous auxin can improve rooting percentages as well as the speed, quality and uniformity of the roots produced (de Assis et al. 2004). The type, concentration, form and time of auxin application varies with the species involved, the type of cutting selected and their age. The type of auxin required to promote rooting of a particular species depends on the affinity for the auxin receptor protein involved in rooting, the concentration of free auxin that is able to reach competent cells, the amount of endogenous auxin and its metabolic stability (de Klerk et al. 1999; Gaspar et al. 2003; Pop et al. 2011). de Almeida et al. (2007) determined that *E. cloeziana* cuttings responded to IBA and not NAA. Similarly, with *E. grandis* x *E. urophylla* cuttings, the application of IBA was more efficient at promoting rooting than NAA (Goulart et al. 2008). Indole-3-butyric acid is considered the most efficient auxin for root initiation and it is therefore the standard active ingredient in commercial rooting substances. For the commercial application of IBA, there are three common delivery methods utilised, *viz.* the basal quick-dip (concentrated-solution dip), dilute soak (dilute-solution soaking) and powder (talc) (Blythe et al. 2007). Lana et al. (2008) determined that there were no significant differences between powder and paste applications of IBA to *E. globulus* cuttings. de Almeida et al. (2007) demonstrated that applying IBA either via liquid or paste forms produced similar rooting results for *E. cloeziana* cuttings.

The optimum concentration of each auxin is dependent on the species and type of cutting (Lana et al. 2008, Titon et al. 2003), as illustrated in Table 1. As mentioned previously, the maturation status of the parent plant can negatively affect the rooting ability of cuttings. However, de Almeida et al. (2007) showed that by increasing the concentration of IBA applied to *E. cloeziana* macrocuttings taken from 15 year old trees, increased survival and rooting percentages were obtained. In contrast, macrocuttings taken from 5 year old trees displayed higher survival and rooting percentages in the absence of IBA. In some cases cuttings taken from mature trees failed to root even with the application of exogenous auxin (Reuveni et al. 1990; Maile and Nieuwenhuis 1996).

According to Wilson (1994a), 6000 – 8000 mg l^{-1} IBA promotes optimal rooting in *E. grandis* stem cuttings. However, Lana et al. (2008) determined that as IBA concentration increased, growth and rooting of *E. globulus* cuttings decreased. The optimum concentration was 2000 mg l^{-1} IBA while 8000 mg l^{-1} IBA decreased the biomass of the roots suggesting that the high IBA dosage became toxic to the cuttings (Lana et al. 2008). Further evidence for the toxic effect of a high concentration (8000 mg l^{-1}) of IBA on rooting ability was observed in *E. nitens* macrocuttings (Maile and Nieuwenhuis 1996).

Minicuttings, being more juvenile than conventional macrocuttings, may possess sufficient endogenous auxin required to initiate the rooting process; in that case, an application of exogenous auxin may introduce excessive and toxic concentrations of that auxin, thus inhibiting rooting and reducing the survival of cuttings (Blythe et al. 2007). The rooting percentages of *E. grandis* minicuttings did not increase with IBA application (Table 1), suggesting that the cuttings had sufficient endogenous auxin for root initiation (Wendling and Xavier 2005). Further, IBA concentrations above 500 mg l^{-1} became toxic to the cuttings (Wendling and Xavier 2005) and similar trends were observed for *E. globulus* hybrid cuttings (Borges et al. 2011)

Table 1 Examples of published effects of varying concentrations of auxins (mg l^{-1}) on percentage rooting in selected *Eucalyptus* species and hybrids.

Species	Type of Cutting (macro, mini or micro)	Auxin	Result (% rooting)	Reference
E. nitens	Macrocuttings	None	66.7	Maile and Nieuwenhuis (1996)
		IBA [8000]	23.3	
E. grandis	Microcuttings	None	90	Titon et al. (2003)
		IBA [1000]	90	
		IBA [2000]	88	
		IBA [4000]	70	
E. grandis	Minicuttings	None	78	
		IBA [1000]	80	
		IBA [2000]	60	
		IBA [4000]	30	
E. grandis	Minicuttings	None	83	Wendling and Xavier (2005)
		IBA [500]	81	
		IBA [1500]	74	
		IBA [3000]	80	
E. cloeziana	Macrocuttings (juvenile)	None	82.5	de Almeida et al. (2007)
	(from 5 year old trees)	IBA [6000]	65	
E. cloeziana	Macrocuttings (mature)	None	20	
	(from 15 year old trees)	IBA [1500]	40	
		IBA [3000]	10	
		IBA [6000]	82.5	
E. cloeziana	Minicuttings	None	81	de Almeida et al. (2007)
		IBA [1500]	82.5	
		IBA [3000]	81	
		IBA [6000]	80	
E. cloeziana	Minicuttings	None	82.5	
		NAA [3000]	80	
		NAA [6000]	83	
E. grandis x *E. urophylla*	Minicuttings	None	93	Goulart et al. (2008)
		IBA [500]	89	
		IBA [1000]	85	
		IBA [2000]	77	
		IBA [4000]	61	
E. benthamii x *E. dunnii*	Minicuttings	None	40	Brondani et al. (2010)
		IBA [2000]	43	
		IBA [4000]	47	
		IBA [6000]	57	
		IBA [8000]	50	

A study conducted by Titon et al. (2003) compared survival and rooting predisposition between *E. grandis* cuttings obtained from macro- and micropropagated parent plants. The results indicated that for microcuttings, the application of exogenous IBA was not required for root initiation but for minicuttings, maximum rooting was obtained with the application of 1000 – 2000 mg l^{-1} IBA. In the presence of 4000 mg l^{-1} IBA, microcutting survival (90%) and rooting (90%) were reduced to 65% and 75%, respectively. Similarly, the survival (80%) and rooting (90%) of minicuttings were both reduced to 30%.

Within *Eucalyptus* there exists both easy-to-root and difficult-to-root (recalcitrant to rooting) species and clones and several explanations have been hypothesised to account for this. Ford et al. (2001) postulated that compared with easy-to-root species, difficult-to-root species may exhibit either 1) a lower rate of basipetal auxin transport or that auxin is metabolised faster thereby reducing basal free auxin, or 2) they may possess a greater concentration of rooting inhibitors or cells with reduced sensitivity to auxin or even less competent for re-differentiation. These speculations could account for the observed differences between easy-to-root clones of *E. cloeziana* cuttings, which responded positively to low IBA concentrations while difficult-to-root clones required a higher IBA concentration to induce rooting (de Almeida et al. 2007).

The endogenous levels of auxins change with the different phases of rooting (Pop et al. 2011). In various plant species, a high auxin concentration is required to promote adventitious rooting only during the induction phase while a high auxin concentration during the initiation and expression phase inhibits further root development (de Klerk et al. 1999). Since each phase of the rooting process has its own particular hormone requirements, an auxin concentration that is optimal for one phase may be suboptimal or even inhibit rooting processes in another phase (de Klerk et al. 1999). The timing of exogenously applied auxin is, therefore, crucial (Blythe et al. 2007). There is also evidence suggesting that tissue sensitivity to auxin changes throughout the rooting process and that maximal sensitivity to auxin does not occur directly after taking a cutting but is delayed (Gaspar and Hofinger 1988, de Klerk et al. 1999)

Although the exogenous application of auxin generally occurs at the initial placement of the cuttings, there is evidence to suggest that exogenously supplied auxin is rapidly conjugated or transported away from the root zone (Blakesley et al. 1991; Blythe et al. 2007). Consequently, when auxin is required for adventitious root induction it may no longer be present in the root zone

(Blakesley et al. 1991; Blythe et al. 2007). Luckman and Menary (2002) investigated the effect of different application times of auxin on the rooting of *E. nitens* cuttings. Applying auxin at the initial setting of cuttings (week 0) achieved the lowest rooting percentage while the delayed auxin application, 4 – 5 weeks after cuttings were set, significantly improved rooting (Luckman and Menary 2002). That study demonstrated that by coordinating the timing of exogenous auxin in relation to root initiation, optimal rooting of cuttings can be achieved.

2.3.4 Environmental factors

The rooting of cuttings may be significantly influenced by the application of auxin but their initial survival is directly dependent on the environmental conditions. Optimum light conditions, temperatures and relative humidity are all essential to promote survival and rooting of cuttings (Hartmann et al. 1997; Cunha et al. 2009a). Since cuttings initially have no roots, they have no means of replacing transpired water, and it is therefore essential to maintain an atmosphere with a low evaporative demand to ensure that transpiration from the cuttings is minimised (Loach 1988). According to that author: 1) maintaining an adequate turgor pressure is also imperative so that cells at the site of root initiation are able to facilitate the regeneration process; 2) the propagation environment must be regulated at the temperature that stimulates metabolism at the base of the cutting and promotes adventitious root formation; and 3) temperatures exceeding the optimum may pose deleterious consequences such as increasing transpiration which subsequently creates tissue water deficits. Irradiance within the propagation environment must be sufficient to enable photosynthesis to occur as the formation of adventitious roots is a carbohydrate dependent process (Veierskov 1988). Intermittent mist application has proven effective as the evaporation of the applied film of water cools the surface of the leaf and minimises transpiration, thereby maintaining a turgor pressure conducive to the induction of adventitious roots (de Assis et al. 2004).

The effect of season, with changing temperature and light intensity, photoperiod and humidity, can significantly affect the survival and rooting of cuttings. Wilson (1998b) recommended that to achieve maximum rooting the temperature of the rooting environment should be between 20°C – 30°C. However, *E. benthamii* x *E. dunnii* minicuttings displayed the highest rooting results (19 – 56%) in the colder seasons and poor rooting (5 – 9%) during the warmer seasons (Brondani et al. 2010). Maile and Nieuwenhuis (1996) demonstrated that *E. nitens* macrocuttings had 30% and 56% rooting for March (end of summer) and September (spring), respectively. Knowledge of the effect of season on each species with regards to rooting can lead to improved management strategies.

The humidity within the rooting environment needs to be high to reduce transpiration through the leaves (Hartmann et al. 1997). However, excessively high humidity could be deleterious to cuttings by impairing gas exchange and facilitating the proliferation of diseases. Cunha et al. (2009b) determined that for *E. grandis, E. urophylla* and *E. grandis* x *E. urophylla*, increases in light intensity and decreased relative humidity promoted the rooting of cuttings.

2.4 *In vitro* propagation of *Eucalyptus*

2.4.1 Routes of regeneration

Plant tissue culture is the aseptic culture of cells, tissues and organs under controlled physical and chemical conditions *in vitro* (Merkle and Nairn 2005; Thorpe 2007; George et al. 2008). Although the biological concept of totipotency was initially postulated in the Cell Theory of Schleiden (1838) and Schwann (1839) (*loc. cit.* Vasil 2008), it was only at the beginning of the 20th century after the required technologies became available, that the opportunity arose for Haberlandt (1902) (*loc. cit.* Vasil 2008), to attempt the first experiments to culture plant cells *in vitro*. However, due to poor choice of explant material, his experiments were unsuccessful and it was only in the late 1960s that Vasil and Hildebrandt (1965, 1967) provided unequivocal evidence of the totipotency of plant cells (Sussex 2008; Vasil 2008). During the 1970s and 1980s the application of *in vitro* culture techniques to various problems in basic biology, agriculture, horticulture and forestry was investigated and throughout the 1990s, *in vitro* technologies expanded to include a wide range of plant species (Thorpe 2007; Vasil 2008).

Since the first reports on *in vitro* cultures of *Eucalyptus* species by Sussex (1965) and Aneja and Atal (1969), numerous species and hybrids have been successfully established *in vitro* (reviews by le Roux and van Staden 1991a, Watt et al. 2003) (Table 2). The various applications of tissue culture systems include the mass production of selected genotypes, those that have proven difficult to propagate via conventional methods, bulking up of hybrid genotypes and the rejuvenation of physiologically old shoots (le Roux and van Staden 1991a; Watt et al. 2003; Delaporte and Sedgley 2004; Nehra et al. 2005; Brondani et al. 2012a).

Table 2 Combinations of PGRs (mg l^{-1}) required by different *Eucalyptus* species and hybrids for which direct organogenic protocols were established within the last decade. BAP = benzylaminopurine, IAA = indole-3-acetic acid, IBA = indole-3-butyric acid, NAA = α-naphthalene acetic acid, GA_3 = gibberellic acid, GA_4 = isomer of gibberellic acid, NR = Not Reported.

Species	**Explant**	**Multiplication PGRs**	**Elongation PGRs**	**Rooting PGRs**	**Reference**
E. nitens	Shoot tips and nodal segments	BAP [0.2] NAA [0.01]	BAP [0.1] NAA [0.01] GA_3 [0.1]	IAA [0 – 3] or IBA [0 – 3]	Gomes and Canhoto (2003)
E. tereticornis x *E. grandis*	Nodal segments	BAP [1.0] NAA [1.0]	No PGRs	IBA [1.0]	Joshi et al. (2003)
E. impensa	Nodal segments	BAP [0.05] kinetin [0.5]	GA_4 [0.2 – 0.3] zeatin [0.1]	IBA [1.0] NAA [0.1]	Bunn (2005)
E. grandis	Nodal segments	BAP [200] for 1-2 hours	NR	NR	de Andrade et al. (2006)
E. erythronema x *E. stricklandii*	Axillary shoots	BAP [0.5] NAA [0.2]	BAP [0.5] NAA [0.2] GA_3 [0.5]	IBA [4.0] over a 7 day pulse followed by subculture to IBA free medium	Glocke et al. (2006b)

Table 2 continued

Species	Explant	Multiplication PGRs	Elongation PGRs	Rooting PGRs	Reference
E. maidenii	Shoot tips and nodal segments	BAP [0.2] IBA [0.02]	BAP [0.1] IBA [0.5]	IBA [1 – 2]	Sotelo and Monza (2007)
E. urophylla x *E. grandis*	Nodal segments	BAP [0.1] NAA [0.01]	Combined with multiplication	IBA [1.0] or NAA [2.0]	Nourissier and Monteuuis (2008)
E. camaldulensis x *E. tereticornis*	Nodal segments	BAP [1.0] IBA [0.1]	Combined with multiplication	IBA [1.0]	Arya et al. (2009)
E. torelliana x *E. citriodora*	Nodal Segments	BAP [1.0]	Combined with multiplication	IBA [0.5]	Arya et al. (2009)
E. benthamii x *E. dunnii*	Nodal segments	BAP [0.25 – 0.5]	NAA [0.25 – 0.75] BAP [0.05]	NR	Brondani et al. (2009)
E. urophylla x *E. grandis*	Nodal segments	BAP [1.0] NAA [0.01]	Combined with multiplication	IBA [1.0] or NAA [2.0]	Mankessi et al. (2009)
E. globulus	Nodal segments	BAP [0.5] NAA [0.01]	NR	NR	Borges et al. (2011)

Table 2 continued

Species	Explant	Multiplication PGRs	Elongation PGRs	Rooting PGRs	Reference
E. benthamii x *E. dunnii*	Nodal segments	BAP [0.5] NAA [0.05]	BAP [0.1] GA_3 [0.1 – 0.2]	IBA [2.0]	Brondani et al. (2011)
E. torelliana x *E. citriodora*	Shoots	Presence or absence of NAA [0.01]	Combined with multiplication	IBA [4.0]	Hung and Trueman (2011)
E. tereticornis	Nodal segments	BAP [0.5] NAA [0.1]	BAP [0.02]	IBA [1.0]	Aggarwal et al. (2012)
E. benthamii	Nodal segments	BAP [0.5] NAA [0.05]	IBA [0.1]	NAA [0.2] IBA [0.2]	Brondani et al. (2012a)
E. camaldulensis	Nodal segments	BAP [2.0] NAA [0.1]	BAP [0.5]	IBA [1.0]	Girijashankar (2012)

Tissue culture techniques depend on two fundamental morphogenic processes, *viz.* somatic embryogenesis and organogenesis. The former is the formation of embryos from somatic cells, which then develop into plants (García-Gonzáles et al. 2010; Iliev et al. 2010), whereas the latter is the formation of plant organs, which can be used to form plants or specific organs of interest (García-Gonzáles et al. 2010; Iliev et al. 2010). Both processes can proceed either directly or indirectly via an intervening callus stage (García-Gonzáles et al. 2010; Iliev et al. 2010). Somatic embryos in *Eucalyptus* are induced on media containing a relatively high concentration of auxins, either 1 – 5 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) or 0.5 – 5 mg l^{-1} α-naphthalene acetic acid (NAA) (Watt et al. 1999; George et al. 2008). Once embryogenic cells develop, they continue to proliferate on the high auxin media forming proembryogenic masses (PEMs) (George et al. 2008). As high auxin concentration inhibits the development of PEMs into somatic embryos, it is then necessary for the embryogenic cultures to be transferred onto auxin-free media (George et al. 2008). The maturation of somatic embryos occurs on media with a low osmotic potential, created by the addition of osmotic agents such as organic salts or polyethylene glycol (PEG) (George et al. 2008). Mature embryos are then transferred to PGR-free media for further germination (George et al. 2008). Somatic embryogenic protocols have been described for *E. camaldulensis* (Prakash and Gurumurthi 2010; Girijashankar 2012), *E. citrodora* (Muralidharan and Mascarenhas 1995), *E. dunnii* (Termignoni et al. 1996), *E. globulus* (Nugent et al. 2001b; Pinto et al. 2002; Gómez et al. 2006), *E. grandis* (Watt et al. 1991; Titon et al. 2007) and *E. tereticornis* (Prakash and Gurumurthi 2005) albeit with varying degrees of regenerative success. Although regeneration of plants via somatic embryogenesis has the potential to mass produce plants, currently this is not commercially viable for several *Eucalyptus* species and hybrids due to poor embryo production and the low frequency of embryo conversion into plants (Titon et al. 2007; Ducos et al. 2009). However, somatic embryogenesis continues to receive considerable attention as embryogenic cultures are excellent targets for gene insertion for the production of transgenic plants (Watt et al. 2003; Moyo et al. 2011).

