# TOWARD A ROOTING PROTOCOL FOR *EUCALYPTUS DUNNII*

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# ABSTRACT

*Eucalyptus dunnii* exhibits fast growth, low lignin and high cellulose contents, traits that are highly desirable for pulp production and are therefore favoured by the paper industry. In this study, *E. dunnii* roots were produced by the various methods practiced by commercial forestry *viz.* macrocuttings, microcuttings and seedlings. Roots produced were compared and characterized in order to understand the distinctions between *Eucalyptus* roots produced by these methods.

Seedlings and clonal mini-cuttings were sourced from the Trahar Technology Centre, Mondi (South Africa). Plant shoots were established in culture and multiplied before the induction of roots *in vitro*. An optimal growth medium was determined for *E. dunnii* multiplication, which was 4.42g.$l^{-1}$ MS (with vitamins), 25g.$l^{-1}$ sucrose, 0.2g.$l^{-1}$ benzyl aminopurine, 0.01g.$l^{-1}$ naphthaleneacetic acid, 0.1g.$l^{-1}$ biotin, 0.1g.$l^{-1}$ calcium pantothenate, and 3g.$l^{-1}$ gelrite (pH of 5.6-5.8). Due to the high (68%) levels of vitrification, tests were conducted to reduce or reverse this phenomenon with no success. Only healthy material was selected for rooting experiments.

*In vitro* rooting was tested on medium containing 1.105 g.$l^{-1}$ MS (with vitamins), 15 g.$l^{-1}$ sucrose, 0.1 g.$l^{-1}$ biotin, 0.1 g.$l^{-1}$ calcium pantothenate, 4 g.$l^{-1}$ gelrite and pH of 4.5-5.8 and 0.1-1mg.$l^{-1}$ IBA without success. *In vitro* rooting (4%) was achieved on peat:vermiculite (1:2) probably due to less humidity.

Comparison of micropropagated plantlets and seedlings of the same height range showed that root architecture of main roots, main side roots, number of side root and shoot masses were all statistically similar.

Mini-cuttings underwent treatments before placement into seedling tray inserts containing coir:perlite (1:2). With the first treatment, the lower excised end of the shoot was dipped in 20mg.$l^{-1}$ IBA, for 48 hours while being incubated in a dark and humid environment; the cutting was then placed into the insert. With the second treatment, mini-cuttings were placed directly into the prepared inserts for four weeks. Thereafter, the shoots were carefully removed and underwent the same process as with treatment one. The third treatment involved dipping mini-cuttings in Seradix 1

(0.1% IBA) and placement into the prepared inserts. Treatment 4 involved three pulse treatments which were tested by placing mini-cuttings into various concentrations of IBA (20mg.$l^{-1}$, 200mg.$l^{-1}$, 2000mg.$l^{-1}$) in solution for 150 seconds before placement into the insert. Mini-cuttings devoid of any treatment had approximately the same percentage success (26%) as those treated with Seradix 1 (25%). Mini-cuttings that underwent the 200mg.$l^{-1}$pulse treatments showed the greatest percentage success (30%).

Mini-cuttings treated with Seradix 1 and seedlings of various sizes (7.5-70cm) were obtained from the Trahar Technology Centre and the root architecture was analyzed and quantified. Mini-cutting derived plantlets and seedlings of the same size had approximately the same shoot:root ratio. Mini-cutting plantlets had a shoot:root ratio of 2.2:1 and seedlings, 2.8:1 in the 5-7cm height range. In the 7.1-9cm height range, cutting plantlets had a shoot:root ratio of 2.5:1; and seedlings, 2.4:1. The number of lateral roots of both seedlings and mini-cutting derived plantlets in each height range was found to be statistically similar. In the comparison of seedlings of the same height range as the mini-cutting plantlets sampled, it was observed that the root architecture differed in root length and structure.

With the comparison of shoot and root growth of different heights (20 to 70cm, increasing in increments of 10cm) of *E. dunnii* seedlings, the length of the main roots of seedlings within all height ranges were found to be similar. The same trend was noted in lateral-root lengths and numbers. While some shoot dry masses were not significantly different from others. Shoot dry mass of seedlings gradually increased in accordance with an increase in the height of shoots. With every 10cm increase in shoot height, shoot mass would increase by approximately 50%.

Although *in vitro* plantlets, mini-cutting plantlets and seedlings of the same height range seemed similar in shoot:root ratios and root lengths, no direct comparisons could be drawn from the study due to the varying growth conditions of the samples before analysis, as well as restrictions on root growth by containers. To fully understand root architecture and growth patterns, it is suggested that more field work is required to obtain an accurate representation at different stages of growth.

# PREFACE

The experimental work described in this thesis was conducted at the School of Animal, Plant and Environmental Sciences, University of the Witwatersrand, from February 2006 to January 2007, under the supervision of Professors David Mycock and Paula Watt.

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 was made of the work of others, it has been duly acknowledged in the text.

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# 1. CONTEXT OF STUDY

## 1.1 IMPORTANCE OF EUCALYPTUS PROPAGATION

Eucalypts are the most widely planted hardwood species in the world as they are highly successful in industrial monocultures. They are cultivated by the Forestry Industry for timber, paper pulp, poles, firewood charcoal, essential oils, fibre-board, and extractable tannins; they can also be planted as windbreaks and are becoming increasingly desirable for their ornamental value (Turnbull, 1999; Kataria *et al*., 2013).

More than five hundred species of *Eucalypts* can be found in Australia and its surrounding islands. The genus is highly adaptable, succeeding in both hot, humid tropical lowlands and cool temperate highlands. Optimal growth is favoured on acidic soil, in the presence of adequate moisture and suitable temperatures (Table 1). This growth range (Table 1) has allowed for different species to be adapted for mass propagation on various continents according to their climate requirements. For example, *Eucalyptus urophylla, E. deglupta* and *E. pellita* grow well under the tropical conditions associated with the Congo and Indonesia, while *E. camaldulensis* and *E. globulus* grow well under the temperate climate provided by parts of Brazil, Chile, China, Morocco and South Africa (Turnbull, 1999).

In particular, *E. dunnii* is grown prolifically in Southern Brazil, Uruguay, China, Australia and more recently, South Africa (Jovanovic *et al*., 2000). The species has fast growth, low lignin and high cellulose contents. These traits are highly desirable for pulp production and are therefore favoured by the paper industry. The species is resistant to frost and can tolerate the tropical Brazilian environment (Termignoni *et al.,* 1996) and the temperate climate of Uruguay (Hernández *et al*., 2009).

*Table 1: Site criteria for the preferred growth of *Eucalyptus* species and hybrids in South Africa

| Species | ERD (cm) | MAP (mm) | MAT (˚C) |
|---|---|---|---|
| *E. badjensis* | > 30 | > 800 | 14.5-17 |
| *E. benthamii* | >45 | >800 | 14.5-17.5 |
| *E. dunnii* | > 50 | > 850 | > 15 |
| *E. grandis* | > 60 | > 900 | >16 |
| *E. grandis* x *E. nitens* | >40 | >830 | 14-19 |
| *E. grandis* x *E. camaldulensis* | >40 | >830 | 18.5-22 |
| *E. grandis* x *E. terecornis* | >40 | >830 | 18.5-22 |
| *E. grandis* x *E. urophylla* | >40 | >920 | 17.5-22 |
| *E. macarthurii* | > 40 | > 850 | <17.5 |
| *E. nitens* | > 75 | > 900 | 13-15.5 |
| *E. saligna* | > 55 | > 900 | >15.5 |
| *E. smithii* | > 70 | > 900 | >15 |

ERD = minimum effective rooting depth; MAP = minimum mean annual precipitation; MAT = optimum mean annual temperature
* Adapted from Herbert, 1996 and Swain and Gardener, 2003

South Africa has over a million hectares of forest plantations, with *Eucalyptus* species being the predominant hardwood propagated and planted in the country (Meadows, 1999). The industry is continually improving, with new techniques emerging to improve propagation success. Such an example to the possible advancement of *Eucalyptus* propagation is the use of genomic selection, which allows for the capturing of complex traits of plant material between *Eucalyptus* populations (Resende *et al*., 2012).