Although indirect organogenic protocols have been established for a host of *Eucalyptus* species and hybrids including *E. camaldulensis* (Rahim et al. 2003; Dibax et al. 2010), *E. erythronema* (Glocke et al. 2006a), *E. grandis* (Hajari et al. 2006), *E. grandis* x *E. urophylla* (Carvalho Alves et al. 2004; Hajari et al. 2006, de Alcantara et al. 2011), *E. globulus* (Nugent et al. 2001a; Degenhardt-Goldbach et al. 2011), *E. gunnii* (Hervé et al. 2001), *E. phylacis* (Bunn et al. 2005), *E. nitens* (Bandyopadhyay et al. 1999), *E. urophylla* (Huang et al. 2010). *E. urophylla* x *E. grandis* (Ouyang

et al. 2012), *E. saligna* (Degenhardt-Goldbach et al. 2011), *E. stricklandii* (Glocke et al. 2006a) and *E. tereticornis* (Aggarwal et al. 2010), poor acclimatisation of plantlets and the risk of somaclonal variation have limited their implementation in the clonal forestry industry (Watt et al. 2003; Bairu et al. 2011).

Within the last decade, significant improvements have been made with regards to plant regeneration via organogenesis and somatic embryogenesis for economically important *Eucalyptus* species and hybrids but since this study focuses on direct organogenesis, only this will be discussed further. Direct organogenesis via axillary shoot proliferation is currently the most frequently utilised micropropagation technique for the clonal mass production of commercially important *Eucalyptus* (Iliev et al. 2010). Usually the process of organogenesis comprises of stages which include sterilisation, culture establishment, shoot multiplication, shoot elongation, root formation and acclimatisation which are discussed below.

2.4.2 Direct Organogenesis

a) Sterilisation and Culture Establishment

The nutritional composition of the culture medium comprises of various combinations of macro-and micronutrients, vitamins, amino acids, carbohydrates and plant growth regulators which together with environmental conditions such as light intensity, photoperiod and temperature govern the growth and development of explants (George et al. 2008). The majority of *Eucalyptus* species and hybrids are grown on Murashige and Skoog (MS) (1962) or modified MS media (Gomes and Canhoto 2003; Joshi et al. 2003; Arya et al. 2009; Girijashankar 2012).

To avoid contamination of cultures by pathogens such as fungi and bacteria, surface sterilisation of explants, involving calcium hypochlorite or sodium hypochlorite and/or mercuric chloride followed by several rinses in distilled water, is a prerequisite (Hartmann et al. 1997; Iliev et al. 2010). Contamination problems can be reduced by ensuring the stock plants from which the explants are sourced are maintained indoors and subjected to a regular fungicide regime (Watt et al. 2006). Gomes and Canhoto (2003) revealed that the degree of contamination can be related to the type of explant selected since shoot tips and nodal segments from *E. nitens* displayed $< 2\%$ and $> 50\%$ contamination, respectively.

As discussed previously, cuttings obtained from mature sources are more difficult to propagate. Similarly, establishing *in vitro* cultures from mature trees is particularly challenging hence, the selection of *Eucalyptus* explants for *in vitro* culture should be carefully considered (George et al. 2008). Juvenile explant material can be sourced from specific zones within the complex architecture of the tree, such as basal epicormic shoots and the zones near floral and apical meristems (le Roux and van Staden 1991a). Nodal segments and shoot tips are the most commonly used explants for establishing *Eucalyptus* species and hybrids *in vitro* (Table 2). Cytokinins are included in bud induction media as they have the ability to break apical dominance thereby enabling the emergence of axillary buds which are then transferred to shoot multiplication media (Iliev et al. 2010; Nakhooda et al. 2012).

b) Shoot Multiplication and Elongation

For the majority of *Eucalyptus* species and hybrids, benzylaminopurine (BAP) and/or α-naphthalene acetic acid (NAA) are used during the multiplication stage (Table 2) since they promote cell division, shoot multiplication and axillary bud formation (Glocke et al. 2006a). It has been reported that the addition of BAP to the multiplication medium, rather than the rooting medium, has a beneficial effect on the rooting of *Eucalyptus* (Bennett et al. 1994; Yang et al. 1995; Nourissier and Monteuuis 2008). In *E. grandis* nodal segments, exposure to 200 mg l^{-1} BAP for 1 – 2 hours stimulated shoot proliferation which resulted in nearly double the number of shoots produced when compared to the control treatment (de Andrade et al. 2006). In the presence of 1 mg l^{-1} BAP and 1 mg l^{-1} NAA, *E. tereticornis* x *E. grandis* produced 20 – 25 shoots/explant (Joshi et al. 2003) and similar results were obtained for *E. tereticornis* (Sharma and Ramamurthy 2000).

Although the multiplication stage can produce shoots long enough (> 2.5 cm) for the rooting stage, for some *Eucalyptus* species and hybrids it is necessary for shoots to first undergo an elongation stage before rooting is attempted (Hartmann et al. 1997; Brondani et al. 2012a). Gibberellic acid (GA_3) has been added to media to elongate shoots of various *Eucalyptus* (Table 2). *Eucalyptus erythronema* x *E. stricklandii* shoots exposed to GA_3 elongated to a length of 6.5 mm compared to the 3.3 mm reached in its absence (Glocke et al. 2006b). However, for *E. benthamii*, the best shoot elongation occurred in 0.1 mg l^{-1} IBA while BAP and GA_3 proved unsuitable as they promoted etiolated, fragile shoots with reduced leaves (Brondani et al. 2012a). Further, shoots subjected to GA_3 during the elongation stage subsequently failed to initiate roots (Brondani et al. 2012a). Joshi et al. (2003) obtained shoot elongation in the absence of PGRs with the subsequent rooting in 1.0

mg l^{-1} IBA occurring without callus formation. *E. impensa* shoots displayed elongation only when exposed to GA_4, an isomer of GA_3 (Bunn 2005). As demonstrated by Nakhooda et al. (2011) on *E. grandis*, the addition of auxins during the multiplication and elongation stages may persist through to the rooting stage, thereby having a subsequent effect on root induction and development.

Micropropagation in semi-solid media requires the aseptic division of plant tissues by skilled personnel and periodic subculturing of plant material onto fresh media (Etienne and Berthouly 2002). New techniques are required to automate procedures which will reduce production costs, improve productivity and reduce the time taken to multiply commercially important material (Etienne and Berthouly 2002). Temporary immersion systems such as RITA® enable cycling of the liquid culture medium thus exposing the plant tissues to the medium intermittently rather than continuously (Etienne and Berthouly 2002; Ducos et al. 2009). Advantages of the temporary immersion system include adequate oxygen available to the plant tissues, reduced contamination and consumable costs, low labour cost due to reduced transfer times and automation which result in improved micropropagule quality thereby enabling easier acclimatisation (Etienne and Berthouly 2002; McAlister et al. 2005; Ducos et al. 2009). Using the temporary immersion bioreactor system, McAlister et al. (2005) reported four to sixfold yield for six *Eucalyptus* clones in half the time when compared to shoot proliferation on semi-solid media.

c) Root formation and acclimatisation

The rooting of shoots may be the most difficult stage in obtaining micropropagated plants and it may limit the viability of the micropropagation process (Nehra et al. 2005; Arya et al. 2009; Hung and Trueman 2011). *In vitro* shoots of *Eucalyptus* display variable rooting abilities with the genetic predisposition of the species or hybrid to form adventitious roots, the combination and exposure time to plant growth regulators, nutrient salt concentrations and environmental factors such as light, temperature, and culture vessel being amongst the contributing factors (McComb and Bennett 1986; Williams, 1999). The discovery of the rhizogenic effects of auxin in the 1930s was a major breakthrough for commercial plant propagation which led to IBA becoming the most frequently utilised auxin to promote *in vitro* adventitious root formation in *Eucalyptus* (le Roux and van Staden 1991a; Blythe et al. 2007). However, as shown in Table 2, the type and concentration of exogenously applied PGRs are specific to a particular *Eucalyptus* species, hybrid or clone and auxin concentrations outside the optimal range may induce excessive callus formation or inhibit shoot

proliferation and root formation (Mokotedi et al. 2000; Joshi et al. 2003; Bunn et al. 2005; Blythe et al. 2007).

As mentioned earlier (Section 2.3), *in vitro* culture systems offer the opportunity to administer and replenish nutrients and carbohydrates to rejuvenate mature plant material. Trindade and Pais (1997) and Sharma and Ramamurthy (2000) have reported the rejuvenating effect of repeated subculturing on BAP-enriched medium for *E. globulus* and *E. tereticornis*, respectively. In a study conducted by Mankessi et al. (2009) on *E. grandis* x *E. urophylla*, 22 year old clones maintained in culture (on multiplication medium supplemented with 0.1 mg l^{-1} BAP and 0.01 mg l^{-1} NAA for six years displayed superior rooting abilities when compared to a one year old clone maintained under the same culture conditions for a year. Mature (30 – 32 year old) *E. camaldulensis* x *E. tereticornis* and *E. torelliana* x *E. citriodora* trees were successfully established *in vitro* with 87.5% and 91.6% rooting in 1.0 mg l^{-1} IBA, respectively (Arya et al. 2009). Similarly, shoots established *in vitro* from 30 year old *E. tereticornis* x *E. grandis* trees achieved 75% rooting (Joshi et al. 2003). In contrast, Gomes and Canhoto (2003) successfully induced roots from *E. nitens* shoots obtained from four, eight and ten week old seedlings but not from a one year old plant. Further, rooting occurred even in the absence of PGRs in the rooting medium (Gomes and Canhoto 2003).

Fogaça and Fett-Neto (2005) conducted a comparative analysis on the effect of auxins on adventitious root formation in *Eucalyptus saligna* (easy-to-root species) and *Eucalyptus globulus* (difficult-to-root species). The control treatment, devoid of auxin, yielded 60% and 0% rooting for *E. saligna* and *E. globulus,* respectively. Both species displayed higher rooting percentages, shorter mean rooting time, greater root density and number of roots per shoot when exposed to IBA than IAA or NAA (Fogaça and Fett-Neto 2005). Those authors suggested that the ability of IBA to be converted to IAA *in vivo*, functioning as a slow release reservoir of a more easily metabolised auxin, may account for the results obtained. The low rooting percentages observed in the NAA treatment could be due to NAA having a longer persistence time than IAA and IBA thereby remaining in tissues in the free form and inhibiting root emergence (Fogaça and Fett-Neto 2005). Working on the same species, Fett-Neto et al. (2001) revealed that rooting of *E. saligna* was promoted with low concentrations of IBA (< 10 mg l^{-1}), whereas *E. globulus* required higher concentrations to initiate roots (> 10 mg l^{-1}). This concept of difficult-to-root species requiring the application of more IBA and easy-to-root species requiring less IBA was also demonstrated in cuttings. Nakhooda et al. (2011) determined that for an easy-to-root *E. grandis* clone, the

combination of endogenous and exogenous auxins added during the multiplication and elongation stages, were sufficient to induce roots on auxin-free rooting medium. In addition, excess exogenous auxin induced the formation of basal callus which subsequently delayed root emergence (Nakhooda et al. 2011). As discussed in the previous section on cuttings, an auxin concentration above the optimal may cause excessive root and callus formation on the shoot which may lead to mortality.

Exposing *Eucalyptus* shoots to a high auxin concentration for a relatively short period of time, (acute treatment) compared to continuous exposure to a lower auxin concentration (chronic treatment) can potentially improve root induction and development (le Roux and van Staden 1991a, Glocke et al. 2006b). *E. erythronema* x *E. stricklandii* shoots achieved the highest rooting percentage (62%) when subjected to 4 mg l^{-1} IBA over a 7 day pulse treatment followed by subculturing on PGR-free medium (Glocke et al. 2006b). In addition, reduced basal callus formation was observed with roots developing directly from the stem thereby ensuring a vascular connection between the stem and roots (Glocke et al. 2006b). In contrast, for shoots of *E. grandis* x *E. nitens*, a single 24 hour pulse treatment of 20 mg l^{-1} IBA failed to induce rooting but produced large amounts of callus and leaf abscission while the 28 day chronic treatment of 0.1 mg l^{-1} IBA displayed relatively higher rooting (28%) (Mokotedi et al. 2000).

It has been reported that modifying or reducing the nutrient concentration of the rooting medium can potentially improve rooting in *Eucalyptus* species and hybrids (Mokotedi et al. 2000; Sharma and Ramamurthy 2000; Glocke et al. 2006b). Mokotedi et al. (2000) found that by reducing the MS nutrient concentration from full strength to ¼ strength and adding ¾ strength concentrations of calcium and magnesium, callus formation decreased from 100% to 48% and subsequently improved rooting from 0% to 52% in *E. grandis* x *E. nitens* shoots. In addition, the roots obtained from reduced MS nutrient concentration ($^1/_3$ and $^1/_4$ strength) were long and thick with well-developed lateral roots compared to the shorter roots obtained in $^1/_2$ and full strength MS (Mokotedi et al. 2000).

E. grandis x *E. nitens* (McAlister et al. 2005) and *E. grandis* x *E. urophylla* (McAlister et al. 2005; de Oliveira et al. 2011) clones displayed greater rooting ability when produced via the RITA® temporary immersion system than in semi-solid media. In addition, plantlets displayed minimal callus formation and were hardier which enabled easier acclimatisation (McAlister et al. 2005).

Acclimatising plantlets from *in vitro* (heterotrophic conditions) to an outdoor (autotrophic) environment is the crucial, final stage in the micropropagation process (Hartmann et al. 1997). Micropropagated plants are relatively fragile and should initially be maintained at a very high humidity with gradual exposure to outdoor conditions. The leaves and stomata of micropropagated plants are morphologically different from those of seedlings as they have reduced epicuticular waxes and stomata which are initially unable to close, making these plants particularly prone to desiccation (Hartmann et al. 1997; George et al. 2008).

Generally, for the acclimatisation of *Eucalyptus* species and hybrids, rooted shoots are transferred to inserts in trays or pots containing sterile, rooting mixtures comprising of various combinations of soil, perlite, peat, vermiculite, pine park, coir etc. (Mokotedi et al. 2000; Gomes and Canhoto 2003; Hajari et al. 2006; Dibax et al. 2010; Nakhooda et al. 2011). Some acclimatisation protocols include supplementing the rooting mixture with $^{1}/_{3}$ strength MS salts and vitamins (Hajari et al. 2006; Nakhooda et al. 2011). Acclimatisation of plantlets can occur in a growth room or a greenhouse and usually, individual plants are sealed with transparent, plastic bags to maintain high humidity (Mokotedi et al. 2000; Gomes and Canhoto 2003; Hajari et al. 2006). After 1 – 2 weeks the humidity is gradually reduced by punching holes in the plastic bags and after another 1 – 2 weeks, the bags are removed completely (Mokotedi et al. 2000; Hajari et al. 2006). A high humidity environment can also be obtained by maintaining the plants in a greenhouse under intermittent mist conditions (Dibax et al. 2010; Nakhooda et al. 2011). Acclimatisation usually takes 1 – 2 months, after which the plants can be maintained under standard greenhouse conditions or transferred to a shadehouse (Mokotedi et al. 2000; Hajari et al. 2006; Nakhooda et al. 2011).

3. MATERIALS AND METHODS

3.1 Plant Material

Studies which involved cuttings taken from selected *Eucalyptus grandis* x *Eucalyptus nitens* clones (Clones 1, 2 and 3) were conducted at Sunshine Seedlings Services (29° 31.709'S; 30° 28.583'E), a commercial nursery in KwaZulu-Natal, South Africa. Parent plants, initiated from rooted stem cuttings, formed clonal mini-hedges (~ 15 cm tall) and were approximately three years old when these studies were undertaken (Figure 1). For the *in vitro* studies, parent plants of these three clones were donated by Sunshine Seedlings Services to the University of KwaZulu-Natal (UKZN) for purposes of this research. The parent plants were maintained in a shadehouse at UKZN with an automated watering system and subjected to stringent fungicide and fertilizer regimes.

Figure 1 *Eucalyptus grandis* x *Eucalyptus nitens* parent plants from which coppice was harvested to make cuttings.

3.2 Standard nursery practices

Coppice was harvested every two weeks in summer and every three to four weeks in winter. Coppice shoots (5 – 7 cm long) harvested from clonal mini-hedges were collected and placed in

water to prevent desiccation. They were made into minicuttings (3.5 – 4 cm long) (hereafter referred to as cuttings) comprising of an apical meristem and a pair of leaves reduced to $^{1}/_{3}$ of its original surface area to minimise water loss through transpiration (Figure 2). Cuttings were placed in a solution of Nufilm® (Miller Chemical and Fertiliser Corporation, Pennsylvania, USA) (active ingredient, poly-1-p-menthene (875 g l^{-1})) before being planted. Nufilm® is a sticking-extending agent designed to lengthen the lifespan of foliar insecticides and fungicides applied to cuttings even in the presence of overhead misting. Cuttings were set in Unigro 98® trays (65 cm x 33 cm x 10.5 cm) which contained 128 removable inserts with growth medium comprising of coir: vermiculite: perlite (5:3:2). The practice at Sunshine Seedling Services is to recycle growth medium through decontamination by steaming at 80 °C. Trays were placed in a randomised complete block design. All results were recorded five weeks after cuttings were initially set and evaluated parameters for each minicutting included survival, rooting and callus formation.

Figure 2 A typical cutting (3.5 – 4 cm long) comprising of an apical meristem and a pair of leaves reduced to $^{1}/_{3}$ of their original surface area.

Cuttings set in Unigro 98® trays were placed in polyethylene rooting tunnels (Figure 3) with the air temperature regulated between 28 – 38 °C by thermostatically controlled fans. In addition, root zone temperature was maintained at 25 °C by bed heaters. The humidity within the rooting tunnel was

Figure 3 *Eucalyptus* cuttings set in Unigro 98® trays and placed in a polyethylene tunnel with thermostatically controlled fans and an automated overhead misting system, with a) and without b) mist.

kept as close to 100% as possible by an automated overhead misting system, which was set to continuously release mist for 10 sec every 4 min. The incorporation of chlorine into the mist (0.4 g l^{-1}) was aimed at reducing algal and fungal proliferation. There were no artificial light sources present within the rooting tunnel, and light intensity ranged from ~ 300 µmol s^{-2} m^{-1} in winter to ~1900 µmol s^{-2} m^{-1} during summer. Cuttings remained under these conditions in the rooting tunnel for a period of four weeks, after which rooted cuttings were transferred to a pre-hardening off tunnel (39 – 42˚C, 90 – 95% humidity and 350 – 850 µmol s^{-2} m^{-1} light intensity in summer) and then hardened off in a greenhouse (39 – 42˚C, 85 – 90% humidity and 500 – 1100 µmol s^{-2} m^{-1} in summer). In winter, the pre-hardening off tunnel and greenhouse displayed similar growing conditions (27˚C, 65% humidity and 320 µmol s^{-2} m^{-1}). Since this study focused on survival and root induction, all results were recorded after five weeks in the rooting tunnel. Standard nursery practices involved the application of the commercial root promoting powder, Seradix 2 (Bayer Crop Science, Leverkusen, Germany) (active ingredient, 3 g kg^{-1} IBA) to the basal end of *Eucalyptus* cuttings immediately before placement in the growth medium. For the initial study, cuttings (n = 100) from two *Eucalyptus grandis* x *Eucalyptus nitens* clones (Clone 1 and 2) were dipped in Seradix 2 to determine percentage survival and rooting as per standard nursery practices.

3.3 Cutting types

Two types of cuttings were used, soft and hard. Coppice shoots harvested from parent plants (Clones 1 and 2) every two weeks were used to make 'soft' cuttings (n = 100) (standard nursery practice). Coppice shoots harvested from parent plants after four weeks of growth served as the more mature, hard cuttings (n = 100). There was a considerable size difference between soft (average length = 48.9 mm, average diameter = 1.25 mm) and hard coppice (average length = 85.0 mm, average diameter = 1.58 mm) of Clone 1 (Figure 4). The size difference in soft (average length = 38.7 mm and average diameter = 0.93 mm) and hard (average length = 57.1 mm, average diameter = 1.46 mm) coppice of Clone 2 was less pronounced (Figure 4). Length and diameter measurements were recorded using digital calipers.

3.4 Mode of application of Seradix

a) Powder Substrate

This study consisted of three treatments: a control (no Seradix or talcum powder applied), cuttings dipped in talcum powder and cuttings dipped in Seradix 2. There were five replicates for each treatment with 24 cuttings in each replicate (n = 120).

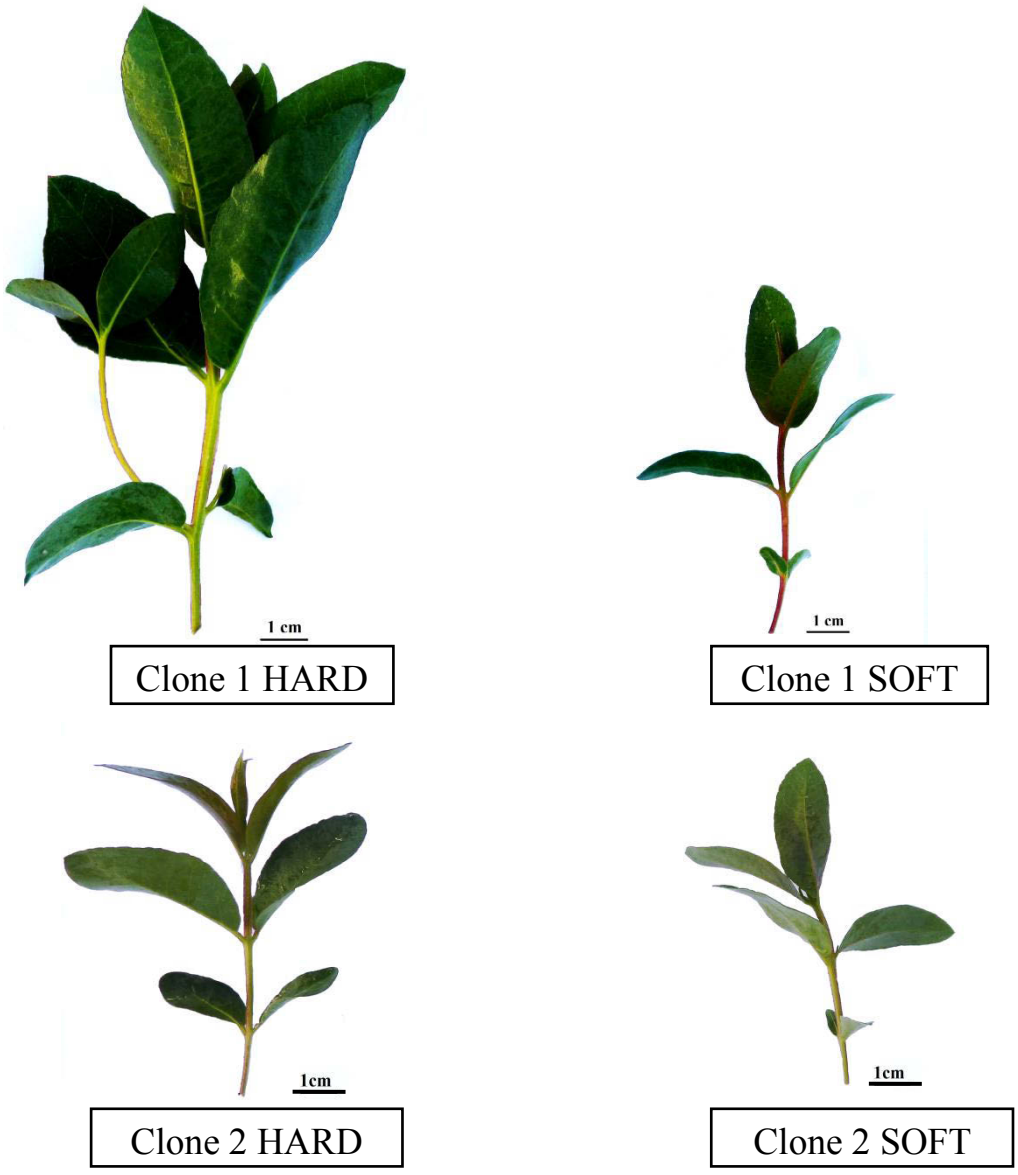


Figure 4 Size differences between mature, hardy coppice and juvenile, soft coppice harvested after four and two weeks respectively, from three year old Clone 1 and Clone 2 parent plants.

b) IBA solution

An IBA solution, 3 g l^{-1} was made up by first dissolving IBA in ethanol and then distilled water. The solution was adjusted to a pH of 5.6 – 5.8 and then decontaminated by autoclaving at 121°C for 20 min. There were two different exposure times to the IBA solution, 10 sec and 5 hr. For the 10 sec IBA treatment, the base of each cutting was held in the IBA solution (at a depth of 0.5 cm) for

10 sec and then placed in the growth medium. For the 5 hr IBA treatment, the prepared IBA solution was mixed with clean vermiculite and placed in containers. This was done to provide a support medium thereby ensuring that only the base of cuttings of Clones 1 and 2 were exposed to the IBA solution for the required 5 hr period before being planted out in the growth medium. Each treatment had five replicates with 24 cuttings in each replicate (n = 120).

3.5 Different Seradix concentrations and time of application

Seradix 1 (1 g kg^{-1} IBA), 2 (3 g kg^{-1} IBA) and 3 (8 g kg^{-1} IBA) were commercially available. To obtain a lower concentration of Seradix than was commercially available, Seradix 1 was mixed with talcum powder in equal proportions to obtain Seradix 0.5 (0.5 g kg^{-1} IBA). Untreated cuttings of Clones 1, 2 and 3 (i.e. no Seradix) were planted to serve as the control. Cuttings were supplied with the various concentrations of Seradix at either initial placement (T_{0weeks}) or two weeks after cuttings were set (T_{2weeks}). For the latter, cuttings were carefully removed from the growth medium after two weeks. If callus was present at the base of the cutting, it was gently removed. The basal part of the cutting was dipped in clean water, to allow Seradix to adhere to the cutting which was then replanted in the growth medium. In both studies, there were five treatments consisting of five replicates with 20 cuttings in each (n = 100).

3.6 *In vitro* studies

A diagrammatic representation of experimental design for the *in vitro* studies is shown in Figure 5.

3.6.1 Decontamination and culture establishment

Shoots were taken from the parent plants of Clones 1, 2 and 3 and placed in a solution of 1g l^{-1} methyl N-(1-butylcarbamoyl-2-benzimidazole) carbamate (Benlate, Volcano Agroscience (Pty) Ltd, South Africa), 1g l^{-1} boric acid , 0.5 ml l^{-1} chlorothalonil (Bravo 500, Volcano Agroscience (Pty) Ltd, South Africa**)** and a drop of Tween® -20, placed on a shaker for 30 minutes and then transferred to the laminar flow. After the shoots were rinsed with sterile, distilled water they were immersed in 0.2 g l^{-1} $HgCl_2$ and a drop of Tween® -20 for two minutes. The shoots were then rinsed three times in sterile, distilled water and then treated with 10 g l^{-1} calcium hypochlorite and a drop of Tween® -20 for another two minutes. After three rinses of distilled water, each shoot was sectioned into 2 – 3 cm long explants with a trimmed pair of leaves and then placed on bud induction medium.

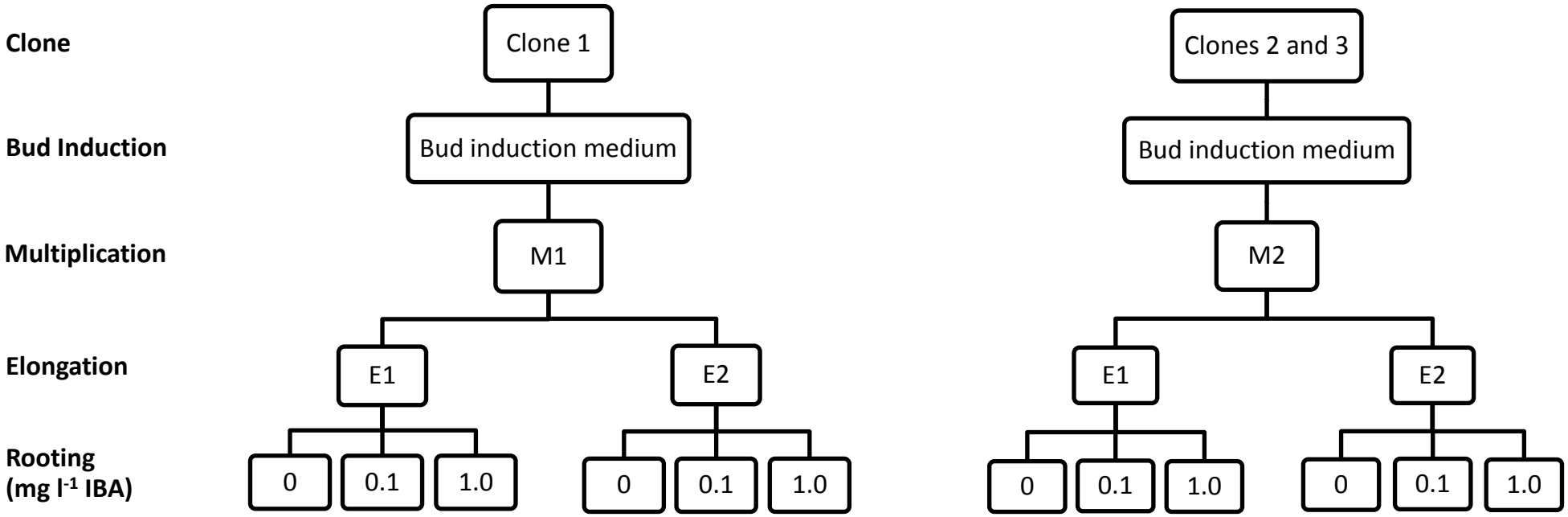
3.6.2 Bud induction and multiplication

After decontamination, each explant was placed on 10 ml of bud induction medium in a 50 ml culture tube for two weeks. This medium contained MS salts and vitamins (Murashige and Skoog, 1962), 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.04 mg l^{-1} NAA, 0.1 mg l^{-1} BAP, 0.05 mg l^{-1} kinetin, 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite®. Two multiplication media were tested, designated M1 and M2. M2 was used as shoots of Clones 2 and 3 did not respond to M1. M1 had the same components as the bud induction medium. M2 contained MS salts and vitamins, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.04 mg l^{-1} IAA and 0.3 mg l^{-1} *trans*-zeatin, 20 g l^{-1} sucrose and 4g l^{-1} Gelrite®. Shoots (5 per bottle) were maintained in 100 ml culture bottles in 20 ml of medium for a period of 3 – 4 weeks. All media used for each of the culture stages were adjusted to a pH of 5.6 – 5.8 and then autoclaved at 121 °C for 20 minutes. All cultures were maintained in a plant growth room with a 16 hour light/ 8 hours dark photoperiod (200 μmol m^{-2} s^{-1}) at 25°C and 23°C, respectively.

3.6.3 Elongation and Rooting

Two elongation media were tested, designated E1 and E2. E1 contained MS, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 0.35 mg l^{-1} NAA, 0.2 mg l^{-1} kinetin, 0.05 mg l^{-1} IBA, 20 g l^{-1} sucrose and 4g l^{-1} Gelrite®. The components of E2 were the same as E1, except that the auxins NAA and IBA were substituted with 0.37 mg l^{-1} IAA. Shoots were elongated in 100 ml culture bottles with 20 ml of medium for a period of 3 – 4 weeks.

For the rooting studies, shoots ≥ 1.5 cm elongated on E1 and E2 were placed on 10 ml of rooting medium within a 50 ml culture tube. The rooting medium contained ¼ MS, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 15 g l^{-1} sucrose, 4 g l^{-1} Gelrite® and the tested concentration of IBA. The concentrations of IBA (0, 0.1 and 1.0 mg l^{-1} IBA) were selected to typify the range of Seradix concentrations applied to cuttings (i.e. no IBA, low and high IBA concentrations). Each treatment had a sample size of 20.



All bud induction, multiplication and elongation media contained MS, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 20 g l^{-1} sucrose and 4 g l^{-1} Gelrite® but different PGRs.