Macro- and micropropagation are used to propagate *Eucalyptus* on a commercial scale. The most common method is macropropagation, where the rooting of axillary material from stem-cuttings produces plantlets. Growth conditions and plant growth regulators may be manipulated for optimal production by this method. The resultant plantlets are then planted in the field. Plant material may be obtained by different sources, such as field hedges and indoor hydroponic mini-hedges (de Assis *et al*., 2004). This plant material can also provide the genetic material for the micropropagation process (Chinnaraj and Malimuthu, 2011).

Micropropagation involves the production of plant material under *in vitro* conditions, using various media and plant growth regulators to manipulate the development process for optimal growth. This control is advantageous in species that may be difficult to propagate under conventional macro-propagation conditions. Both shoots and roots can be generated from seeds, shoots, seedlings, flowers, and callus material. The resultant plantlets are acclimatised to the external environment and planted in the field (George, 1993; Kataria *et al*., 2013).

## 1.2 DISTRIBUTION OF EUCALYPT PLANTATIONS IN SOUTH AFRICA

The South African forestry industry is distributed along the coastal belt of the Southern Cape, KwaZulu-Natal and the eastern escarpment of the Drakensburg from the Eastern Cape to Gauteng. *E. dunnii* can be found in the colder, drier regions within this distribution and can surpass *E. grandis* in performance under short rotations (Jovanovic *et al*., 2000).

As is employed internationally, to maximise productivity, numerous *Eucalpyts* are matched with the differing South African climates, based on the temperature and rainfall patterns (Schönau and Purnell, 1987). With their diverse growth range (Table 1), Eucalypt species grow extremely well under South African conditions. Herbert (2000) recognised that there are three important factors to consider when matching a site to a species. These are air temperature, which is derived from mean annual temperature (MAT); rainfall which is defined by the mean annual precipitation (MAP) and effective rooting depth (ERD) of the soil (which is the minimal depth required for effective rooting). Table 1 provides an indication of these factors that form the minimum growth requirements of the *Eucalyptus* species propagated in South Africa.

A particular species/clone may be selected over others in certain climatic zones for mass propagation if it can grow under the same conditions and to equal size and value in a shorter period. Some *Eucalyptus* clones have even displayed low water use, making them highly desirable, since South Africa is a water-limited country (Albaugh *et al*., 2013). This would mean that a particular *Eucalyptus* species costs less to produce and is therefore more profitable. In South Africa the majority of the plantations are comprised of *E. grandis* and hybrids thereof, but other species including *E. dunnii* are grown commercially. *E. dunnii* is suited to the temperate, high-altitude regions of South Africa due to the species' coppicing ability as well as its cold tolerance and frost-resistance (Herbert, 1996; Little and Gardner, 2003).

## 1.3 *E. dunnii* PROPAGATION IN SOUTH AFRICA

Due to its commercial value to the paper industry in South Africa, propagation advancements are being investigated for *E. dunnii*. Macro- and mini-cuttings are currently the sole method of commercial propagation. This has proven to be difficult as the species does not root readily.

The objectives of this study were therefore to:

- Establish *E. dunnii in vitro* and investigate methods of micropropagation to produce sufficient material for experiments;
- work toward rooting of *E. dunnii in vitro*;
- research alternatives toward improving the rooting success of mini-cuttings; and
- compare the architecture and morphology of roots derived from the different propagation (macro- and micropropagation) methods with roots that are developed naturally (from seedlings).

# 2. INTRODUCTION

## 2.1. PROPAGATION OF EUCALYPTS FROM MINI-CUTTINGS AND SEEDLINGS

The process of propagation of superior tree stock has improved over time through technological and operational advancements (Denison and Kietzka, 1993; de Assis *et al*., 2004). Conventionally, field-grown clonal hedges provide the production facilities with young vegetative material that are used to produce mini-cuttings. These are produced by excising pieces of stems with one or more nodes from the clonal hedges. Rooting is induced from these mini-cuttings either by the presence of exogenous and/or endogenous auxin; and rooted mini-cuttings are transferred to the plantations (Watt *et al.,* 2003; de Assis *et al*., 2004). This method of vegetative propagation has been successfully used for a number of eucalypt species on a commercial scale around the world (Herbert, 2000).

While previously only limited numbers of shoots could be produced from each clonal hedge plant (McComb and Bennet, 1986), advancements such as the use of indoor hydroponic clonal hedges have transformed the industry (de Assis *et al*., 2004). Indoor systems utilizing either intermittent flooding of the clonal hedge containers with a nutrient-containing solution, or the use of drip-irrigated sand beds have become widely used for propagation (Campinhos *et al*., 2000). The advantages of these systems include control over the growth environment, reduced cost due to less labour being required and lower consumption of growth chemicals and water (de Assis *et al*., 2004).

Indoor hydroponic clonal hedges are more efficient than normal propagation techniques and were found to increase productivity in Brazil by 350-fold as cuttings could be harvested from stock material at a faster rate (de Assis *et al*., 2004).

The success of the development of cuttings post-harvest is dependent on their treatment as well as the species being propagated. For example, cuttings from species such as *E. saligna* are more difficult to root when compared with the hybrid *E. grandis* x *E. urophylla*, whose production is double that of the pure species (deAssis *et al*., 2004).

Control over growth conditions could provide an advantage for improved productivity of all species. The control over environmental and growth factors such as temperature, light intensity and nutrition that the indoor systems/greenhouses provide, allows for material to be harvested all year round (de Assis *et al*., 2004). Consequently, every effort is made to ensure that optimal rooting is achieved, from the preparation of harvested cuttings, to the environment in which they are subsequently housed.

### 2.1.1. Mini-cutting Preparation

Propagation from mini-cuttings requires only the development of a root system, as a potential shoot system is already present in the form of axillary and/or apical buds (Hartmann *et al*., 2002). Since mini-cuttings of certain species of eucalypts do not readily form roots, it is best to treat the base of the cutting with a rooting plant growth regulator (Fett-Netto *et al*., 2011).

Indole-3-butyric acid (IBA) has been found to be most successful of the plant growth regulators for inducing rooting in *Eucalyptus* mini-cuttings (de Assis *et al*., 2004). Commercially, auxin (IBA)-based rooting powders such as Seradix® are used to promote rooting in many forestry trees, including *Eucalyptus* (Leakey *et al*., 1990). More recent investigations into combining the auxin IBA with other treatments (such as anti-ethylene treatments) have shown promise in improving vegetative propagation of eucalypt hybrids (Kilkenny *et al*., 2012).

The variation within *Eucalyptus* clones can also influence the rate of adventitious root development (Brondani *et al*., 2012a). Brondani *et al*. (2012a) found this to be true of three clones of *Eucalyptus benthamii* x *Eucalyptus dunnii*. Clones were subjected to a uniform IBA application process, yet each clone had different rates of root development.

It is therefore apparent that multiple influences, including environmental factors and mini-cutting preparation may impact on root development. For example, timing of IBA application may maximize growth of the roots formed (Luckman and Menary, 2002). Luckman and Menary (2002) found that with *E. nitens*, dipping the cutting in an auxin concentrate ($20mg.l^{-1}$) in the fourth or fifth week after its harvest, resulted in greater rooting percentages than those mini-cuttings that had been dipped immediately after harvest.

As with cuttings from micropropagated plant material (called micro-cuttings), the age of mini-cuttings has been found to impact rooting success, as well as the type of cuttings taken. Juvenile plant material was found to have a greater capacity for shoot and adventitious root formation when compared with mature plant material (Naghmouchi *et al.,* 2008).

Another factor to consider may be the type and size of the container used for rooting, which may impact on the the growth and development of root systems as well as success of transplanting and field performance (NeSmith and Duval, 1998; Gordon, 2007; George *et* al., 2008; Al-Zalzaleh, 2009). Container size and type, such as the smaller-celled trays used by the commercial forestry industries, may improve transplant efficiency but may cause restrictions to root growth (NeSmith and Duval, 1998). It has been suggested that distortions of the root system that occur at, and as a result of the primary propagation stage can affect the plant for the remainder of its existence (Gordon, 2007). These factors (containers and rooting medium), therefore, need to be carefully considered for *Eucalyptus* rooting.

### 2.1.2. Growth Medium

Various substrates and mixtures of materials are used for germinating seeds and rooting micro- and mini-cuttings (Hartmann *et al*., 2002). Porous substrates allow for better aeration and water drainage, but the medium must be firm and dense enough to hold the cuttings or seeds in place during rooting or germination (Hartmann *et al*., 2002; Shi *et al.,* 2007).

Some aerated substrates that have been used successfully in place of potting soil and gelling agents are carbonised rice husk and vermiculite (1:1) (Teixeira *et al*., 2007), and sawdust (Agbo and Omaliko, 2006).

Changes to plant nutrition can impact root initiation and development. Gomes *et al.* (2012), found that roots of *E. camaldulensis* planted in substrates of varying cadmium concentrations exhibited increased biomass and epi- and endo-dermal thickness, in response to the increased cadmium concentrations. Trueman *et al.* (2013) discovered that addition of boron promoted adventitious root formation.

External factors such as environmental conditions can also limit success of tree growth. To enhance the process of propagation, hormone-treated mini-cuttings are usually placed in greenhouses where the microclimate (temperature and humidity) is controlled for maximum production (Hartmann *et al*., 2002). Optimal success for root development within the greenhouse environment can vary based on the genetic material (Brondani *et al*., 2012c) e.g. for *Eucalyptus benthamii* clones this varied between 35 to 42 days.

### 2.1.3. Root Development

There are four phases in the rooting process.

1. Induction: Molecular and biochemical events occur prior to any visible morphological changes. The presence of endogenous or exogenous auxin (Indole-3-butyric acid is most commonly used for *Eucalyptus* rooting) is required in this phase (Cheng *et al.,* 1992a; Corrêa *et al.,* 2005).

   With mini-cuttings, the wounds induced during the excision process damages cell compartments and this causes the injured cells to die. A necrotic plate forms which protects the cut surface from desiccation (Hartmann *et al*., 2002) and pathogens (Eyles *et al*., 2003). The wound is then sealed with suberin. The associated xylem may also become plugged. Catabolic enzymes such as peroxidases are released or synthesized during this time. The role of the enzymes is believed to enhance the tissues response to plant growth regulators such as auxin (De Klerk *et al*., 1999). Sucrose and glucose have been found to be beneficial for *Eucalyptus saligna, Eucalyptus globulus* and *Eucalyptus sideroxylon* root development when applied under *in vitro* conditions (Cheng *et al.,* 1992a; Corrêa *et al.,* 2005), along with dark treatments executed at this stage (George, 1993; Mokotedi *et al.,* 2000; Corrêa *et al.,* 2005).

2. Initiation: Increased cell division occurs (these ultimately result in the organisation of the root meristem and radicle primordia from the seed). The changes become visible, in the form of callus and root initials. The callus develops into a wound periderm (Calderón Baltierra *et al.,* 2004).

   The presence of auxin necessary for root initiation is coupled with an increase in the activity of the enzyme peroxidase, which aids rooting (George, 1993; De Klerk *et al*., 1999; Calderón Baltierra *et al.,* 2004; Corrêa *et al.,* 2005; Schwambach *et al.,* 2008).

3. Organisation: Cells in the vicinity of the vascular cambium and phloem of the stem begin to divide and expand into new adventitious roots (Hartmann *et al*., 2002).

   Root primordia can be histologically distinguished, the vascular tissues of which become connected to those of the explant (Metivier *et al.,* 2007) and elongation occurs (George, 1993; Corrêa *et al.,* 2005).

4. Growth: The primordia undergoes cell division and develop into roots (George, 1993). Peroxidase activity declines with the external appearance of roots (Schwambach *et al.,* 2008). The role of the root cap is to guide the penetration and direction of root growth in response to gravity, the soil/medium and microbial environment surrounding the root (Chen *et al*., 1999; Hawes, *et al.,* 2003).

## 2.2 PROPAGATION OF EUCALYPTS THROUGH MICROPROPAGATION

Forestry industries employ tree-breeding programs for which only those trees that yield superior quality wood are selected. The superior *Eucalyptus* trees possess the desired characteristics, such as a straight and clear bole, disease and pest resistance, drought tolerance, fast growth, etc. (Sharma and Ramamurthy, 2000). Arborea are used to house the genetic stock of the *Eucalyptus* species in the form of clonal hedges (Mycock *et al.,* 2004).

The micropropagation process has been divided into several stages (George, 1993; Thiart, 2003):

I. Selection of the mother plant, and decontamination of selected plant material;
II. establishment of aseptic culture *in vitro*;
III. propagation of *in vitro* material;
IV. preparation for growth *ex vitro*; and
V. transfer of material to greenhouse/external conditions.

## Stage I – Selection of the Mother Plant, and Decontamination of Selected Plant Material

At the nurseries of Mondi Group South Africa, *Eucalyptus* clonal hedges (produced by mini-cuttings and micropropagated plants) are formed by regular pruning of plant material which results in young shoots being continually produced (Watt *et al*. 1991, 1995). The young material is then used for propagation in the field (macropropagation) and under *in vitro* conditions (micropropagation) (Watt *et al*., 1997).

As with the establishment of clonal hedges, only improved clones are selected for the micropropagation process. Sections of the explant are excised from mature trees and coppice material; and decontaminated in preparation for micropropagation (Le Roux and Van Staden, 1991). Decontamination is necessary as plants are exposed to a number of contaminants and can become infested both internally and externally with bacteria, fungi, yeasts and animal pests. These contaminants pose a problem for material that is to be used for *in vitro* cultures as the environment is ideal for their proliferation. The use of disinfectants along with fungicides, bactericides, and insecticides prior to excision, along with only selecting the most vigorously growing plants minimize this possibility (De Fossard *et al.,* 1977, Neto *et al*., 2013). Antibiotic solutions administered to eucalypt tissues in culture may be used to further protect plant tissues/cells against potential contaminations (Watt *et al.,* 1991). As well as protecting plant tissues, some fungicides have been found to promote growth in culture. In this regard fungicides such as Amphotericin B and Standak Top® had a positive effect on the development of *E. grandis* shoots and seeds in culture (Watt *et al*., 1996; Neto *et al*., 2013).

**Stage II – Establishment of Aseptic Culture *In Vitro***

After decontamination, the cells, tissues or organs are placed onto a culture medium. Due to their vigorous growth, axillary buds are commercially used for the *in vitro* multiplication of the eucalypt species and are the most common micropropagation explant used for *Eucalyptus* propagation (Boulay, 1987; George, 1993). The direct growth of new organs from the explants is referred to as direct organogenesis as new growth directly from the axillary buds will result in the development of new shoots and buds (Le Roux and Van Staden, 1991). With indirect organogenesis, once the explant is established in culture, callus or a suspension culture is induced from the explant (George, 1993). Plant growth regulators are used to manipulate this process, as well as the subsequent growth of shoots or roots from the callus/suspension culture (Thiart, 2003).

The composition of the culture medium also has an effect on direct and indirect organogenesis (Watt *et al.,* 1991). The growth medium contains macro- and micronutrients, various vitamins, amino acids, a gelling agent, carbon and often other additives (George, 1993). The growth conditions are manipulated to allow for optimal development, growth and multiplication of the material. Mineral nutrition is important for the induction of organogenesis (Brondani *et al*., 2012b). The choice of medium must be appropriate for the species of plant, the organ/tissue to be cultured and the desired result. When considering a new species, it is therefore best to use the medium or media and methods which have been found to be successful for members of the same genus (George, 1993). In this regard, it has been found that growth can be more dependent on plant genotype than the constituents of the medium (George *et al.,* 2008). The most effective medium can be empirically determined by placing the plant material onto the likely media and comparing growth and multiplication over time.

**Stage III – Propagation of *In Vitro* Material**

Once the best protocol is established, the culture can be maintained by periodic sub-culturing, which involves the placement of *in vitro* plant material onto fresh semi-solid or liquid medium (George, 1993).