Bud induction medium = 0.04 mg l^{-1} NAA, 0.1 mg l^{-1} BAP and 0.05 mg l^{-1} kinetin

M1 = 0.04 mg l^{-1} NAA, 0.1 mg l^{-1} BAP and 0.05 mg l^{-1} kinetin

M2 = 0.04 mg l^{-1} IAA and 0.3 mg l^{-1} *trans*-zeatin

E1 = 0.35 mg l^{-1} NAA, 0.2 mg l^{-1} kinetin and 0.05 mg l^{-1} IBA

E2 = 0.37 mg l^{-1} IAA and 0.2 mg l^{-1} kinetin

All rooting media contained ¼ MS, 0.1 mg l^{-1} biotin, 0.1 mg l^{-1} calcium pantothenate, 15 g l^{-1} sucrose, 4 g l^{-1} Gelrite® and the tested concentration of IBA (mg l^{-1})

Figure 5 Experimental design of the *in vitro* studies undertaken with the three *Eucalyptus grandis* x *E. nitens* clones. Two different multiplication media were used because shoots of Clones 2 and 3 did not respond to M1.

3.7 Statistical analyses

All statistical analyses were completed using IBM SPSS Statistics (Version 21). An analysis of variance (ANOVA) test was performed to test for significant differences amongst treatments ($p < 0.05$). Clonal differences with regards to a specific treatment were also examined. If significant differences were observed, a Tukey's HSD (honestly significant different) test was then implemented. The assumptions for ANOVA were tested and if they were not satisfied, the non-parametric data were log-transformed and analyses were performed on the transformed data.

4. RESULTS

4.1 Macropropagation of three *Eucalyptus grandis* x *E. nitens* clones

4.1.1 Survival and rooting under standard nursery practices

In this study, root induction on soft cuttings of two *Eucalyptus grandis* x *E. nitens* clones (Clones 1 and 2) was initiated with Seradix 2 (active ingredient, 3 g kg^{-1} IBA), and the percentage of cuttings that survived, rooted and developed callus was determined (Table 3). This was done to test the standard nursery practices which reportedly resulted in variable rooting abilities of Clone 1 and 2 cuttings. Cuttings which were green and healthy after five weeks in the rooting tunnel were considered to have 'survived'. Although results for survival and rooting were low (< 50%) for both clones, cuttings of Clone 2 survived (48%) and rooted (36%) significantly better than those of Clone 1 (21% and 15%, respectively). The results were clearly different depending on how rooting percentage was calculated. For instance, cuttings of Clones 1 and 2 displayed low rooting percentages (15% and 36%, respectively) if rooting was calculated as a percentage of the total number of cuttings set, whereas rooting percentage was considerably higher for cuttings of Clone 1 (71%) and Clone 2 (75%) if rooting was calculated as a percentage of only the cuttings that survived (Table 3). Similarly, the percentage of cuttings with callus was low for Clones 1 and 2 (18% and 37%, respectively) when calculated as a percentage of the total number of cuttings planted, whereas callus formation in Clone 1 (86%) and Clone 2 (77%) was much higher when calculated as a percentage of only the cuttings that survived. Percentage callus was recorded because the formation of callus at the base of cuttings occurs in response to wounding or as a result of unbalanced PGRs, in particular, an excess of auxin (Hartmann et al. 1997). Subsequent studies provided further elucidation on the relationship between rooting and callus formation.

The results from this study (Table 3) revealed that the standard nursery practice of applying Seradix 2 to cuttings of Clones 1 and 2 was not successful in supplying sufficient numbers of rooted shoots. The main reason was high mortality and, in fact, the majority of the cuttings that survived, subsequently rooted. Consequently, ensuing studies focused on improving the survival percentage of cuttings.

Table 3 Survival (%), rooting (%) and callus formation (%) of cuttings of two *Eucalyptus grandis* x *E. nitens* clones treated with Seradix 2. Results were recorded five weeks after placement of cuttings. Means ± standard errors within columns followed by different letters are significantly different ($p < 0.05$) (n = 100).

Clone	Survival (%)	Rooting (as % total cuttings)	Rooting (as % cuttings that survived)	Callus (as % total cuttings)	Callus (as % cuttings that survived)
Clone 1	21 ± 1.871^{A}	15 ± 2.739^{A}	71 ± 11.165^{A}	18 ± 2.000^{A}	86 ± 8.307^{A}
Clone 2	48 ± 7.517^{B}	36 ± 5.568^{B}	75 ± 2.462^{A}	37 ± 5.385^{B}	77 ± 5.706^{A}

4.1.2 Effect of type of cuttings on their survival and rooting potential

The cuttings used in the previous study were 'soft' cuttings as per nursery practices. This could have accounted for the high mortality exhibited (Table 3) since soft cuttings have a low probability of survival (Wilson 1993). To test this hypothesis, two types of cuttings (soft and hard, obtained from two week old and four week old parent plants, respectively, Figure 4) were investigated in terms of their survival and rooting potential. Seradix 2 was not applied to any of the cuttings due to the high mortality observed earlier (Table 3).

The difference between the survival of soft and hard cuttings was significant for Clone 1 (95% and 62%, respectively) but not for Clone 2 (87% and 71%, respectively) (Table 4). However, soft cuttings of Clones 1 and 2 displayed significantly higher rooting percentage (32% and 29%, respectively) than hard cuttings (2% and 8%, respectively). However, there were no significant differences between types of cuttings of either clone in terms of callus production by cuttings that survived, with > 90% of Clone 1 cuttings and > 67% of Clone 2 cuttings recorded with basal callus formation (Table 4). Since the majority of the cuttings which survived developed callus in the absence of Seradix 2, it is suggested that callus formation was not a result of exogenous IBA application. Unlike the previous investigation where the majority of the cuttings that survived, rooted and developed callus (Table 3), in this investigation (Table 4) rooting of the cuttings that survived was low (< 32%) but percentage callus formation remained high (> 67%). From those results, it was established that soft cuttings of both clones survived and rooted better than hard cuttings. The results obtained in this study validate the preferential use of soft cuttings over hard *Eucalyptus* cuttings by the nursery.

A comparison between the clones with respect to type of cuttings revealed that a significantly higher percentage of Clone 1 soft cuttings developed callus than Clone 2 soft cuttings, which suggested that the former may have had a greater concentration of endogenous auxin than Clone 2 cuttings. No significant differences were observed between hard cuttings of both clones, for the various parameters investigated. It must be noted that there was considerable size difference (both in length and diameter) between soft and hard coppice produced from Clone 1 parent plants (Figure 4), and that could possibly account for the significant differences observed with regards to survival and rooting. The size differences between soft and hard coppice of Clone 2 (Figure 4) were less distinct, which could explain the lack of significant differences in the percentage of cuttings that survived.

By comparing these results of soft cuttings from this study (Table 4) with those shown in Table 3, cuttings of both clones demonstrated higher survival in the absence of Seradix 2. This suggests that the application of Seradix 2 inhibited the survival of cuttings, which was investigated in the subsequent study.

Table 4 The effect of type of cuttings (soft vs. hard) on survival (%), rooting (%) and callus formation (%) of cuttings of *Eucalyptus grandis* x *E. nitens* clones in the absence of an exogenous rooting stimulant. All results were recorded five weeks after placement of cuttings. Means ± standard errors within columns followed by lowercase letters are significantly different between types of cuttings within each clone; uppercase letters denote significant differences between the clones ($p < 0.05$) (n = 100).

Clone	Cutting Type	Survival (%)	Rooting (as % total cuttings)	Rooting (as % cuttings that survived)	Callus (as % total cuttings)	Callus (as % cuttings that survived)
Clone 1	Soft	95 ±2.739$^{a, A}$	32 ± 9.301$^{a, A}$	33 ± 9.129$^{a, A}$	89 ± 2.916$^{a, A}$	94 ± 2.905$^{a, A}$
	Hard	62 ± 5.148$^{b, A}$	2 ± 1.225$^{b, A}$	3 ± 2.111$^{b, A}$	56 ± 5.788$^{b, A}$	90 ± 3.353$^{a, A}$
Clone 2	Soft	87 ± 6.042$^{a, A}$	29 ± 5.099$^{a, A}$	33 ± 4.418$^{a, A}$	58 ± 3.391$^{a, B}$	67 ± 3.431$^{a, B}$
	Hard	71 ± 2.916$^{a, A}$	8 ± 1.225$^{b, A}$	11 ± 1.417$^{b, A}$	55 ± 2.739$^{a, A}$	77 ± 4.894$^{a, A}$

4.1.3 Effect of mode of auxin application

a) Seradix powder

Having established that mortality was not due to the cuttings being soft (Table 4), this study investigated the properties of Seradix 2 which could have caused the high mortality in Clone 1 and 2 cuttings (Table 3). Seradix 2 is a rooting promoter containing the PGR IBA that is incorporated into a powder substrate (talcum) for ease of application. Consequently, it was important to distinguish the effect of such a substrate and that of the PGR on the survival and rooting potential of cuttings. Towards this end, the cuttings were dipped in either Seradix 2 or talcum powder. The purpose of supplying cuttings with talcum powder was to determine if the powder substrate of Seradix created a physical barrier to the uptake of water and nutrients thereby inhibiting survival of the cuttings. In addition, cuttings without the application of Seradix 2 or talcum powder were planted to serve as a control.

The results indicated that Seradix 2 adversely affected the survival and rooting of cuttings for both clones (Table 5). Since the application of talcum powder did not influence the survival or rooting of the cuttings, the negative effect of Seradix 2 on the cuttings could be attributed to the IBA present in Seradix. For Clone 1 cuttings, the control and talcum powder treatments resulted in 89% and 88% survival, respectively. Similarly, Clone 2 cuttings displayed 79% survival in both the control and talcum powder treatments. In the presence of Seradix 2, the survival of both Clone 1 and Clone 2 cuttings were significantly reduced to 43% and 33%, respectively. Further, of the total cuttings set for Clone 1, 56% and 55% rooted successfully in control and talcum powder treatments, respectively, while only 18% rooted in the presence of Seradix 2 ($p < 0.05$). Rooting percentage of Clone 2 cuttings was low (< 27%) in all three treatments. Although cuttings of Clone 1 achieved almost identical survival and rooting percentages in the control and talcum powder treatments, the percentage of cuttings that developed callus (45% and 28%, respectively) differed significantly. These results indicate that the application of talcum powder significantly reduced percentage callus formation of Clone 1 cuttings. In contrast, Clone 2 cuttings displayed no significant differences with regards to callus formation (as percentage cuttings that survived). When rooting was considered as a percentage of the cuttings that survived, no significant differences were recorded amongst the three treatments for Clones 1 and 2. This strongly suggested that the detrimental effect of Seradix 2 was on survival and not rooting.

The comparison of survival, rooting and callus formation between clones for the control treatment revealed significant differences in all parameters, except survival (Table 5). Rooting

and callus formation (as percentage total cuttings set and as percentage cuttings that survived) were significantly higher in cuttings of Clone 1 than in those of Clone 2 (Table 5). The talcum powder treatment resulted in significant differences only with respect to rooting (as percentage total cuttings set and as percentage cuttings that survived) between Clone 1 (55% and 63%, respectively) and Clone 2 (26% and 33%, respectively). After exposure to Seradix 2, Clones 1 (12%) and 2 (33%) only demonstrated significant differences with regards to the formation of callus (as percentage cuttings that survived).

Table 5 The effect of no treatment (control), talcum powder (talc) and Seradix 2 treatments on survival (%), rooting (%) and callus formation (%) of cuttings of two *Eucalyptus grandis* x *E. nitens* clones. All results were recorded five weeks after placement of cuttings. Means ± standard errors within columns followed by lowercase letters are significantly different amongst the treatments within each clone; uppercase letters denote significant differences between the clones ($p < 0.05$) (n = 120).

Clone	Treatment	Survival (%)	Rooting (as % total cuttings)	Rooting (as % cuttings that survived)	Callus (as % total cuttings)	Callus (as % cuttings that survived)
Clone 1	Control	$89 \pm 4.532^{a, A}$	$56 \pm 4.652^{a, A}$	$63 \pm 6.345^{a, A}$	$45 \pm 2.332^{a, A}$	$51 \pm 3.000^{a, A}$
	Talc	$88 \pm 4.468^{a, A}$	$55 \pm 5.119^{a, A}$	$63 \pm 4.739^{a, A}$	$28 \pm 3.092^{b, A}$	$32 \pm 2.846^{b, A}$
	Seradix 2	$43 \pm 15.217^{b, A}$	$18 \pm 6.414^{b, A}$	$44 \pm 10.805^{a, A}$	$5 \pm 2.490^{c, A}$	$12 \pm 4.960^{c, A}$
Clone 2	Control	$79 \pm 6.038^{a, A}$	$15 \pm 1.600^{ab, B}$	$19 \pm 1.965^{a, B}$	$21 \pm 4.695^{a, B}$	$26 \pm 5.678^{a, B}$
	Talc	$79 \pm 3.747^{a, A}$	$26 \pm 3.441^{a, B}$	$33 \pm 4.748^{a, B}$	$28 \pm 4.823^{a, A}$	$36 \pm 5.006^{a, A}$
	Seradix 2	$33 \pm 7.047^{b, A}$	$8 \pm 3.043^{b, A}$	$24 \pm 7.305^{a, A}$	$11 \pm 3.917^{b, A}$	$33 \pm 7.440^{a, B}$

b) IBA solution

To further investigate the possible inhibitory effect of IBA (contained in Seradix) on the survival and subsequent rooting of cuttings of Clones 1 and 2, a study was devised to determine if IBA supplied in a liquid medium to the basal end of cuttings also inhibited survival and

rooting. Further, there is evidence for tissue sensitivity to auxin changing throughout the rooting process with maximal sensitivity to auxin not occurring directly after taking a cutting (Gaspar and Hofinger 1988; de Klerk et al. 1999; Luckman and Menary 2002). Consequently, the second objective was to determine the effect on survival and rooting of cuttings subjected to a quick-dip vs. prolonged exposure to IBA in a liquid medium.

The concentration of the analytical grade IBA solution used was 3 g kg^{-1} IBA, the same found in Seradix 2. Cuttings were exposed to the IBA solution over two different time periods (10 seconds and 5 hours) to determine the effect of prolonged exposure to IBA on the survival and rooting of cuttings. Exposure to the IBA solution at both time intervals adversely affected the survival of cuttings of Clones 1 and 2 (Table 6) ($p < 0.05$). Only 11% of Clone 1 cuttings survived the 10 second IBA treatment, while the 5 hours exposure resulted in 0% survival. However, when rooting was considered for only the cuttings that survived, there were no significant differences between the control (63%) and the 10 second (54%) IBA treatments.

Table 6 Survival (%) and rooting (%) of cuttings of two *Eucalyptus grandis* x *E. nitens* clones exposed to a control (no IBA) and IBA solution (3 g kg^{-1} IBA) for 10 seconds and 5 hours. Controls are the same as Table 5. Means ± standard errors within columns followed by lowercase letters are significantly different amongst the treatments within each clone; uppercase letters denote significant differences between the clones ($p < 0.05$) (n = 120). NB: Rooting (%) was calculated from the number of cuttings that survived.

Clone	IBA Treatment	Survival (%)	Rooting (%)
Clone 1	Control	$89 \pm 4.532^{a, A}$	$63 \pm 6.345^{a, A}$
	10 s	$11 \pm 4.722^{b, A}$	$54 \pm 14.845^{a, A}$
	5 h	$0 \pm 0.000^{b, A}$	-
Clone 2	Control	$79 \pm 6.038^{a, A}$	$19 \pm 1.965^{ac, B}$
	10 s	$5 \pm 3.184^{b, A}$	$67 \pm 16.412^{b, A}$
	5 h	$17 \pm 3.292^{b, B}$	25 ± 8.944^{c}

These results substantiated the previous observations that IBA negatively affected the survival and not rooting of Clone 1 cuttings. The percentage rooting of Clone 2 cuttings which survived was variable amongst the treatments, with a relatively high rooting percentage observed in the 10 second IBA treatment only. As with Clone 1, the exposure of Clone 2 cuttings to IBA negatively affected only the survival and not the rooting of cuttings. There were no significant differences, in terms of survival and rooting, between the two clones treated with the IBA solution for 10 seconds. However, none of the Clone 1 cuttings survived the 5 hour exposure to IBA while 17% of Clone 2 cuttings did ($p < 0.05$).

4.1.4 Effect of Seradix concentration

a) Seradix applied at initial setting

The results from previous investigations (Tables 3, 5 and 6) indicated that an IBA concentration of 3 g kg^{-1} inhibited survival of cuttings. Hence the objective of this study was to ascertain the concentration of IBA that would promote root formation without inhibiting survival, thereby resulting in a greater number of successfully rooted cuttings. For this study, Clone 3 (of which there was limited material available for the earlier studies) was also used.