A superior alternative to the conventional method of multiplication on semi-solid medium is the use of Temporary Immersion Bioreactor Systems such as the RITA® system (Berthouly and Etienne, 2002). The RITA® system is capable of producing greater yields of *Eucalyptus* than the semi-solid system (McAlister *et al.,* 2005). It works by periodically exposing the entire explant to liquid medium, thereby allowing a greater surface area for the diffusion of nutrients and gases. Benefits of this method include less equipment and labour, lower quantities of medium, as well as bulk handling thereby making this system worth investigation for commercial multiplication (Berthouly and Etienne, 2002; George *et al*., 2008).

During the multiplication phase, plant material can become hyperhydric (Ivanova and Van Staden, 2011), i.e. abnormal, waterlogged organs that are glassy in appearance, with shoots producing few stomata and plasmodesmata, low photosynthetic capacity and poor root growth (Louro *et al*., 1999; Kevers *et al*., 2004; George *et al*., 2008). It is imperative then, that only healthy material is selected for the multiplication phase.

The growth and development of organs in culture (formation of roots and shoots) is dependent on the addition of plant growth regulators to the medium. Plant hormones (called plant growth regulators) are important in micropropagation due to their ability to regulate plant function and development. The most common- and widely-used plant growth regulators used in micropropagation to control morphogenesis are cytokinins and auxins (Hartmann *et al*., 2002).

Cytokinins delay leaf senescence while promoting cell division and dormant bud activation. Their addition to the medium therefore promotes shoot development (dependant on concentration). Auxins have been found to have multiple roles in plant development including phototropism, apical dominance and root induction. Their addition to the medium promotes adventitious rooting (Hartmann *et al*., 2002). The auxin most commonly used for *eucalyptus* rooting is Indole-3-butyric acid (IBA) (Leakey *et al*., 1990; de Assis *et al*., 2004).

**Stage IV – Preparation for Growth *Ex Vitro***

Roots are necessary if the plant is to sustain itself in the natural environment. Auxin is used to induce adventitious rooting (Hartmann *et al*., 2002). The quantities and types of the auxin present in the medium impact the morphology and quality of the roots produced (Hartmann *et al*., 2002). Similarly, the concentration and type of medium also has an effect on organ development (George, 1993). The medium generally used for the growth and multiplication of *Eucalyptus* shoots *in vitro* is Murashige and Skoog (1962) (MS). The concentration of MS required for shoot growth has been found to inhibit root formation in some species (George, 1993). Lower concentrations (half or one quarter of the concentration) have been found to successfully promote rooting in eucalypt species (Mokotedi, 2000; Sharma and Ramamurthy, 2000; McAlister *et al.,* 2005).

Aeration of the substrate has been found to improve rooting *in vitro*, and substrates like agar (a potential hypoxic environment) which adheres to roots and may cause problems when plantlets are transferred to soil (Newell *et al*., 2006; George *et al.*, 2008).

Manipulation of the medium components such as pH, plant growth regulators and the environmental conditions has allowed for a greater degree of control and success in the rooting process *in vitro*. For example, a slightly acidic pH (approximately 5.5) has

been found to induce rooting in certain Australian and *Eucalyptus* species (Gupta *et al.,* 1983; Williams *et al.,* 1985).

The concentration of phytohormones utilized in media may impact on the quality of roots produced. High levels of auxin have been found to produce large quantities of callus while lower concentrations may not induce growth (Nourissier and Monteuuis, 2008).

Warm culture environments and exposure to light have been shown to encourage root growth in *Eucalyptus grandis* x *nitens* and *Eucalyptus urophylla* x *grandis* respectively (Mokotedi *et al.,* 2000; Nourissier and Monteuuis, 2008).

Roots can be produced from excised *in vitro* shoots by the micropropagation process (referred to as micro-cuttings for the purpose of this study) under *in vitro* conditions, or *in vitro* shoots can be transferred to the external environment where rooting is then induced. The ability of certain *Eucalyptus* clones to root successfully *in vitro* is variable. The age of the material used for propagation has an impact on success of the *Eucalyptus* rooting process. For example, with *E. marninata*, when plants were micropropagated from explants taken from the crown of mature trees, the initial shoot and root morphology was different from that derived from seedling explants (Bennet, *et al*., 1986). The *in vitro* environment can affect root production. For example, roots that are induced may lack root hairs (Rogers and Smith, 1992). Due to callus formation that often arises at the base of stems, the resultant roots may have incomplete vascular connections between the roots and shoots (de Fossard *et al.,* 1978; Gregory, 2006). Only plantlets that exhibit complete connections then should be selected for the acclimatisation process.

**Stage V – Transfer to Material to Greenhouse/External Conditions**

After the formation of roots, it may be advantageous to pot the plantlet onto a substrate other than potting soil. Porous substances allow for easy diffusion of oxygen. Substrates that have been used with success is a mixture of perlite and coir (Hajari, 2004), or a mixture of river sand and pine bark (Mokotedi *et al.,* 2000). The closed environment of the culture vessel makes it necessary to gradually accustom the delicate plants to the external environmental conditions, to ensure their survival in the field (George, 1993). As the environment within the culture vessel is highly humid, the easiest way in which to acclimatize the plant is to decrease the humidity over time.

For successful acclimatization with *Eucalyptus* under laboratory conditions (on a small scale), the potted-out plantlet should be covered in a plastic bag and the humidity can be decreased by either removing the bag for progressively longer periods or by punching an increasing number of holes in the bag. Eventually, the plant can simply be removed from the bag and will be able to survive *ex vitro* (Warren, 1991; Mokotedi *et al.,* 2000; Hajari, 2004). On a large scale, *Eucalyptus* plants can be potted-out in sterile soil and placed in a greenhouse, where the high relative humidity can be maintained by use of a misting system (George, 1993). Over time, humidity can be reduced and plants transferred to the natural environment.

The advantages of propagation make it necessary to further investigate the growth of *Eucalyptus in vitro* and to find an appropriate method with which to induce the best possible root growth.

## 2.3. THE RELATIONSHIP BETWEEN ROOT STRUCTURE AND FUNCTION

### 2.3.1 Nutrient Uptake

Nutrient uptake, in part, involves the interception of a nutrient source by the root. Nutrients are dissolved in water from the surrounding substrate and transported via diffusion (from the bulk soil to root surface) to the root system (Marschner, 1995; Jungk, 2002). Overall tree growth is therefore affected by the availability of soil water, nutrients, organic matter and soil friability to the tree's roots (Turnbull, 1999).

In an assessment of a forest of *Eucalyptus* and also specifically, *E. marginata* and *E. globulus*, root growth was found to be most abundant at the soil surface, where organic matter and nutrients were concentrated (Carbon *et al*., 1980; Fabião *et al*., 1987; Laclau *et al*., 2001). While growth was abundant in this upper layer, low rooting densities were recorded for *E. marginata* and *E. dunnii*, hypothesized as being due to a high nutrient supply (*E. marginata)*, as well as poorly-drained soils which can be problematic for *E. dunnii* (Carbon *et al*., 1980; Herbert, 2000; Grant *et al*., 2012). The opposite was observed by Laclau *et al*., (2001), who found that *Eucalyptus* roots within the plantation environment decreased in density as depth increased. However, competition for nutrients in the soil as well as soil density plays a role with competition and decreased soil density causing an increase in root penetration and density (Fabião *et al*., 1987; Craine, 2006). Seasonal effects have also been shown to impact on the anatomical traits of *Eucalyptus camaldulensis* roots (Chaudhry, 1994). Similarly increased day/night temperatures have been found to greatly increase rooting success of mini-cuttings of *Eucalyptus cloeziana* (Trueman *et al*., 2013). Collectively, those findings indicate that acquisition capacity of the root, nutrient availability of the substrate, and environmental conditions influence root development (Yamauchi, 2001; Graciano *et al*., 2009). Apart from responding to the available resources, root growth is also affected by other stimuli (e.g. gravity, light, touch and microorganisms (Takashi, 1997).).

### 2.3.2 Root Architecture

Root architecture has been defined as the "spatial configuration of a root system in the soil" (Yamauchi *et al*., 1996; Gregory, 2006). While a plant root system consists of roots that differ in morphology and functions (Wang *et al*., 2006), reproduction of the root system pattern excludes fine details such as root hairs (Lynch, 1995). From their architecture, the branching patterns (topology) of the root and the root distribution can be determined. Soil characteristics and plant genetics are also regulators of root architecture (Robinson, 1994).

Both root morphology (i.e. length and radius) and architecture (i.e. branching pattern) influence the acquisition of resources (Yamauchi *et al*., 1996) and can also be influenced by the resource allocation in the environment (Fabião *et al.,* 1990/91; Baňoc *et al*., 2000; Yamauchi, 2001; da Silva *et al*., 2011; Duursma *et al*., 2011). Root architecture may be less sensitive to resource availability than root morphology (Cortina *et al*., 2008).

The response of root architecture to nutrient supply has been investigated using phosphorus, the most extensively studied nutrient element (Gregory, 2006). Phosphorus is relatively immobile in soil (with highest concentrations present in topsoil), and therefore, the architecture of root adaptations for acquisition can be tracked (Gregory, 2006). Lynch and Brown (2001) suggested that root architecture for enhanced phosphorus exploration included a more horizontal basal-root growth angle and enhanced lateral root formation, both resulting in shallower root systems. This was coupled with increased root hair density and length, which is plausible as finer roots increase the surface area of the root system and can therefore increase the nutrient uptake per unit root mass (Gregory, 2006). In the root system architecture of *Eucalyptus grandis* it was found that nitrogen, potassium and calcium tracers at varying soil depths yielded different uptake rates related to the depth of the nutrient (da Silva *et al*., 2011). These findings indicate that roots are specialised to effectively source the required nutrients for the development of the plant. The difference in

architecture may possibly have an impact on plant growth rates (Rockwood and Warrag, 1994).

Macropropagation alters the root architecture of plants of the same species from that of their physically-unmodified counterparts. A study by Sasse and Sands (1997) on *Eucalyptus globulus* found that there was a difference between root architecture of seedlings and plants derived from cuttings. Seedlings had gravitropic tap- and primary-roots while cuttings did not develop tap roots and did not develop primary roots during the study. Similar differences were observed by Rockwood and Warrag (1994) in their comparison of field-grown *E. grandis* micropropagated plantlets, macropropagated plantlets and seedlings.

### 2.3.3 The Relationship between Roots and Shoots

The growth and functioning of roots are regulated by the shoot through nutrient cycling between the two, via root-sourced xylem and leaf-sourced phloem (Dodd, 2005; Wang *et al*., 2006). For instance, cytokinins are synthesized primarily in root tips and transported to shoots via the xylem where they regulate cell division and the rate of leaf senescence in mature plants (Wang *et al*., 2006). Conversely, the auxins are produced in actively-growing shoot tips (leaf primordia and young leaves) and transported to the root from the shoot via the vascular system (Torrey, 1976, Nakhooda *et al*., 2012). As well as being involved in growth, their role is to control the growth response of root tips to gravity (Rashotte *et al*., 2000). This was tested when plants were decapitated to observe the resultant effect on root growth. Such plants were found to produce less auxin, and also had decreased rates of root growth. Both impacts on auxin production and root growth were reversed by the application of auxin to the decapitated shoot tip (Dolan and Davies, 2004). Nakhooda *et al*. (2011) made a similar observation with the *in vitro* environment. Shoots produced roots on auxin-free medium, after having had auxin exposure through preceding multiplication and elongation phases. However, when auxin was omitted from the

multiplication phase and restricted in the elongation phase, rooting on the auxin-free medium was significantly reduced (Nakhooda *et al*., 2011).

The close interactions between both shoot and root systems show that for successful plant growth, each cannot be sustained independently. It also indicates that root system formation proceeds in close coordination with shoot growth (Tatsumi and Kono, 1980; Ogawa *et al*., 2005; Wang *et al*., 2006).

Hence, studies of roots should highlight the dynamic relationships between roots and shoots, to capture their complex interactions (Gregory, 2006). For example, a comparison of *E. globulus* seedlings with cuttings showed that while both displayed similar heights and growth rates, shoot:root ratios showed that cuttings had significantly larger shoot systems and relatively smaller root systems than seedlings, implying that root development was poorer in cuttings (Sasse and Sands, 1997). Mokotedi *et al*. (2010) found that field-grown micropropagated *E. grandis* x *E. nitens* trees had a less-developed root system when compared with seed-derived trees and had less resistance to uprooting.

Root:shoot ratios can also act as indicators (Mokany *et al*., 2006). The root:shoot ratio of seedlings (*E. nitens*) have been indicators of their vulnerability to transplanting (Wilson and Clark, 1998), and adaptations to soil and atmospheric water deficits (*E. dumosa* and *E. hypochlamydea*) (Warren and Adams, 2005). Comparison of hardwood seedling root volume with shoot height has been successfully used to predict subsequent growth rates and tree dimensions (height and diameter) (Jacobs *et al*., 2005).

While root:shoot mass ratios cannot account for the total investment made by plants in their root systems (due to flux of assimilates in the system), it does give a good indication of the investment of plants in their root systems at any point in time through the plant's basic biomass allocation (Gregory, 2006; Mokany *et al*., 2006). For example, when mineral elements are scarce, biomass allocation favours the root

system (Hermans *et al*., 2006). The different ratios (i.e. for same species of same size) can therefore be compared with trends such as root longevity, rates of root renewal and rates of metabolic activity (Gregory, 2006).

The method used for propagation (macro- versus micropropagation) may not only then impact rooting, but also the relationship between roots and shoots which ultimately determines successful development. Therefore, this study not only compared root development (architecture and morphology) of the differing propagation methods, but also considered the shoot development associated with these roots.

# 3. MATERIALS & METHODS

## 3.1 ESTABLISHMENT OF A MICROPROPAGATION PROTOCOL FOR *E. dunnii*

### 3.1.1 *E. dunnii* Seeds

Seeds of *Eucalyptus dunnii* were sourced from Mondi Group South Africa. Decontamination of seeds was according to the protocol developed by Watt *et al.* (1991). Seeds were first separated from the husks by placing the seeds in a beaker with a small quantity of water. The husks floated to the surface of the water and were removed using a sterile spatula. Seeds were then placed in 1% (w/v) sodium hypochlorite solution for 15 minutes. The seeds were rinsed five times in sterile distilled water and placed in $10g.l^{-1}$ mercuric chloride containing one drop of Tween-20® per litre solution for ten minutes. Once again, thorough rinsing in sterile distilled water followed. Antibiotic solution was required to minimize the possibility of contamination. Seeds were therefore immersed in $10ml.l^{-1}$ of Antibiotic and Antimycotic solution (Sigma® active ingredients: penicillin, streptomycin sulphate, amphotericin B) for ten minutes, before being transferred to germination medium (Table 2) and incubated in darkness for six days. Seeds were thereafter placed under standard growth room conditions (25±3°C under a 16 hour light photoperiod at $200\mu mol/m^2/s^1$ photosynthetic photon flux density). Once germinated, shoots were excised from roots and placed onto *E. grandis* and *E. camaldulensis* and *E. grandis* x *E. nitens* multiplication media (Table 3).

Table 2: Germination Medium for *Eucalyptus* seeds

| Medium Components | $g.l^{-1}$ |
|---|---|
| MS (with vitamins) | 4.42 |
| Sucrose | 10 |
| Casein Hydrolysate | 1 |
| Benomyl | 0.1 |
| Gelrite® | 4 |

MS – Murashige and Skoog (1962); pH of medium range between 5.6-5.8.

Table 3: Multiplication Media

| Medium Components | *E.grandis* & *camaldulensis* | *E. grandis*x*E.nitens* | other *Eucalyptus* clones | |
|---|---|---|---|---|
| | | | semi-solid medium | liquid medium |
| Sucrose ($g.l^{-1}$) | 25 | 30 | 25 | 25 |
| MS (with vitamins g. $l^{-1}$) | 4.42 | 4.42 | 4.42 | 4.42 |
| BAP (mg. $l^{-1}$) | 0.2 | 0.1 | 0.2 | 0.2 |
| NAA (mg $l^{-1}$.) | 0.01 | 0.01 | 0.01 | 0.01 |
| Biotin (mg. $l^{-1}$) | 0.1 | 0.1 | 0.1 | 0.1 |
| Calcium Pantothenate (mg. $l^{-1}$) | 0.1 | 0.1 | 0.1 | 0.1 |
| Gelrite® ($g.l^{-1}$) | 3 | 4 | 2.3 | A |

MS – Murashige and Skoog (1962); BAP – Benzyl aminopurine; NAA – Naphthaleneacetic acid; A - Absent from medium. pH of all media range between 5.6-5.8.