The results confirmed the negative effect of Seradix on the survival of Clone 1 cuttings (Table 7). The highest survival percentage for cuttings of Clone 1 (74%) was obtained in the absence of Seradix. As the concentration of Seradix increased, the survival of Clone 1 cuttings decreased significantly. The application of Seradix 0.5 (0.5 g kg^{-1} IBA) resulted in 70% survival which was not significantly different from the control treatment. However, the survival of cuttings subjected to Seradix 1 (42%), 2 (21%) and 3 (6%) was significantly lower than the control. When rooting was considered as a percentage of the cuttings that survived, it was not significantly different amongst the various treatments. Further, when considering only the cuttings that survived, high rooting percentages (67 – 92%) were achieved across all the treatments. These results indicate that despite the poor survival due to high concentrations of Seradix, the majority of the cuttings that did survive, rooted successfully. The percentage of cuttings that survived and produced callus was variable amongst the different treatments and no trends were observed.

For cuttings of Clone 2 there were no significant differences amongst the control (72%), Seradix 0.5 (83%), Seradix 1 (72%) and Seradix 2 (48%) treatments with regards to survival. However, the application of Seradix 3 significantly reduced the percentage survival of Clone 2 cuttings (33%). With respect to the rooting of Clone 2 cuttings, there were no significant differences

when calculated as a percentage of total cuttings placed. There were no significant differences amongst the different treatments with regards to callus formation. When comparing rooting and callus as a percentage of the total cuttings planted, the majority of the cuttings that formed callus also rooted.

For Clone 3 cuttings, there were no significant differences in survival (67 – 80%), rooting (26 – 48%) and callus formation (37 – 47%) amongst the various treatments. For all treatments, the majority of Clone 3 cuttings that developed callus also rooted.

No significant differences were recorded for all tested parameters amongst the control treatment of all three clones. When subjected to Seradix 0.5, there were significant differences between the rooting (as percentage total cuttings) of Clone 1 (64%) and Clone 3 (37%) cuttings. Significant differences were also observed between percentage rooting of Clone 1 (92%) and Clone 3 (48%) cuttings, but only if considering the cuttings that survived. Cuttings of Clone 1 subjected to treatment with Seradix 1, displayed significantly higher rooting (percentage cuttings that survived) and lower callus formation (as percentage total cuttings and as percentage cuttings that survived) than cuttings of Clones 2 and 3 (Table 7). Clone 1 cuttings in the Seradix 2 treatment exhibited significantly lower survival and rooting (as percentage total cuttings) than those of Clones 2 and 3. However, when rooting was taken as a percentage of only the cuttings that survived, there were no significant differences observed amongst the three clones. Similarly, callus formation (as percentage total cuttings) amongst the clones was significantly lower for Clone 1 cuttings (18%). In contrast, when only the cuttings that survived were considered, Clone 1 (86%) and 2 (77%) cuttings had significantly higher callus formation than Clone 3 (59%) cuttings. While the application of Seradix 3 proved inhibitory in terms of survival to cuttings of Clones 1 and 2, Clone 3 cuttings were relatively unaffected. Consequently, when subjected to Seradix 3, Clone 3 cuttings displayed significantly higher survival, rooting and callus formation (the latter two as percentage total cuttings) when compared to Clone 1 and 2 cuttings. On the other hand, when considering only the cuttings that survived, there were no significant differences observed amongst the three clones with regards to rooting and callus formation.

Table 7 The effect of different concentrations of Seradix applied at placement of cuttings of three *Eucalyptus grandis* x *E. nitens* clones, on survival (%), rooting (%) and callus formation (%). Control = no Seradix, Seradix 0.5 = 0.5 g kg^{-1} IBA, Seradix 1 = 1 g kg^{-1} IBA, Seradix 2 = 3 g kg^{-1} IBA and Seradix 3 = 8 g kg^{-1} IBA. Results were recorded five weeks after placement of cuttings. Means ± standard errors within columns followed by lowercase letters are significantly different amongst treatments within clones; uppercase letters denote significant differences amongst the clones ($p < 0.05$) (n = 100).

Clone	Treatment	Survival (%)	Rooting (as % total cuttings)	Rooting (as % cuttings that survived)	Callus (as % total cuttings)	Callus (as % cuttings that survived)
Clone 1	Control	$74 \pm 3.889^{a, A}$	$52 \pm 6.009^{a, A}$	$70 \pm 7.389^{a, A}$	$44 \pm 5.256^{a, A}$	$59 \pm 5.403^{ab, A}$
	Seradix 0.5	$70 \pm 7.416^{a, A}$	$64 \pm 6.595^{a, A}$	$92 \pm 3.564^{a, A}$	$31 \pm 6.403^{ab, A}$	$44 \pm 7.358^{bc, A}$
	Seradix 1	$42 \pm 5.148^{b, A}$	$39 \pm 5.788^{ab, A}$	$92 \pm 3.816^{a, A}$	$12 \pm 4.637^{bc, A}$	$29 \pm 9.363^{c, A}$
	Seradix 2	$21 \pm 1.871^{bc, A}$	$15 \pm 2.739^{bc, A}$	$71 \pm 11.165^{a, A}$	$18 \pm 2.000^{bc, A}$	$86 \pm 8.307^{a, A}$
	Seradix 3	$6 \pm 0.000^{c, A}$	$4 \pm 0.000^{c, A}$	$67 \pm 0.000^{a, A}$	$4 \pm 0.000^{c, A}$	$67 \pm 0.000^{ac, A}$
Clone 2	Control	$72 \pm 7.151^{a, A}$	$45 \pm 4.113^{a, A}$	$63 \pm 6.103^{ab, A}$	$34 \pm 2.986^{a, A}$	$47 \pm 6.137^{a, A}$
	Seradix 0.5	$83 \pm 4.062^{a, A}$	$40 \pm 5.701^{a, AB}$	$48 \pm 6.332^{a, B}$	$40 \pm 2.739^{a, A}$	$48 \pm 3.382^{a, A}$
	Seradix 1	$72 \pm 8.155^{a, A}$	$39 \pm 5.099^{a, A}$	$54 \pm 3.302^{ab, B}$	$40 \pm 8.515^{a, B}$	$55 \pm 8.346^{a, AB}$
	Seradix 2	$48 \pm 7.517^{ab, B}$	$36 \pm 5.568^{a, B}$	$75 \pm 2.462^{ab, A}$	$37 \pm 5.385^{a, B}$	$77 \pm 5.706^{a, AB}$
	Seradix 3	$33 \pm 8.959^{b, A}$	$28 \pm 9.184^{a, AB}$	$85 \pm 8.721^{b, A}$	$24 \pm 9.573^{a, A}$	$73 \pm 12.55^{a, A}$
Clone 3	Control 1	$71 \pm 3.675^{a, A}$	$47 \pm 3.227^{a, A}$	$67 \pm 4.004^{a, A}$	$37 \pm 4.573^{a, A}$	$52 \pm 5.201^{a, A}$
	Seradix 0.5	$75 \pm 8.367^{a, A}$	$37 \pm 7.517^{a, B}$	$48 \pm 5.468^{ab, B}$	$39 \pm 6.205^{a, A}$	$53 \pm 6.380^{a, A}$
	Seradix 1	$73 \pm 4.062^{a, A}$	$26 \pm 6.964^{a, A}$	$34 \pm 8.959^{b, B}$	$47 \pm 7.842^{a, B}$	$64 \pm 9.524^{a, B}$
	Seradix 2	$67 \pm 4.637^{a, B}$	$38 \pm 6.633^{a, B}$	$55 \pm 6.414^{ab, A}$	$40 \pm 6.325^{a, B}$	$59 \pm 7.675^{a, B}$
	Seradix 3	$80 \pm 3.536^{a, B}$	$48 \pm 4.637^{a, B}$	$60 \pm 4.919^{ab, A}$	$47 \pm 2.550^{a, B}$	$59 \pm 3.886^{a, A}$

b) Delayed application of Seradix

As discussed in Section 1.3, there is evidence which suggests that exogenously supplied auxin is rapidly conjugated or transported away from the rooting zone (Blakesley et al. 1991; Blythe et al. 2007). Consequently, in cuttings of some genotypes, auxin may be unavailable when required to initiate rooting. According to Luckman and Menary (2002), the delayed application of exogenous rooting PGRs can potentially improve the survival and rooting of *Eucalyptus* cuttings. The present study tested that hypothesis using the same Seradix concentrations as for the previous study (Table 7) but applied after two weeks of setting the cuttings (Table 8).

Regarding Clone 1 cuttings, the results indicated that there were no significant differences in percentage survival of the control, Seradix 0.5, 1, and 2 treatments (Table 8). The high IBA concentration present in Seradix 3 inhibited survival (48%) significantly when compared to the control (74%). Percentage shoot survival decreased with increasing Seradix concentration for cuttings (Table 8), similarly to when Seradix was applied on the day that cuttings were set (Table 7). The percentage rooting, when considered as percentage of the cuttings that survived, was not significantly different amongst the various tested treatments. Callus formation amongst treatments was variable with no discernible trend. For this clone the results indicate that Seradix supplied two weeks after cuttings were set (Table 8) had the same effect on the parameters measured as when it was supplied on the day that cuttings were set (Table 7).

For cuttings of Clone 2, despite relatively high survival percentages (42 – 91%) across the treatments, rooting (as percentage total cuttings) was only 22 – 45% (Table 8). These results support those observed in the previous study (Table 7), which indicated recalcitrance to rooting of Clone 2 cuttings. Cuttings of Clone 3 displayed high survival percentages (71 – 81%) amongst the treatments but, unlike in the previous study (Table 7), there were significant differences in rooting amongst the treatments. When compared to the control, Seradix 2 and 3, cuttings exposed to Seradix 0.5 and Seradix 1 yielded significantly lower rooting (10% and 16%, respectively) (Table 8). The percentage of Clone 3 cuttings which developed callus was variable amongst the treatments (Table 8).

The comparison amongst the clones indicated that, in general, cuttings of Clones 2 and 3 rooted less and developed more callus than those of Clone 1 when subjected to Seradix 0.5 (Table 8). Cuttings of Clone 1 subjected to Seradix 1 displayed significantly higher rooting and lower callus formation percentages than both Clones 2 and 3 cuttings. The comparison amongst the

Table 8 The effects of different concentrations of Seradix, when applied two weeks after the placement of cuttings of three *Eucalyptus grandis* x *E. nitens* clones, on survival (%), rooting (%) and callus formation (%). Control = no Seradix, Seradix 0.5 = 0.5 g kg^{-1} IBA, Seradix 1 = 1 g kg^{-1} IBA, Seradix 2 = 3 g kg^{-1} IBA and Seradix 3 = 8 g kg^{-1} IBA. All results were recorded 5 weeks after placement of cuttings. Means ± standard errors within columns followed by lowercase letters are significantly different amongst treatments within each clone; uppercase letters denotes significant differences amongst the clones ($p < 0.05$) (n = 100).

Clone	Treatment	Survival (%)	Rooting (as % total cuttings)	Rooting (as % cuttings that survived)	Callus (as % total cuttings)	Callus (as % cuttings that survived)
Clone 1	Control	74 ± 3.889 $^{a, A}$	52 ± 6.009 $^{ab, A}$	70 ± 7.389 $^{a, A}$	44 ± 5.256 $^{a, A}$	59 ± 5.403 $^{a, A}$
	Seradix 0.5	72 ± 2.550 $^{ab, A}$	64 ± 6.595 $^{a, A}$	88 ± 7.082 $^{a, A}$	28 ± 3.391 $^{ab, A}$	39 ± 5.046 $^{ab, A}$
	Seradix 1	69 ± 5.788 $^{ab, A}$	61 ± 6.595 $^{ab, A}$	88 ± 2.577 $^{a, A}$	22 ± 3.000 $^{b, A}$	31 ± 2.059 $^{b, A}$
	Seradix 2	58 ± 8.874 $^{ab, AB}$	41 ± 4.994 $^{ab, A}$	71 ± 7.486 $^{a, A}$	30 ± 2.236 $^{ab, A}$	52 ± 8.237 $^{ab, A}$
	Seradix 3	48 ± 6.442 $^{b, A}$	33 ± 6.442 $^{b, A}$	69 ± 3.950 $^{a, A}$	31 ± 2.915 $^{ab, A}$	65 ± 9.319 $^{a, A}$
Clone 2	Control	72 ± 7.151 $^{ab, A}$	45 ± 4.113 $^{a, A}$	63 ± 6.103 $^{a, A}$	34 ± 2.986 $^{ac, A}$	47 ± 6.137 $^{ab, A}$
	Seradix 0.5	73 ± 6.245 $^{ac, A}$	22 ± 2.550 $^{b, B}$	31 ± 4.521 $^{b, B}$	43 ± 8.456 $^{ab, AB}$	59 ± 8.004 $^{ab, A}$
	Seradix 1	91 ± 2.915 $^{a, B}$	29 ± 3.674 $^{ab, B}$	32 ± 3.734 $^{b, B}$	58 ± 5.385 $^{b, B}$	64 ± 5.499 $^{a, B}$
	Seradix 2	44 ± 7.314 $^{bc, A}$	24 ± 5.788 $^{bc, A}$	55 ± 8.358 $^{ab, A}$	17 ± 4.637 $^{c, A}$	39 ± 7.033 $^{ab, A}$
	Seradix 3	42 ± 6.042 $^{c, A}$	28 ± 5.831 $^{ab, A}$	67 ± 5.537 $^{a, A}$	11 ± 1.871 $^{c, B}$	26 ± 9.014 $^{b, B}$
Clone 3	Control	71 ± 3.675 $^{a, A}$	47 ± 3.227 $^{a, A}$	67 ± 4.004 $^{a, A}$	37 ± 4.573 $^{ac, A}$	52 ± 5.201 $^{a, A}$
	Seradix 0.5	79 ± 6.205 $^{a, A}$	10 ± 3.162 $^{b, B}$	13 ± 3.470 $^{b, B}$	62 ± 5.385 $^{b, B}$	79 ± 6.875 $^{b, B}$
	Seradix 1	81 ± 5.099 $^{a, AB}$	16 ± 4.000 $^{b, B}$	20 ± 6.573 $^{b, B}$	50 ± 4.183 $^{ab, B}$	62 ± 3.121 $^{ab, B}$
	Seradix 2	74 ± 4.301 $^{a, B}$	36 ± 7.969 $^{a, A}$	48 ± 9.708 $^{a, A}$	34 ± 6.595 $^{ac, A}$	45 ± 7.710 $^{a, A}$
	Seradix 3	74 ± 4.848 $^{a, B}$	38 ± 2.550 $^{a, A}$	52 ± 4.176 $^{a, A}$	27 ± 3.742 $^{c, A}$	36 ± 7.036 $^{a, AB}$

three clones treated with Seradix 2 showed that cuttings of Clones 2 and 3 differed significantly in terms of survival only. Cuttings of Clones 1 and 2 exposed to Seradix 3 exhibited significantly lower survival than those of Clone 3. Cuttings of Clone 2 subjected to Seradix 3 produced significantly less callus (as percentage total cuttings) than those of Clones 1 and 3. However, when callus formation was calculated from only the cuttings that survived, significant differences were only observed between Clone 1 and 2 cuttings (Table 8).

c) Comparison of the effects of Seradix applied at different times

The general practice of applying Seradix immediately after harvesting cuttings (T_{0weeks}) (Table 7) and the delayed application of Seradix after two weeks of placing cuttings (T_{2weeks}) (Table 8) was compared in terms of survival, rooting and callus formation (Table 9).

There was no significant difference in terms of percentage survival when cuttings of Clone 1 were supplied with Seradix 0.5 at initial placement (T_{0weeks}) compared with Seradix 0.5 applied two weeks after placement (T_{2weeks}) (Table 9). There were significant differences in percentage survival of cuttings depending on the different application times of Seradix 1, 2 and 3. However, only cuttings treated with Seradix 2 exhibited significantly different percentage rooting. Cuttings of Clone 1 supplied with Seradix 2 and 3 at T_{0weeks} and T_{2weeks} displayed significant differences in percentage callus formation (Table 9).

Regarding cuttings of Clone 2, there were no significant differences observed in percentage survival when cuttings were supplied with Seradix at different times in any of the treatments. However, percentage rooting of cuttings was significantly lower when Seradix 0.5, 1 and 2 were supplied at T_{2weeks} than when supplied at T_{0weeks}. As a result of the different application times of Seradix 2 and 3, cuttings of Clone 2 displayed significantly different percentage callus formation.

Cuttings of Clone 3 subjected to Seradix 0.5 at T_{0weeks} and T_{2weeks} differed significantly in all measured parameters except percentage survival. There were no significant differences observed between Seradix 1 and 2 supplied at T_{0weeks} and T_{2weeks} amongst any of the tested parameters. Cuttings of Clone 3 supplied with Seradix 3 at different times only showed significant differences with respect to callus formation.