### 3.1.2 *E. dunnii* Clones

*E. dunnii* clones (pure species), D127 (clone A), D95 (clone B) and D104 (clone C) were transplanted into Culterra Potting Soil® and placed in the greenhouse (Figure 1) at the University of the Witwatersrand, Johannesburg, where they were sprayed weekly with a fertilizer and fungicide. Fertilizers ($10ml.l^{-1}$ Trelmix, $1g.l^{-1}$ Mondi Orange and $0.3\ g.l^{-1}$ Calmag) and one fungicide mixture ($1ml.l^{-1}$ Bravo and $2g.l^{-1}$ Dithane) were applied as a foliar spray, while the second fungicide mixture ($1.25ml.l^{-1}$ Folicur and $1ml.l^{-1}$ Sporgon) was applied to the soil.

Figure 1: *Eucalyptus* clones in greenhouse, at the University of the Witwatersrand, Johannesburg

After six weeks of treatment (application of fertilizers and fungicides), freshly sprouted lateral branches (with both apical and axillary buds), were excised and decontaminated in $0.2g.l^{-1}$ mercuric chloride with 1 drop of Tween-20® detergent per litre solution for ten minutes. Branches were rinsed three times with sterile distilled water and placed in $0.2g.l^{-1}$ calcium hypochlorite for ten minutes. Shoots were then rinsed three times with sterile distilled water. The branches were trimmed into nodal sections, and leaves were halved. Nodes were plated into Magenta jars (400ml in volume) containing 50ml of multiplication medium (Table 3) (Watt *et al.,* 1991). The procedure for selection of the appropriate medium is described in section 3.1.3. The vessels were incubated in a growth room at a temperature of 25°C under a 16-hour light photoperiod at $200\mu E.m^{-2}.s^{-1}$. Once growth from the axillary buds was achieved, the new shoots (individual stems) were excised from shoot clusters and placed upon fresh multiplication medium, where subsequent multiplication of material was conducted on a monthly basis. Fresh plant material was brought *in vitro* from clonal material monthly over a seven-month period.

### 3.1.3 Multiplication of *In Vitro* Material

Different types of media were tested for the multiplication process to determine the optimal multiplication medium for *E. dunnii.* Media used in the past to successfully multiply *E. grandis* and *E. camaldulensis* and *E. grandis* x *E. nitens* (Table 3), formed the basis of this investigation. Once plants had been established *in vitro,* multiplication of plant material was attempted using both a semi-solid system and the Temporary Immersion Bioreactor System - RITA® (McAlister *et al.,* 2005).

#### 3.1.3.1 Semi-Solid System

Equal numbers of shoots (3 replicates [*n*=20]) were placed into Magenta jars (volume 400ml) containing 50ml of *E. grandis* and *E. camaldulensis* and *E. grandis* x *E. nitens* (Table 2) multiplication medium. After a four-week period, clusters (multiple shoots arising from original shoot) were sub-cultured onto fresh medium. Cluster development was monitored weekly, with the number of new buds produced being recorded as well as the overall appearance and quality of the cluster (cluster colour, callus formation and the production of hyperhydric material). Statistical analysis was conducted using the two-sample t-test due to the comparison of two normally-distributed populations (SAS® software version 9.1).

#### 3.1.3.2 Temporary Immersion Bioreactor System (RITA®)

After a three-month period on semi-solid medium, only healthy, contaminant-free material was visually selected for placement into the Temporary Immersion Bioreactor System - RITA®. The liquid medium employed by McAlister *et al.* (2005) (Table 2) was used for the *E. dunnii* species. This medium was selected as it had been found to form healthy shoots (approximately 6cm) in other cold tolerant clones, and also yielding a four- to six-fold increase in shoot multiplication compared with the semi-solid method (McAlister *et al.,* 2005). Statistical analysis was conducted using

the two-sample t-test due to the comparison of two normally-distributed populations (SAS® software version 9.1).

Healthy plant clusters (approximately 3-4 shoots on each) underwent an antibiotic pre-treatment of $0.1g.l^{-1}$ rifampicin on a shaker for seven days at 70rpm. Contaminant-free shoots were selected and placed into RITA® vessels (Figure 2) (50 clusters per vessel). The material was then subjected to 30-second flushes every 10 minutes (McAlister *et al.,* 2005), under standard growth room conditions.


Figure 2: *E. dunnii* clusters in RITA® vessel.

### 3.1.3.3 Tests to Reduce Hyperhydricity

After approximately eight weeks, plant multiplication was hampered by the progression of material into a hyperhydric state. Since most of the material was affected, a variety of trials were undertaken to reduce/reverse the hyperhydric state of the plant tissues:

Test One – Lower Relative Humidity in the Culture Vessel

Plastic boats (10ml in volume) containing activated silica gel were placed into the Magenta jars containing *E. grandis* and *E. camaldulensis* medium (George, 1993). Ten hyperhydric shoots were introduced per jar with a total sample size of 60 shoots tested. The hyperhydric state of plants was monitored over a four-week period and silica gel was replaced every four days to maintain effectiveness.

Test Two – Isolation and Re-culture of Non-hyperhydric Tissue

Upon closer examination, the tops of the hyperhydric clusters were found to have "normal" healthy shoot growth. The buds of this material were therefore isolated and placed upon Petri dishes containing multiplication medium (Table 3). Sixty healthy buds and sixty buds from the tops of hyperhydric material were isolated and plated, and monitored over a two-week period for hyperhydricity.

Test Three – Increased Gelling Agent Concentration to Reduce Water Availability

The quantity of Gelrite® in the multiplication medium was manipulated and hyperhydric material was sub-cultured onto this modified medium. Three Gelrite® concentrations were tested, 3, 3.5 and 4g.$l^{-1}$. A sample size of sixty shoots (with approximately 7 buds) was used for each concentration. Bud number was recorded weekly as well as hyperhydric status.

### 3.1.4 Elongation of *In Vitro* Material

Once sufficient numbers of clusters were obtained, the healthy shoots were sub-cultured onto elongation medium (Table 4) in preparation for rooting. The medium utilized was that reportedly effective for *E.grandis* and *E. camaldulensis* elongation (Padaychee, 2000).

Table 4: Elongation Medium

| Medium Components | |
|---|---|
| Sucrose ($g.l^{-1}$) | 25 |
| MS (vitamins) ($g.l^{-1}$) | 4.42 |
| IBA ($mg.l^{-1}$) | 0.1 |
| NAA ($mg.l^{-1}$) | 0.35 |
| Biotin ($mg.l^{-1}$) | 0.1 |
| Calcium Pantothenate ($mg.l^{-1}$) | 0.1 |
| Kinetin ($mg.l^{-1}$) | 0.1 |
| Gelrite® ($g.l^{-1}$) | 3 |

MS – Murashige and Skoog (1962); IBA – Indole-3-butyric acid; NAA – Naphthaleneacetic acid; pH of medium ranges between 5.6-5.8.

## 3.2 APPROACHES TO ROOT PRODUCTION

### 3.2.1 Production of Roots *In Vitro*

#### 3.2.1.1 Induction of Rooting by Alteration of pH Levels

Three sets of multiplication medium (Table 2) with varying pH levels (4, 4.5, 5.0 and 5.5) were prepared to determine the effect of pH on the *E. dunnii* shoots (3 replications [$n$=20]), and to determine if rooting could be triggered by a change in pH.

Shoots (with approximately 4 leaves each) grown (under standard growth room conditions) on the various pHs were compared by counting axillary bud production weekly, with those grown in the pH range of 5.6-5.8. Statistical analysis was conducted using the Bonferroni ANOVA due to the comparison of multiple sample groups (SAS® software version 9.1).