Table 9 *p* values obtained from t-test analyses which compared the application of different concentrations of Seradix at the initial placement of the cuttings (T_{0weeks}) (Table 7) and a delayed application two weeks after placement (T_{2weeks}) (Table 8). Significant differences ($p < 0.05$) amongst the two different application times (T_{0weeks} and T_{2weeks}) for that particular concentration of Seradix for a specific clone are indicated by a *.

Clone	Treatment	Survival (%)	Rooting (as % total cuttings)	Rooting (as % cuttings that survived)	Callus (as % total cuttings)	Callus (as % cuttings that survived)
Clone 1	Seradix 0.5	0.805	1.000	0.662	0.690	0.635
	Seradix 1	0.008*	0.037	0.433	0.108	0.856
	Seradix 2	0.003*	0.002*	0.741	0.004*	0.028*
	Seradix 3	0.011*	0.043	1.000	0.003*	0.961
Clone 2	Seradix 0.5	0.216	0.020*	0.063	0.744	0.371
	Seradix 1	0.060	0.150	0.002*	0.112	0.301
	Seradix 2	0.713	0.173	0.024*	0.023*	0.002*
	Seradix 3	0.410	0.943	0.275	0.213	0.037*
Clone 3	Seradix 0.5	0.711	0.011*	0.001*	0.023*	0.022*
	Seradix 1	0.255	0.248	0.257	0.744	0.814
	Seradix 2	0.301	0.852	0.547	0.530	0.243
	Seradix 3	0.347	0.095	0.244	0.002*	0.030*

4.1.5 Seasonal effects on the survival and rooting of cuttings

Season, with related changes in temperature, light intensity, photoperiod and humidity can significantly affect the survival and rooting of cuttings (Hartmann et al. 1997). The seasonal effects on survival and subsequent rooting of cuttings of Clones 1 and 2 (Clone 3 was not tested as cuttings were limited throughout the year) were derived from data collected from previous studies. In addition, a subsidiary study was completed in March 2012 and the data set was included here (Table 10). Only data obtained for cuttings not treated with Seradix were used.

For cuttings of Clone 1, survival during Summer 2011 (74%) was significantly lower than in all the other monitored seasons (Table 10). Cuttings of Clone 1 set in Winter 2011 (July) had high percentage survival (95%) but only 33% (as a percentage of the cuttings that survived) rooted. Further, these cuttings had the highest percentage of callus formation (94%). Cuttings of Clone 2 survived best in Summer 2012 (99%). Percentage rooting of cuttings of Clone 2 was considerably higher in summer than winter. Although percentage survival was different in Summer 2011 and 2012, percentage rooting was similar (66% and 65%) when calculated as percentage of the cuttings that survived. Percentage callus formation amongst the cuttings of Clones 1 and 2 set in different seasons was variable. However, both clones recorded the highest callus formation when cuttings were set in Winter 2011 (July). Both clones displayed the highest survival and rooting when set in Summer (2012).

A comparison between the two clones revealed that there were no significant differences in all tested parameters between cuttings set in Summer 2011. For cuttings set during Winter 2011 (June) and Summer 2012 (March), Clone 1 had significantly higher percentages in all tested parameters except survival. In Winter 2011 (July) the cuttings of the two clones differed significantly only with regard to callus formation.

Table 10 The effect of summer and winter seasons on the survival (%), rooting (%) and callus formation (%) of cuttings of two *Eucalyptus grandis* x *E. nitens* clones in the absence of Seradix. Means ± standard errors within columns followed by lowercase letters were significantly different amongst the months within each clone; uppercase letters denote significant differences between the clones ($p < 0.05$) (n = 100).

Clone	Month	Survival (%)	Rooting (as % total cuttings)	Rooting (as % cuttings that survived)	Callus (as % total cuttings)	Callus (as % cuttings that survived)
Clone 1	Summer 2011 (March)	$74 \pm 3.889^{a, A}$	$52 \pm 6.009^{a, A}$	$70 \pm 7.389^{a, A}$	$44 \pm 5.256^{a, A}$	$59 \pm 5.403^{ac, A}$
	Winter 2011 (June)	$89 \pm 4.488^{b, A}$	$56 \pm 4.652^{ab, A}$	$64 \pm 6.345^{ab, A}$	$45 \pm 2.332^{a, A}$	$51 \pm 2.982^{a, A}$
	Winter 2011 (July)	$95 \pm 2.739^{b, A}$	$32 \pm 9.301^{a, A}$	$33 \pm 9.129^{b, A}$	$89 \pm 2.915^{b, A}$	$94 \pm 2.905^{b, A}$
	Summer 2012 (March)	$95 \pm 1.288^{b, A}$	$83 \pm 2.550^{b, A}$	$87 \pm 3.507^{a, A}$	$72 \pm 4.445^{b, A}$	$76 \pm 5.229^{bc, A}$
Clone 2	Summer 2011 (March)	$72 \pm 7.151^{a, A}$	$45 \pm 4.113^{a, A}$	$66 \pm 6.103^{a, A}$	$34 \pm 2.986^{ac, A}$	$51 \pm 6.137^{ac, A}$
	Winter 2011 (June)	$79 \pm 6.038^{a, A}$	$15 \pm 1.600^{b, B}$	$19 \pm 1.965^{b, B}$	$21 \pm 4.695^{a, B}$	$26 \pm 5.671^{b, B}$
	Winter 2011 (July)	$87 \pm 6.041^{a, A}$	$29 \pm 5.099^{ab, A}$	$32 \pm 4.422^{b, A}$	$58 \pm 3.391^{b, B}$	$67 \pm 3.441^{a, B}$
	Summer 2012 (March)	$99 \pm 1.000^{b, A}$	$64 \pm 4.848^{c, B}$	$65 \pm 4.760^{a, B}$	$39 \pm 4.583^{c, B}$	$39 \pm 4.400^{bc, B}$

According to the nursery's records, during 2011/12 Clone 1, 2 and 3 averaged 52%, 37% and 39% rooting, respectively. The studies reported here provided evidence of an inhibitory effect of high concentrations of Seradix (Seradix 2 and 3) on the survival of cuttings of Clones 1 and 2. In contrast, Clone 3 cuttings were unaffected by the application of Seradix 3. Since the application of Seradix did not significantly improve the percentage rooting of cuttings in any of the clones, it is recommended that cuttings are set in the absence of Seradix. Cuttings of Clones 1, 2 (Table 10) and 3 (Table 7) set in the absence of Seradix had considerably higher survival (95%, 99% and 71%, respectively) and rooting (83%, 64% and 47%, respectively, when considered as a percentage of the total cuttings set) when compared to the nursery's standard rooting practices of using Seradix 2.

4.2 Micropropagation of three *Eucalyptus* clones

4.2.1 Bud induction, multiplication and elongation

The objective of this study was to determine the survival and rooting characteristics of the clones *in vitro* as a means to investigate genotypic variations under more controlled conditions than those of cuttings. Bud break was significantly higher in Clones 1 (100%) and 3 (95%) than in Clone 2 (67%) (Table 11).

Table 11 Average bud break (%) from shoots of three clones of *Eucalyptus grandis* x *E. nitens* clones after two weeks on *bud induction medium. Mean ± standard error followed by different letters denote significant differences amongst the clones ($p < 0.05$) (n = 20).

Clone	Bud Break (%)
Clone 1	100 ± 0.000^{A}
Clone 2	67 ± 4.715^{B}
Clone 3	95 ± 5.000^{A}

*bud induction medium = MS + 0.1 mg l^{-1} biotin + 0.1 mg l^{-1} calcium pantothenate + 0.04 mg l^{-1} NAA + 0.1 mg l^{-1} BAP + 0.05 mg l^{-1} kinetin

The multiplication medium (designated M1), consisting of PGRs NAA, BAP and kinetin, has been used in our laboratory for the multiplication of a variety of *Eucalyptus* clones (Mokotedi et al. 2000; Nakhooda et al. 2011; Nakhooda et al. 2012). While Clone 1 shoots multiplied successfully on M1 (3 – 4 shoots/ explant), shoots of Clones 2 and 3 did not (Table 12) and indicated symptoms of hyperhydricity (not shown). The medium was then modified to reduce cytokinins as this has been reported to alleviate hyperhydricity (Durand-Cresswell et al. 1982; Iliev et al. 2010). The cytokinins BAP and kinetin were substituted with the less stable, more easily metabolised *trans*-zeatin (George et al. 2008) and NAA was substituted with the more easily metabolised, natural auxin IAA. This new medium (M2) resulted in 3-4 shoots/ explant for Clones 2 and 3 (Table 12). However, when Clone 1 shoots were subjected to M2, they failed to multiply, which was attributed to the large callus formation at the base, which may have inhibited the uptake of nutrients and PGRs from the medium.

Table 12 Average number of shoots produced per individual shoot of three *Eucalyptus grandis* x *E. nitens* clones when subjected to two different multiplication media formulations (*M1 and M2) over a period of four weeks (n = 50).

Clone	M1 (shoots/explant)	M2 (shoots/explant)
Clone 1	3-4	1
Clone 2	0	3-4
Clone 3	0	3-4

*M1 = MS + 0.1 mg l^{-1} biotin + 0.1 mg l^{-1} calcium pantothenate + 0.04 mg l^{-1} NAA + 0.1 mg l^{-1} BAP + 0.05 mg l^{-1} kinetin
M2 = MS + 0.1 mg l^{-1} biotin + 0.1 mg l^{-1} calcium pantothenate + 0.04 mg l^{-1} IAA + 0.3 mg l^{-1} *trans*-zeatin

Before the rooting of shoots is attempted, it is usually necessary that shoots are ≥ 1.5 cm to ensure a greater probability of survival therefore an elongation stage is required (Hartmann et al. 1997; Brondani et al. 2012a). In our laboratory, the standard elongation medium (designated E1) comprising of NAA, IBA and kinetin has been used successfully for a variety of *Eucalyptus* shoots (Nakhooda et al. 2012). However, as the elongation of shoots on E1 proved challenging for the present set of clones, a different elongation medium formulation (E2) with IAA and kinetin was tested. For all three clones, 100% elongation of shoots was achieved on E2 (Table 13).

Table 13 Shoots of three *Eucalyptus grandis* x *E. nitens* elongated (%) on two elongation media formulations (*E1 and E2) over four weeks. Means ± standard errors followed by different lowercase letters (across rows) denote significant differences between the elongation media; uppercase letters (within columns) denote significant differences amongst clones ($p < 0.05$) (n = 100).

Clone	E1 (%)	E2 (%)
Clone 1	$62 \pm 5.249^{a, A}$	$100 \pm 0.000^{b, A}$
Clone 2	$28 \pm 6.110^{a, B}$	$100 \pm 0.000^{b, A}$
Clone 3	$23 \pm 9.156^{a, B}$	$100 \pm 0.000^{b, A}$

*E1 = MS + 0.1 mg l^{-1} biotin + 0.1 mg l^{-1} calcium pantothenate + 0.2 mg l^{-1} kinetin + 0.35 mg l^{-1} NAA + 0.05 mg l^{-1} IBA
E2 = MS + 0.1 mg l^{-1} biotin + 0.1 mg l^{-1} calcium pantothenate + 0.2 mg l^{-1} kinetin + 0.37 mg l^{-1} IAA

4.2.2 Rooting

As PGRs supplied during the multiplication and elongation culture stages have a subsequent effect on root induction and development in eucalypts (Nakhooda et al. 2011), shoots elongated on E1 and E2 were used to investigate rooting responses. To determine the best concentration of IBA required to promote *in vitro* rooting of shoots of Clones 1, 2 and 3, three IBA treatments (0, 0.1 and 1.0 mg l^{-1}) were selected to typify the range of Seradix concentrations supplied to cuttings (i.e. no IBA, low and high IBA concentrations).

For all shoots that demonstrated excessive root and callus formation, the callus at the base of the shoots was examined to ascertain if a vascular connection between the roots and the stem existed. If no vascular connection was present the shoots were considered non-viable since those shoots would not survive acclimatisation (le Roux and van Staden 1991b). However, if after the removal of the basal callus a vascular connection between the roots and the stem was observed, those shoots were considered as successfully rooted.

All shoots from all the clones elongated on standard elongation medium (E1) survived in the absence of IBA in the rooting medium (0 mg l^{-1} IBA = control) (Table 12). For all clones, the tested IBA concentrations significantly inhibited survival with no significant difference between the 0.1 mg l^{-1} and 1.0 mg l^{-1} IBA treatments, except for Clone 1. Although 100% of Clone 1 shoots survived in the absence of IBA, none rooted and 55% of shoots displayed basal callus formation. Although only 50% of Clone 1 shoots survived in the 0.1 mg l^{-1} IBA treatment, 80% of the shoots that survived rooted successfully. Of the 50% of Clone 1 shoots that survived, 100% developed callus in 0.1 mg l^{-1} IBA. The application of 1.0 mg l^{-1} IBA caused excessive callus development (Figure 6) which may have contributed to the death of Clone 1 shoots.

Despite 100% survival of Clone 2 shoots in the absence of IBA in the rooting medium, none rooted which suggested that an exogenous supply of IBA was required for root initiation. When shoots were supplied with 0.1 mg l^{-1} IBA, all shoots that survived rooted, but none survived the 1.0 mg l^{-1} IBA treatment. In the absence of IBA, all Clone 3 shoots survived but only 10% rooted. Despite significantly lower percentage survival of shoots of Clone 3 in 0.1 mg l^{-1} IBA (35%) and 1.0 mg l^{-1} IBA (30%) when compared with the control (100%), the former two treatments resulted in significantly higher percentage rooting (71% and 67%, respectively) than the control (10%). Unlike Clones 1 and 2 where 100% of shoots died, 30% of Clone 3 shoots survived exposure to 1.0 mg l^{-1} IBA.

For shoots elongated on E1, and subsequently rooted on IBA-free medium there were significant differences amongst the three clones only with regards to callus formation and none in the 0.1 mg l^{-1} IBA treatment for all tested parameters (Table 14). For all three clones subjected to IBA, 100% of the shoots that rooted developed callus.

Table 14 Survival (%), rooting (%) and callus formation (%) of shoots of three *Eucalyptus grandis* x *E. nitens* clones elongated on E1 and subsequently exposed to three different concentrations of IBA (0, 0.1, 1 mg l^{-1}) in rooting media over four weeks. Rooting and callus formation were calculated only from shoots that survived. Means ± standard errors within columns followed by lowercase letters were significantly different amongst the IBA treatments within each clone; uppercase letters denote significant differences amongst the clones ($p < 0.05$) (n = 20).

Clone	Treatment (mg l^{-1} IBA)	Survival (%)	Rooting (%)	Callus Formation (%)
Clone 1	0	$100 \pm 0.000^{a, A}$	$0 \pm 0.000^{a, A}$	$55 \pm 9.574^{a, A}$
	0.1	$50 \pm 17.321^{b, A}$	$80 \pm 9.574^{b, A}$	$100 \pm 0.000^{b, A}$
	1	$0 \pm 0.000^{c, A}$	-	-
Clone 2	0	$100 \pm 0.000^{a, A}$	$0 \pm 0.000^{a, A}$	$0 \pm 0.000^{a, B}$
	0.1	$10 \pm 5.774^{b, A}$	$100 \pm 0.000^{b, A}$	$100 \pm 0.000^{b, A}$
	1	$0 \pm 0.000^{b, A}$	-	-
Clone 3	0	$100 \pm 0.000^{a, A}$	$10 \pm 5.774^{a, A}$	$100 \pm 0.000^{a, C}$
	0.1	$35 \pm 9.574^{b, A}$	$71 \pm 16.899^{b, A}$	$100 \pm 0.000^{a, A}$
	1	$30 \pm 5.774^{b, B}$	67 ± 23.936^{b}	100 ± 0.000^{a}

Rooting Media = ¼ MS + 0.1 mg l^{-1} biotin + 0.1 mg l^{-1} calcium pantothenate + [*x*] mg l^{-1} IBA, where *x* is the specific concentration of IBA being tested

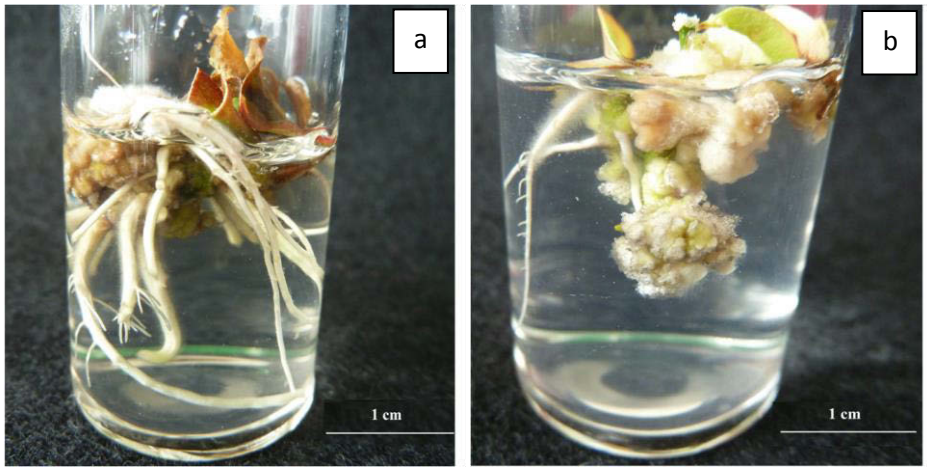


Figure 6 Excessive callus and root production typical of shoots of a) Clone 1 and b) Clone 2, when placed on rooting medium that contained 1.0 mg l^{-1} IBA, following elongation on E1.

For the protocol involving E2 elongation medium, all shoots of Clone 1 placed on IBA-free rooting medium survived but none of them rooted or developed callus (Table 15). When exposed to 0.1 mg l^{-1} IBA, 80% of shoots survived and of those, 50% rooted successfully. Of the shoots that survived, 81% formed callus. On 1.0 mg l^{-1} IBA, only 15% of the shoots survived (Figure 7) which was significantly lower than the 100% survival of shoots in the control and 80% of shoots placed in 0.1 mg l^{-1} IBA. However, of the shoots that survived exposure to 0.1 mg l^{-1} IBA and 1.0 mg l^{-1} IBA, 50% and 67% rooted, respectively. Furthermore, of the shoots that rooted, the majority developed callus.

Shoots of Clone 2 in the control and 0.1 mg l^{-1} IBA treatments achieved significantly higher survival (80% and 100%, respectively) than those subjected to 1.0 mg l^{-1} IBA (50%) (Table 15) (Figure 7). Despite their high survival in the absence of IBA, none of the shoots rooted or formed callus. However, on 0.1 mg l^{-1} IBA and 1.0 mg l^{-1} IBA rooting was significantly higher than in the control. Of the shoots that rooted, 95% and 100% formed callus in the presence of 0.1 mg l^{-1} IBA and 1.0 mg l^{-1} IBA, respectively.

Although shoots of Clone 3 demonstrated similar survival (90% and 100%, respectively) in the control and 0.1 mg l^{-1} IBA treatments the percentage of shoots which rooted (0% and 40%, respectively) and developed callus (0% and 100%, respectively) were significantly different (Table 15). Shoots on 1.0 mg l^{-1} IBA exhibited significantly lower survival (20%) (Figure 7) than the other two treatments but of the shoots that survived, 25% and 100% showed root and callus formation, respectively.

A comparison amongst the clones revealed that in the absence of IBA in the rooting medium, following elongation on E2, there were no significant differences in survival, rooting and callus formation. In the 0.1 mg l^{-1} IBA treatment, shoot survival was 80% for Clone 1 and 100% for Clones 2 and 3 ($p < 0.05$). Despite significantly different survival percentages, rooting and callus formation results indicated no significant differences amongst the clones. Shoots of clones 1, 2 and 3 exposed to 1.0 mg l^{-1} IBA, did not display significant differences in any of the parameters investigated.

Table 15 Survival (%), rooting (%) and callus formation (%) of shoots of three *Eucalyptus grandis* x *E. nitens* clones elongated on E2 and subsequently rooted on different concentrations of IBA (0, 0.1, 1 mg l^{-1}) over four weeks. Rooting and callus formation were calculated only from the shoots that survived. Means ± standard errors within columns followed by lowercase letters were significantly different amongst IBA treatments within each clone; uppercase letters denote significant differences amongst the clones ($p < 0.05$) (n = 20).

Clone	Treatment (mg l^{-1} IBA)	Survival (%)	Rooting (%)	Callus formation (%)
Clone 1	0	$100 \pm 0.000^{a, A}$	$0 \pm 0.000^{a, A}$	$0 \pm 0.000^{a, A}$
	0.1	$80 \pm 8.165^{a, A}$	$50 \pm 14.250^{b, A}$	$81 \pm 7.030^{b, A}$
	1	$15 \pm 5.000^{b, A}$	$67 \pm 28.868^{b, A}$	$100 \pm 0.000^{b, A}$
Clone 2	0	$80 \pm 14.142^{ab, A}$	$0 \pm 0.000^{a, A}$	$0 \pm 0.000^{a, A}$
	0.1	$100 \pm 0.000^{a, B}$	$75 \pm 5.000^{b, A}$	$95 \pm 5.000^{b, A}$
	1	$50 \pm 10.000^{b, A}$	$40 \pm 15.769^{b, A}$	$100 \pm 0.000^{b, A}$
Clone 3	0	$90 \pm 10.000^{a, A}$	$0 \pm 0.000^{a, A}$	$0 \pm 0.000^{a, A}$
	0.1	$100 \pm 0.000^{a, B}$	$40 \pm 8.165^{b, A}$	$100 \pm 0.000^{b, A}$
	1	$20 \pm 11.547^{b, A}$	$25 \pm 17.678^{ab, A}$	$100 \pm 0.000^{b, A}$

Rooting Media = ¼ MS + 0.1 mg l^{-1} biotin + 0.1 mg l^{-1} calcium pantothenate + [*x*] mg l^{-1} IBA, where *x* is the specific concentration of IBA being tested

Figure 7 Prolific callus formation typical of shoots of Clones 1, 2 and 3 rooted on 1.0 mg l^{-1} IBA-containing medium, following elongation on E2. These shoots would not survive acclimatisation.

The effects of the different elongation media (E1 and E2) on survival, rooting and callus formation of shoots of Clones 1, 2 and 3 were compared (Table 16). For Clone 1, in the absence of IBA, there were only significant differences observed between shoots elongated on E1 (Table 14) and E2 (Table 15) with regards to percentage callus formation (Table 16). This suggested that the IBA and NAA included in the E1 medium may have persisted through to the rooting stage and that shoots developed callus due to excess auxin. Since shoots of Clone 1 elongated on E2 medium did not form callus on the IBA-free rooting medium, this implied that the IAA present in that elongation medium was likely to have been metabolised by the shoots prior to rooting. In the 0.1 mg l^{-1} IBA and 1.0 mg l^{-1} IBA treatments no significant differences were observed between shoots elongated on different media.

Regarding shoots of Clone 2, in the absence of IBA, there were no significant differences in any of the tested parameters. However, shoots elongated on E1 medium displayed significantly lower percentage survival in 0.1 mg l^{-1} IBA and 1.0 mg l^{-1} IBA treatments than those elongated on E2 medium.

Shoots of Clone 3 displayed significant differences only in terms of percentage callus formation on IBA-free rooting medium. In the 0.1 mg l^{-1} IBA treatment, 35% of shoots elongated on E1 medium survived while all shoots elongated on E2 survived ($p < 0.05$). This suggested that IBA and NAA supplied during the E1 elongation stage accumulated with IBA supplied during the rooting stage to levels which adversely affected the survival of shoots. There were no significant differences in any of the measured parameters between shoots elongated in different media and then subjected to 1.0 mg l^{-1} IBA in the rooting stage.

Table 16 *p* values obtained from t-test analyses which compared differences in rooting between shoots elongated on E1 (Table 14) and E2 (Table 15). * denotes significant differences between shoot elongated on E1 and E2, for that specific treatment within a particular clone ($p < 0.05$).

Clone	Treatment (mg l^{-1} IBA)	Survival (%)	Rooting (%)	Callus formation (%)
Clone 1	0	1.000	1.000	0.001*
	0.1	0.168	0.257	0.320
	1	0.240	-	-
Clone 2	0	0.207	1.000	1.000
	0.1	0.000*	0.200	0.356
	1	0.002*	-	-
Clone 3	0	0.356	0.134	0.008*
	0.1	0.000*	0.152	1.000
	1	0.468	0.391	1.000

4.3 Comparison of survival and rooting responses of the two vegetative propagation methods

Cuttings and *in vitro* shoots of Clone 1 exhibited significant differences in both percentage survival and rooting in the absence of IBA (Table 17). In the absence of IBA, percentage survival for both cuttings and *in vitro* shoots was high (> 95%). However, cuttings rooted best in the absence of IBA, while *in vitro* shoots failed to root. Despite significantly different percentage survival in the moderate IBA treatment between cuttings (42%) and *in vitro* shoots (80%), percentage rooting was similar (39 and 40%, respectively). When subjected to the high IBA treatment there were no significant differences observed between cuttings and *in vitro* shoots in terms of percentage survival and rooting.

As with Clone 1, cuttings of Clones 2 and 3 rooted best in the absence of IBA while *in vitro* shoots failed to root (Table 17). In the moderate IBA treatment, *in vitro* shoots of Clones 2 and 3 had significantly higher percentage survival that those of cuttings. However, only Clone 2 exhibited differences in percentage rooting with *in vitro* shoots (75%) rooting better than cuttings (39%).

As previously shown high concentrations of IBA significantly reduced the survival of cuttings of Clones 1 and 2, while those of Clone 3 were unaffected (Table 7). However, when *in vitro* shoots were exposed to the high IBA treatment, shoots of all three clones showed reduced survival (Tables 14 and 15). Subsequently, when comparing cuttings and *in vitro* shoots treated with high IBA concentrations, only Clone 3 showed significant differences in terms of survival and rooting percentages (Table 17).

Table 17 Survival (%) and rooting (%) of the three *Eucalyptus grandis* x *E. nitens* clones when propagated as cuttings and *in vitro* and subjected to a range of IBA treatments. *In vitro* shoots elongated on E2 were used. Means ± standard errors across rows followed by different letters denotes significant differences between cuttings and *in vitro* shoots with regards to percentage survival and rooting ($p < 0.05$).

Clone	*IBA Treatment	Survival (%)		Rooting (%)	
		Cuttings	*In vitro* shoots	Cuttings	*In vitro* shoots
Clone 1	None	95 ± 1.288[a]	100 ± 0.000[b]	83 ± 2.550[a]	0 ± 0.000[b]
	Moderate	42 ± 5.148[a]	80 ± 8.165[b]	39 ± 5.788[a]	40 ± 14.142[a]
	High	6 ± 0.000[a]	15 ± 5.000[a]	4 ± 0.000[a]	10 ± 5.774[a]
Clone 2	None	99 ± 1.000[a]	80 ± 14.142[a]	64 ± 4.848[a]	0 ± 0.000[b]
	Moderate	72 ± 8.155[a]	100 ± 0.000[b]	39 ± 5.099[a]	75 ± 5.000[b]
	High	33 ± 8.959[a]	50 ± 10.000[a]	28 ± 9.184[a]	20 ± 8.165[a]
Clone 3	None	91 ± 3.675[a]	90 ± 10.000[a]	47 ± 3.227[a]	0 ± 0.000[b]
	Moderate	73 ± 4.062[a]	100 ± 0.000[b]	26 ± 6.964[a]	40 ± 8.165[a]
	High	80 ± 3.536[a]	20 ± 11.547[b]	48 ± 4.637[a]	5 ± 5.000[b]

*IBA treatments for cuttings = none (no Seradix), moderate (Seradix 1 [1 g kg^{-1} IBA]) and high (Seradix 3 [8 g kg^{-1} IBA])

*IBA treatments for *in vitro* shoots = none (no IBA), moderate (0.1 mg l^{-1} IBA) and high (1.0 mg l^{-1} IBA)

5. DISCUSSION

As previously discussed, the South African Forestry Industry is dependent on the low-cost, fast-growing wood supplied by various *Eucalyptus* species and hybrids. The vegetative propagation of superior selected genotypes via rooted cuttings is the most commonly-used method for the rapid deployment of 'true-to-type' clones (Hartmann et al. 1997; de Assis et al. 2004; Brondani et al. 2010; Borges et al. 2011). However, some *Eucalyptus* genotypes which display desirable growth characteristics and wood properties show recalcitrance to root induction (Bayley and Blakeway 2002). Consequently, the first aim of this study was to improve the protocol used by a commercial nursery for the propagation of three *Eucalyptus grandis* x *Eucalyptus nitens* clones. *Eucalyptus* genotypes which have proven difficult to propagate via conventional methods have indicated greater success when *in vitro* culture methods have been employed (Mokotedi et al. 2000; Yasodha et al. 2004; George et al. 2008). As the tested clones are known to have variable rooting responses when set as cuttings under commercial nursery conditions (Pollard pers. com.[1]), their rooting potential *in vitro* was also investigated.

5.1 Effects of types of cuttings and season on survival and rooting

At Sunshine Seedlings Services nursery, the standard practice for *Eucalyptus* species and hybrids involves supplying soft, apical cuttings harvested from juvenile (approximately three year old parent plants) with Seradix 2 (active ingredient, 3 g kg^{-1} IBA) at placement. Soft cuttings of Clones 1 and 2 rooted (32% and 29%, respectively) significantly better than hard cuttings (2% and 8%, respectively) (Table 4). This validated the use of soft cuttings by the nursery for the propagation of *Eucalyptus* species and hybrids.

In the present study, cuttings of Clones 1 and 2 rooted best in summer (83% and 64%, respectively) when compared with winter (56% and 15%, respectively). Further, cuttings of Clone 2 seemed to be more sensitive to the seasonal fluctuations with respect to rooting than those of Clone 1 (Table 10). According to nursery records, cuttings of Clones 1 and 2 rooted best during winter (Pollard pers. com.[2]) which is in contrast to the results obtained in this study. These contradictory results could be attributed to prolonged storage time and exposure to stress of the cuttings by the nursery. Nursery staff collect and set a much greater quantity of cuttings than set for each individual study reported here. Consequently, cuttings taken by the nursery staff are subjected to longer periods of stress and temporary storage in water before they are

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eventually set in the growth medium in the rooting tunnels. A study conducted by Goulart and Xavier (2008) determined that the best percentage rooting of four clones of *E. grandis* x *E. urophylla* occurred when cuttings were placed in growth medium in a greenhouse immediately after collection from the clonal garden. Further, when cuttings were stored even for a relatively short period of time, percentage rooting was negatively affected (Goulart and Xavier 2008).

5.2 The relationship between the survival and rooting of cuttings and auxin supply

The results obtained from the series of studies conducted revealed that of the tested clones, the survival of cuttings from Clone 1 were the most adversely affected by the application of increasing concentrations of Seradix (> 1 g kg^{-1} IBA) (Table 7). Further, the application of Seradix 0.5 (64%), Seradix 1 (39%), Seradix 2 (15%) and Seradix 3 (4%) did not significantly improve the rooting of cuttings of Clone 1 when compared with the control (52%) (Table 7). Titon et al. (2003) found similar trends when *E. grandis* cuttings were subjected to increasing concentrations of IBA (> 2 g kg^{-1} IBA) which considerably reduced survival. Although the delayed application of Seradix 1, 2 and 3 significantly improved (Table 9) the survival of cuttings of Clone 1 (69%, 58% and 48%, respectively) (Table 8) when compared with the general practice of supplying rooting powder at the initial placement of cuttings (42%, 21% and 6%, respectively) (Table 7), this was inconsequential since the best survival occurred in the absence of Seradix (Table 7). The highest percentage survival and rooting for cuttings of Clone 1 were attained in the absence of Seradix (95% and 83%, respectively) (Table 10). These results indicate that Clone 1 was a good rooter, capable of attaining > 80% rooting which suggested that the endogenous PGR balance was predisposed in favour of root induction. When exogenous auxins were then supplied to cuttings of Clone 1, the endogenous PGR balance changed which probably caused the increased cutting mortality as observed with cuttings of *E. grandis* (Titon et al. 2003).

The survival and subsequent rooting of cuttings of Clone 2 were significantly reduced only when subjected to the highest tested Seradix concentration (8 g kg^{-1} IBA) (Table 7). Other studies, conducted with a variety of *Eucalyptus* species and hybrids, supported the suggestion that if exogenous IBA was required for adventitious root formation in cuttings, it may improve rooting up until a certain concentration, after which a higher IBA concentration may become toxic (Titon et al. 2003; Wendling and Xavier 2005; de Almeida et al. 2007; Goulart et al. 2008; Lana et al. 2008). As with Clone 1, the best rooting results for cuttings of Clone 2 (64%) were obtained in the absence of rooting hormone (Table 10).

The tested concentrations of Seradix did not significantly influence nor improve the percentage survival (67 – 80%) or rooting (26 – 48%) of cuttings of Clone 3 (Table 7). Since there was no significant difference in rooting, and the application of rooting powder is both costly and requires more time and effort, the best treatment would be to set cuttings of Clone 3 in the absence of Seradix.

Cuttings from all the clones survived and rooted best in the absence of exogenous rooting hormone. Despite high percentage survival in all the clones, cuttings of Clone 1 showed the highest percentage rooting (83%) (Table 10) while cuttings of Clones 2 and 3 attained only 64% (Table 10) and 47% (Table 7) respectively, under the control conditions. Since the application of 0.5 to 8 g kg^{-1} of IBA did not improve rooting, it is suggested that these two clones are either genetically limited in terms of rooting predisposition or that they require further optimisation of environmental parameters.