### 3.2.1.2 Indole-3-butyric acid (IBA) Range

Various IBA concentrations (0.1, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0mg.l$^{-1}$) were tested to determine what the optimal concentration would be for *E. dunnii* rooting (3 replications [*n*=20]) (Table 5). Individual shoots with approximately four leaves were placed onto the various media, and monitored weekly.

Table 5: Rooting Medium

| Rooting Medium Components | |
|---|---|
| Sucrose (g.l$^{-1}$) | 15 |
| MS (vitamins) (g.l$^{-1}$) | 1.105 |
| IBA (mg.l$^{-1}$) | 0.1-1.0 |
| Biotin (mg.l$^{-1}$) | 0.1 |
| Calcium Pantothenate (mg.l$^{-1}$) | 0.1 |
| Gelrite® (g.l$^{-1}$) | 4 |

MS – Murashige and Skoog (1962); IBA – Indole-3-butyric acid; pH of medium ranges between 5.6-5.8.

### 3.2.1.3 Rooting Substrate

Due to the high incidences of contamination, an environment that was less humid than the semi-solid medium was considered. A peat/vermiculite (1:2) mixture (30ml) was placed into 200 millilitre glass culture bottles (Figure 3), watered with approximately 15ml of the rooting medium (Table 5, excluding Gelrite®, 0.5g.l$^{-1}$ IBA) and autoclaved. Shoot clusters (approximately 4-5 shoots) were thereafter placed onto the substrate, watered as required (when substrate appeared dry) with the rooting medium, and the shoot clusters were assessed for root growth after eight weeks (3 replications [*n*=40]). No acclimatisation was attempted.


Figure 3: *In Vitro* shoot on alternate rooting substrate watered with liquid rooting medium.

### 3.2.1.4 Antibiotic Treatments

As bacterial contamination was prevalent throughout the use of the RITA® Temporary Immersion Bioreactor System, and the IBA media treatments with various antibiotics had to be administered (Table 6). Plants were removed from the vessels, which were then cleaned and autoclaved, while the clusters were subjected to the various treatments. Explants were placed into flasks containing:

1. A solution of $0.4g.l^{-1}$ rifampicin and $0.5g.l^{-1}$ gentamicin sulphate mixed with sterile, autoclaved MilliQ ultra-pure water and MS with vitamins;
2. $10ml.l^{-1}$ of Antibiotic-Antimycotic (Sigma® active ingredients: penicillin, streptomycin sulphate, amphotericin B) solution;
3. 1% (w/v) sodium hypochlorite in autoclaved MilliQ ultra-pure water (Table 6).

After the treatments, shoots or clusters were then placed into clean, sterile vessels with fresh medium, and returned to the original culture conditions.

Table 6: Antibiotic Treatments Tested on Temporary Immersion Bioreactor System - RITA® (1) and Indole-3-butyric acid (IBA) Range (1-8).

| IBA Range | Plant-Type | Treatment | Concentration | Time-Period of Treatment | Shaker | Plant-type placed on Rooting Medium after Treatment |
|---|---|---|---|---|---|---|
| 1 | Cluster | Rifampicin with | 0.4g.l $^{-1}$ | 15 minutes | + | Shoot |
| | | Gentamycin Sulphate | 0.5g.l $^{-1}$ | | | |
| 2 | Shoot | Rifampicin with | 0.4g.l $^{-1}$ | 5 minutes | + | Shoot |
| | | Gentamycin Sulphate | 0.5g.l $^{-1}$ | | | |
| 3 | Cluster | Antibiotic-Antimycotic Solution | 10ml.l $^{-1}$ | 15 minutes | + | Shoot |
| 4 | Shoot | Antibiotic-Antimycotic Solution | 10ml.l $^{-1}$ | 4 weeks | - | Shoot |
| | | | | Antibiotic placed into Rooting Medium | | |
| 5 | Shoot | Rifampicin with | 0.4g.l $^{-1}$ | 5 minute dip for | - | Shoot |
| | | Gentamycin Sulphate | 0.5g.l $^{-1}$ | 5 consecutive days | | |
| 6 | Cluster | Sodium hypochlorite followed by | 1% | 3 minutes | - | |
| | | Antibiotic-Antimycotic Solution | 10ml.l $^{-1}$ | Rinse | - | Shoot |
| 7 | Cluster | Sodium hypochlorite followed by | 1% | 3 minutes | - | |
| | | Antibiotic-Antimycotic Solution followed by | 10ml.l $^{-1}$ | Rinse | - | Shoot |
| | | Antibiotic-Antimycotic Solution | 10ml.l $^{-1}$ | 4 weeks | - | |
| | | | | Antibiotic placed into Rooting Medium | - | |
| 8 | Cluster | Antibiotic-Antimycotic Solution | 10ml.l $^{-1}$ | 4 weeks | - | Cluster |
| | | | | Antibiotic placed into Rooting Medium | | |

+ Flasks (250ml volume) containing the antibiotic solution and plant material were placed onto a shaker at70rpm

- A shaker was not required

Data Collection

In all cases, shoot growth was compared weekly by monitoring the number of new axillary buds produced by each shoot. Clusters were photographed.

The architecture of the roots (if any) formed was described. The source of roots, primary roots (relative thick roots) and secondary roots (thin "adventitious" roots formed from the primary roots) were recorded, along with respective root lengths, callus, fleshy roots, dead-ends and "kinks". Kinks in roots, i.e. roots displaying deviation from the downward vertical root growth of the main root, horizontal root growth of main and side-roots, and condition of root tips were noted. Root tips were identified as fleshy and white dead root tips or healthy root tips. Percentages of each characteristic were noted and photographed.

Callus was measured on a scale of 0-3. 0 = no callus was present; 1 = callus formation was present but minimal (1-3mm thick). 2 = moderate (4-6mm thick) callus production, and 3 = prolific (greater than 6mm thick) callus formation. Shoot and node production were recorded and the plant was then photographed. Fresh and dry mass of shoots and roots were also determined by weighing material in foil boats before and after drying in an oven at 80˚C for 48 hours. The shoot:root ratio based on the dry mass was also determined.

Statistical Analysis

Results of shoot growth and rooting on the various test media were compared with that of seedlings (3.2.3) within the same height range (2-5cm) using statistical analysis. The Kruskal-Wallis Analysis of Variance (ANOVA) was utilized due to the comparison of non-parametric multiple sample groups (SAS® software version 9.1).

### 3.2.2 Production of Roots from Mini-cuttings

Plant material from Clone A and B was used (equal quantities in each treatment). Shoots (approximately 8cm long) were excised from hydroponic material at Mountain Home, Mondi Group, South Africa. These were then further cut into mini-cuttings (approximately 2.5cm) that contained two nodes. The lower leaves were completely removed, while the upper leaves were trimmed to half their length. In all cases, cuttings were placed into Unigro inserts containing a mixture of coir:perlite (1:2). The rectangular inserts (Figure 4) were approximately 10cm in length, with an area of 16cm$^2$ at the top, tapering to 1cm$^2$ at the base, and a volume of 60ml.


Figure 4: Insert containing rooted-cutting and substrate.

Mini-cuttings were set and grown in a greenhouse at Mountain Home, Mondi Group South Africa. The day temperature ranged between approximately 21°C and 28°C, with a night temperature between 7°C to 14°C during the duration of the study. Humidity ranged between 68% to 80%. The bed temperature was maintained between 27°C- 30°C. No fertilizer or fungal treatments was administered, and cuttings were misted for ten seconds every twenty minutes. Six applications of auxin were executed:

Treatment One

Once mini-cuttings had been prepared, the lower excised end of the shoot was placed in a Magenta jar (400ml in volume) containing 20mg.l$^{-1}$ IBA for 48 hours. The sealed Magenta jars that the mini-cuttings were housed in were covered in tinfoil to create a constant dark environment. Shoots were then planted into inserts (3 replicates [*n*=66]).

Treatment Two

Mini-cuttings were placed directly into the prepared inserts for four weeks. At the end of the fourth week, the shoots were carefully removed and placed in 20mg.l$^{-1}$ IBA for 48 hours in the dark (sealed Magenta jars covered in foil). Shoots (with and without root production) were then rinsed to remove any traces of surface auxin and were re-planted into inserts (Luckman and Menary, 2002) (3 replicates [*n*=66]).