The cuttings of all three clones did not require exogenous application of auxin probably because they were sourced from juvenile (approximately three year old) parent plants. The good rooting ability of cuttings sourced from juvenile parent plants has been well documented in the literature. Mankessi et al. (2011) determined that cuttings taken from juvenile three month old *E. urophylla* x *E. grandis* parent plants outperformed those taken from mature eight year old trees in terms of survival and rooting. In addition, the cuttings obtained from juvenile parent plants rooted successfully in the absence of exogenously supplied PGRs (Mankessi et al. 2011). *E. cloeziana* cuttings from 15 year old trees indicated better rooting in the presence of IBA (1.5 – 6.0 g kg^{-1}) while cuttings sourced from five year old trees rooted better in the absence of IBA (de Almeida et al. 2007). In a study conducted by Maile and Nieuwenhuis (1996), cuttings harvested from mature (11 year old) *E. nitens* trees failed to root while cuttings taken from juvenile, three year old parent plants had 67% rooting in the absence of IBA. Further, when exogenous IBA was supplied, the rooting percentage decreased significantly to 23%. The results obtained in the present investigation support those studies, and suggest that cuttings obtained from juvenile parent plants do not require the exogenous application of auxin for root induction and that in certain clones (e.g. Clone 1), the unnecessary application of exogenous IBA can result in cutting mortality.

5.3 Shoot survival and rooting responses *in vitro*

Regarding shoot proliferation, only shoots of Clone 1 multiplied successfully in medium containing NAA, BAP and kinetin (M1), while those of Clones 2 and 3 displayed

hyperhydricity. Shoots of *E. benthamii* x *E. dunnii* supplied with NAA and BAP also failed to multiply as a result of hyperhydricity (Brondani et al. 2011). The inclusion of > 1 mg l^{-1} BAP in the multiplication medium reduced the number of buds/explants in *E. globulus* (Bennett et al. 1994), *E. tereticornis* x *E. camaldulensis* (Bisht et al. 1999) and *E. tereticornis* (Sharma and Ramamurthy 2000). However, the combination of low concentrations of BAP (0.2 mg l^{-1}) and NAA (0.01 mg l^{-1}) produced the best shoot proliferation in *E. nitens* (Gomes and Canhoto 2003). In this study, replacing NAA, BAP and kinetin (M1) with the more easily metabolised PGRs IAA and *trans*-zeatin (M2) enabled shoot proliferation in Clones 2 and 3.

Furze and Cresswell (1985) found that in shoots of good rooting clones of *E. grandis* and *E. nitens*, multiplication medium containing *trans*-zeatin was unsuitable as large amounts of callus formed at the base of shoots. The results obtained in the present investigation support that study since Clone 1, the good rooter, formed large amounts of basal callus which inhibited bud proliferation while intermediate rooters, Clones 2 and 3, multiplied successfully on *trans*-zeatin (M2) (Table 13). Since the three clones responded differently to the tested multiplication media, it suggests that their endogenous PGR composition may have differed. According to Mokotedi et al. (2000) even different clones from the same parental stock may have different PGR requirements to promote rhizogenesis.

Shoots from all the clones elongated better on E2 (containing kinetin and IAA) than on E1 (containing kinetin, NAA and IBA) (Table 14). It is possible that the shoots responded better to the more easily metabolised auxin IAA than the more stable auxins NAA and IBA. Since metabolically stable PGRs may persist through to subsequent culture stages (Nakhooda et al. 2011), it is plausible that PGRs supplied during the multiplication stage persisted through to the elongation culture stage and accumulated to levels which may have inhibited the successful elongation of shoots. It has been observed that the substitution of the synthetic cytokinin kinetin with the natural, less stable *trans*-zeatin during elongation significantly increased the rooting ability of *E. grandis* x *E. nitens* shoots (Nakhooda et al. 2012). Further, the complete omission of cytokinins during the elongation stage resulted in the best rooting percentages (80.3%) (Nakhooda et al. 2012).

During the rooting stage, shoots of all three clones, irrespective of elongation medium, showed high percentage survival (> 80%) but failed to root in the absence of IBA (Tables 15 and 16). However, when subjected to a high concentration of IBA (1.0 mg l^{-1}) in the rooting medium, shoots of all the clones regardless of elongation on E1 or E2, showed considerably reduced

percentage survival (Tables 15 and 16). When shoots of the three clones were elongated on E1 and rooted in the moderate IBA treatment (0.1 mg l^{-1}), percentage survival was low (< 50%) but of the shoots that survived, majority rooted (> 71%). This suggests that a lower concentration of IBA (< 0.1 mg l^{-1}) may be required to promote rhizogenesis without inhibiting the survival of the shoots.

The highest rooting percentage of shoots of *E. tereticornis* x *E. camaldulensis* (Bisht et al. 1999) and *E. tereticornis* x *E. grandis* (Joshi et al. 2003) was obtained with 1.0 mg l^{-1} IBA in the rooting medium. However, when shoots were rooted on > 1.0 mg l^{-1} IBA, the percentage rooting was lower and callus formed. Similar responses were observed in shoots of *E. benthamii* x *E. dunnii* when rooted on medium containing > 2.0 mg l^{-1} IBA (Brondani et al. 2011). *E. tereticornis* explants obtained from freshly coppiced shoots from ten year old trees (Aggarwal et al. 2012) and *E. camaldulensis* shoots obtained from 18 month old plants rooted best in 1.0 mg l^{-1} IBA (Girijashankar 2012). In contrast, shoots from *E. grandis* x *E. nitens* clones obtained from one year old plants, achieved the highest rooting percentage in 0.1 mg l^{-1} IBA (Mokotedi et al. 2000). Those studies and the results obtained in the present investigation suggest that shoots obtained from juvenile parent plants do not require the exogenous application of high concentrations of IBA to induce rhizogenesis.

As previously discussed, each phase of the rooting process has specific PGR requirements (Pop et al. 2011). In some cases, a high auxin concentration is required only during the induction phase, and continued exposure to a high auxin concentration during the subsequent rooting phases may inhibit rhizogenesis (de Klerk et al. 1999). As reported by Fett-Neto et al. (2001), shoots of difficult-to-root *E. globulus* rooted best when subjected to high IBA concentrations for just four days and then transferred onto IBA-free medium. Shoots of *E. regnans* (Blomstedt et al. 1991) and *E. erythronema* x *E. stricklandii* (Glocke et al. 2006b) exposed to high auxin concentrations for a short period of time (acute treatment) displayed improved percentage rooting and reduced proliferation of callus when compared with shoots which were continually exposed to a lower auxin concentration (chronic treatment). Since, in this study, the chronic IBA treatment was unable to produce sufficient rooted shoots for Clones 1 and 3, an acute IBA treatment could possibly improve rooting and reduce callus formation.

Although auxins are considered the 'rooting' PGR it must be emphasised that the effects of a single PGR cannot be viewed in isolation as there exists a complex interaction with other PGRs which contribute to either the success or failure of a propagule to initiate roots (Hartmann et al.

1997; George et al. 2008). Amongst the PGRs, the antagonistic relationship between auxins and cytokinins is well-known and the general model follows that a high auxin to low cytokinin ratio favours root formation while a high cytokinin to low auxin ratio favours shoot proliferation (Hartmann et al. 1997; George et al. 2008). As mentioned, cytokinins (such as kinetin and BAP) supplied during stages preceding rooting may disrupt the balance of auxins and cytokinins, thereby delaying or even inhibiting root initiation of *in vitro* shoots (Nakhooda et al. 2011).

Since shoots elongated on E1 were exposed to more metabolically stable PGRs than shoots elongated on E2, this could possibly explain the recorded reduced percentage rooting of the former. For all three clones, the best survival and rooting *in vitro* was achieved when shoots were subjected to 0.1 mg l^{-1} IBA in the rooting medium, following elongation on E2 (Table 15). The PGRs supplied during the stages preceding rooting may have persisted through to the rooting stage and disrupted the endogenous PGR balance which was already predisposed in favour of root induction. Consequently, *in vitro* shoots required 0.1 mg l^{-1} IBA supplied during the rooting stage, possibly to counteract the antagonistic effects of cytokinins supplied during the multiplication and elongation stages, and enable adventitious root formation. According to Nakhooda et al. (2012) root induction in poor rooters may be dependent on the depletion of supplied cytokinins. Consequently, it is suggested that PGRs which are easily metabolised are employed to prevent their persistence through to subsequent culture stages.

5.4. Comparison of cuttings and *in vitro* survival and rooting responses

The survival and rooting responses of cuttings and *in vitro* shoots of the three clones differed significantly in particular IBA treatments (Table 17). In the absence of IBA, cuttings and *in vitro* shoots of all the clones had high percentage survival (> 80%). However, while cuttings rooted successfully, *in vitro* shoots failed to root in the absence of IBA. This could be attributed to different PGR balances between cuttings and *in vitro* shoots prior to the rooting stage. As previously discussed, since cuttings of the three clones were sourced from juvenile parent plants, the application of exogenous PGRs was unnecessary as the PGR balance was already predisposed in favour of root induction. However, *in vitro* shoots were exposed to a range of PGRs during the multiplication and elongation culture stages which may have persisted through to the rooting stage, thereby disrupting the PGR balance.

When subjected to the moderate IBA treatment (1 g kg^{-1} IBA for cuttings and 0.1 mg l^{-1} IBA for *in vitro* shoots) percentage survival of *in vitro* shoots was significantly higher than those of cuttings for all three clones. Despite this, only Clone 2 showed significantly different percentage

rooting between cuttings (39%) and *in vitro* shoots (75%). This indicates that of the three clones, Clone 2 responded best to the tested culture protocol.

In the high IBA treatment (8 g kg^{-1} IBA for cuttings and 1.0 mg l^{-1} IBA for *in vitro* shoots) only Clone 3 showed significant differences in survival and rooting responses between cuttings and *in vitro* shoots. Unlike Clones 1 and 2, cuttings of Clone 3 were unaffected by the high (8 g kg^{-1} IBA) Seradix concentration (Table 7). However, *in vitro*, the survival and rooting of Clone 3 shoots were significantly reduced when subjected to the high IBA (1.0 mg l^{-1} IBA) treatment (Table 15). This indicates that while cuttings of Clone 3 were tolerant of the high IBA treatment in the greenhouse, *in vitro,* the range of PGRs supplied during the multiplication and elongation culture stages may have persisted through and combined with the high IBA concentration supplied in the rooting stage, and accumulated to levels which inhibited survival and rooting of the shoots.

In terms of yield, taking into consideration the time and cost factor, cuttings of Clones 1 and 3 would produce a greater number of rooted shoots than micropropagation under the current culture protocol. Since Clone 2 had the highest percentage rooting under *in vitro* conditions, the potential yield of rooted shoots produced through micropropagation is greater than if propagated by cuttings.

According to the literature, many *Eucalyptus* species and hybrids which are difficult-to-root via cuttings can be successfully propagated using *in vitro* culture methods (Mokotedi et al. 2000; Sharma and Ramamurthy 2000; Fett-Neto et al. 2001; Gomes and Canhoto 2003; Nourissier and Monteuuis 2008; Hung and Trueman 2011; Aggarwal et al. 2012; Brondani et al. 2011). Fett-Neto et al. (2001) managed to successfully promote *in vitro* rhizogenesis of difficult-to-root *E. globulus* shoots by following a two-step sequential medium protocol which comprised of a four day induction step on a wide range of IBA concentrations (0 – 100 mg l^{-1}) followed by root formation on IBA-free medium with 1 g l^{-1} of activated charcoal (Fett-Neto et al. 2001). Activated charcoal was included since it is known to improve cell growth and development in tissue cultured material (Thomas 2008). For *E. saligna* shoots, rooting percentage was high (~ 75%) in all IBA treatments. Shoots of *E. globulus* achieved approximately 60% rooting in all IBA treatments but percentage rooting was significantly higher when shoots were exposed to the highest IBA concentrations (10 and 100 mg l^{-1}) during the four day root induction phase (Fett-Neto et al. 2001). This suggested that difficult-to-root clones required higher concentrations of IBA to successfully induce root formation while easy-to-root clones required

less. de Almeida et al. (2007) found similar results with easy-to-root clones of *E. cloeziana* responding better to low IBA concentrations while difficult-to-root clones required a higher concentration of IBA. In the present study, the *in vitro* protocol did not result in survival and rooting percentages greater than those obtained though macropropagation for Clones 1 and 3. However, it is evident that a greater optimisation of the *in vitro* protocol could possibly lead to better rooting responses in the tested clones.

5.5 The relationship between callus formation and rooting

The formation of callus at the base of a propagule occurs in response to wounding or as a result of unbalanced PGRs, particularly excess auxin (Hartmann et al. 1997). In some cases, roots emerge through the callus with a complete vascular connection to the stem and the callus eventually degrades with time (Hartmann et al. 1997). However, it has been observed that the formation of large amounts of basal callus inhibits the effective absorption of water and nutrients suggesting that there is no vascular connection between the roots and the stem (le Roux and van Staden 1991b; Mokotedi et al. 2000; Brondani et al. 2012b; Mycock and Watt 2012).

In this investigation, callus formation in cuttings and *in vitro* shoots of Clones 1, 2 and 3 varied considerably in the different studies. Callus formation in cuttings of Clone 1 were distinctly variable depending on the Seradix concentration used (Table 7) and cuttings subjected to the higher Seradix concentrations (3 g kg^{-1} and 8 g kg^{-1} IBA) tested formed greater amounts of basal callus which may have contributed to survival inhibition. Callus formation in cuttings of Clones 1 and 2, in the absence of exogenous auxin, differed significantly during the year suggesting that the endogenous PGR content changes with the seasons. Since the endogenous PGR content is influenced by environmental factors such as light and temperature (Hartmann et al. 1997; Fett-Neto et al. 2001; de Assis et al. 2004; Corrêa and Fett-Neto 2004; Brondani et al. 2010) it stands to reason that callus formation would differ as a consequence of seasonal change. There was no discernible trend observed between the formation of callus and root induction in cuttings.

For all three clones, the majority of the *in vitro* shoots that survived and rooted also developed callus (Tables 15 and 16). However, when subjected to high concentrations of IBA (1.0 mg l^{-1}) *in vitro*, callus formation in all three clones was prolific and engulfed the shoots (Figures 6 and 7) which ultimately resulted in mortality. Similarly when shoots of *E. tereticornis* x *E. camaldulensis* (Bisht et al. 1999), *E. tereticornis* x *E. grandis* (Joshi et al. 2003) and *E.*

benthamii x *E. dunnii* (Brondani et al. 2011) were subjected to > 1.0 mg l^{-1} IBA the formation of callus occurred. Mokotedi et al. (2000) found that supplementing ¼ strength MS medium with ¾ strength concentrations of calcium and magnesium during the rooting stage prevented the proliferation of callus on shoots *E. grandis* x *E. nitens*. Blomstedt et al. (1991) prevented callus formation on shoots of *E. regnans* by subjecting shoots to pulse treatments of IBA (20 mg l^{-1} IBA for 7 days) as opposed to a chronic treatment of 1.0 mg l^{-1} IBA.

5.6 Concluding remarks

The nursery obtained an average rooting of 52%, 37% and 39% for Clones 1, 2 and 3, respectively, when Seradix 2 (3 g kg^{-1} IBA) was supplied to the cuttings. In the present study, cuttings of Clones 1, 2 and 3 rooted best (83%, 64% and 47%, respectively) in the absence of exogenously supplied rooting hormone which is a considerable improvement on the nursery's rooting results. Further, taking into consideration the time, effort and cost involved in applying Seradix to cuttings, there are financial and productivity benefits to rooting cuttings in its absence.

In vitro, Clones 1, 2 and 3 had the best rooting (40%, 75% and 40%, respectively) in the presence of 0.1 mg l^{-1} IBA. Cuttings rooted best in the absence of IBA while 0.1 mg l^{-1} IBA was required for rhizogenesis *in vitro*. Since the PGR balance of these *Eucalyptus grandis* x *Eucalyptus nitens* clones was already predisposed in favour of rhizogenesis, the exposure to PGRs during the *in vitro* culture stages preceding rooting disrupted this balance. Consequently, 0.1 mg l^{-1} IBA was required by all three clones to restore this balance and promote rhizogenesis. Survival and rooting of both cuttings and *in vitro* shoots were inhibited by the highest IBA treatments (8 g kg^{-1} and 1.0 mg l^{-1}, respectively).

Although micropropagation can potentially produce a greater yield once the culture protocol is optimised, for nursery practices is it recommended that these clones be propagated as cuttings in the absence of exogenous rooting hormone.

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