Treatment Three

As implemented by Mondi Group South Africa, mini-cuttings were dipped in Bayer CropScience Seradix®1 (0.1% IBA) and placed into the prepared inserts (3 replicates [*n*=80]).

Pulse Treatment One

Prepared mini-cuttings were dipped into a solution of 20mg.l$^{-1}$ IBA for 150 seconds before placement into the insert (3 replicates [*n*=10]).

Pulse Treatment Two

Prepared mini-cuttings were dipped into a solution of 200mg.l$^{-1}$ IBA for 150 seconds before placement into the insert (3 replicates [*n*=10]).

Pulse Treatment Three

Prepared mini-cuttings were dipped into a solution of 2000mg.l$^{-1}$ IBA for 150 seconds before placement into the insert (3 replicates [*n*=10]).

Control

Mini-cuttings were placed directly into prepared inserts without undergoing any treatment (3 replicates [*n*=33]).

Statistical Analysis

For each treatment, the number of mini-cuttings that produced roots was compared statistically using the Kruskal-Wallis ANOVA (SAS® software version 9.1).

### 3.2.3 Production of Seedlings

All *E. dunnii* seedlings were sourced from Mountain Home, Mondi Group South Africa, and were grown in seedling trays (60ml insert volume) containing coir:perlite mix (1:2) (Figure 5). Seedlings (120 of each) were grouped based on shoot height as 7.5cm to 15cm in height, and 21cm to 70cm, which were further subdivided into height ranges: 21-30cm, 31-40cm, 41-50cm, 51-60cm and 61-70cm.

Figure 5: Seedlings in seedling tray in greenhouse.

Data Collection

Both seedlings and mini-cuttings were analysed in the same manner (listed below) so comparisons could be drawn.

Mini-cuttings and Seedlings

After an 8 week period, mini-cuttings from the treatments and control were compared and recorded. Only material positive for both shoot and root growth were used for data collection. Once mini-cuttings were 5-9cm in shoot height, data was recorded. For mini-cuttings, the size of the shoot, number of nodes on each shoot, and the original nodes from which they were produced were all noted and compared with seedlings within the same height-range. For root development, the source of root formation (for mini-cuttings), the number of primary roots, secondary roots, approximate length of roots, and callus production were recorded.

Using the same parameters described in section 3.2.1.4, callus growth and other trends were measured, and shoot and root dry mass and ratios were recorded with both mini-cuttings and seedlings.

Statistical Analysis

Shoot and root comparisons of mini-cuttings and seedlings of the same height ranges (5-7cm and 7.1-9cm) were made using statistical analysis. Seedling growth (shoot and root) was compared between the different height ranges (21-30cm, 31-40cm, 41-50cm, 51-60cm, 61-70cm). The Kruskal-Wallis ANOVA was utilized for these comparisons of multiple non-parametric sample groups (SAS® software version 9.1).

# 4. RESULTS AND DISCUSSION

## 4.1 ESTABLISHMENT OF A MICROPROPAGATION PROTOCOL

### 4.1.1. Establishment and Multiplication of *In Vitro* Cultures

#### 4.1.1.1 Comparison of Semi-Solid Test Media

Growth media have to be optimized for different species (George, 1993). In addition, the type of culture has an influence on the medium formulated. When considering the introduction of a new species to culture however, it is sensible to use media and methods that have been found to be successful (George *et al*., 2008). Therefore, *E. dunnii* growth was tested on two established media (*E. grandis* and *E. camaldulensis* medium and *E. grandis* x *E. nitens* medium). Growth was measured by the average number of buds produced each week per shoot, and the appearance of the shoot (Figure 6). Although a greater quantity of buds was observed at week 3 on the *E. grandis* and *E. camaldulensis* medium, there were no statistical differences in the number of buds formed on the different media each week.

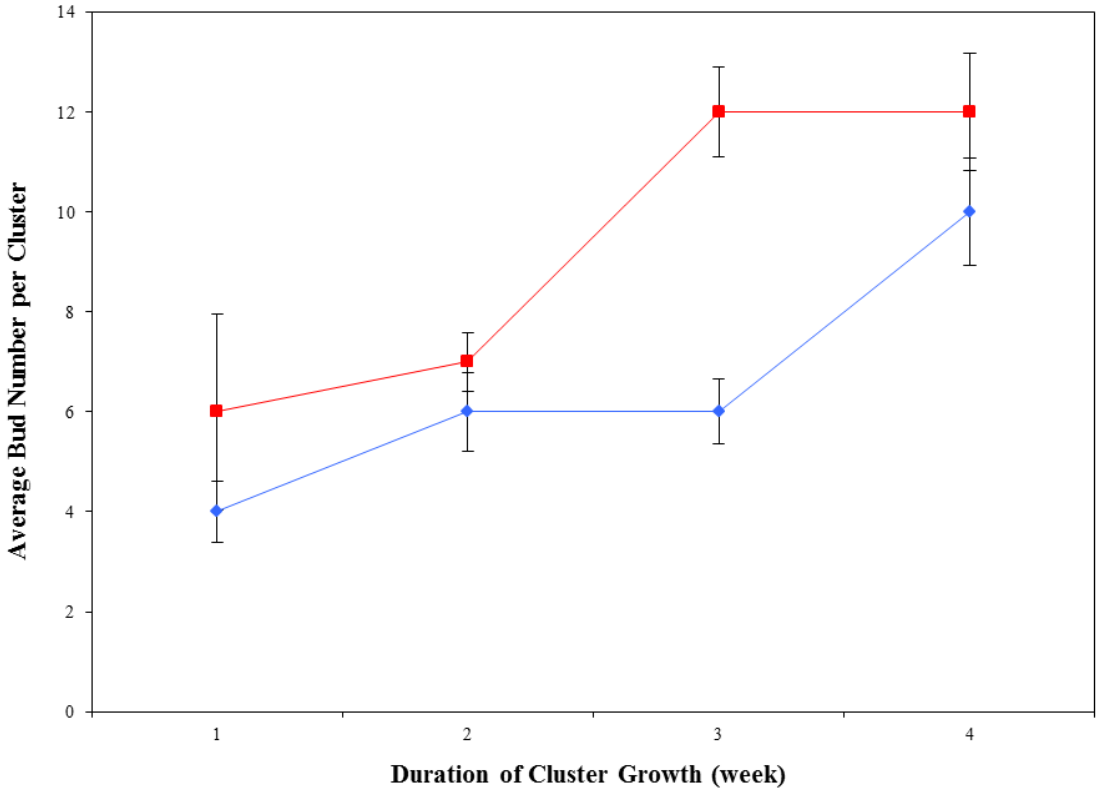



Figure 6: Comparison of *E. dunnii* bud production on *E. grandis* and *E. camaldulensis* multiplication medium (red) with *E. grandis* x *nitens* multiplication medium (blue). Shoots were cultured for 4 weeks, and growth was measured by the number of buds produced by each cluster per week. Bars represent the average ±SE of 3 replicates (*n*=20). At week 4, no significant difference existed between growth on the two media using a two-sample t-test ($P > 0.05$).

While a similar number of buds were produced on each medium, the physical appearance of the plant clusters differed. Although not quantified, it was observed that the shoots on the *E. grandis* x *E. nitens* medium had much shorter stems and internodes and callus formed at the bases of stems. Leaves were much smaller and darker (Figure 7A). A small percentage (14%) of plant material also turned hyperhydric over the test period. However, the majority (68%) of the plant material grown on the *E. grandis* and *E. camaldulensis* medium became hyperhydric.

Hyperhydricity occurs as a result of the minimized gaseous exchange with the external environment and increased humidity of the vessel environment (George *et al*., 2008). The effect of this condition on plant material, which includes low chlorophyll contents, stunted growth, abnormal stomata, deformed guard cells and poor rooting make the subsequent propagation of hyperhydric material not feasible (Zoybayed *et al*., 2000; Jackson, 2002; Kevers *et al*., 2004; Yu *et al*., 2011). However, the remaining 32% of shoots on the *E. grandis* and *E. camaldulensis* medium were "healthy" in appearance, with larger leaves and elongated internodes (Figure 7B), when compared with material established on the *E. grandis* x *E. nitens* medium.

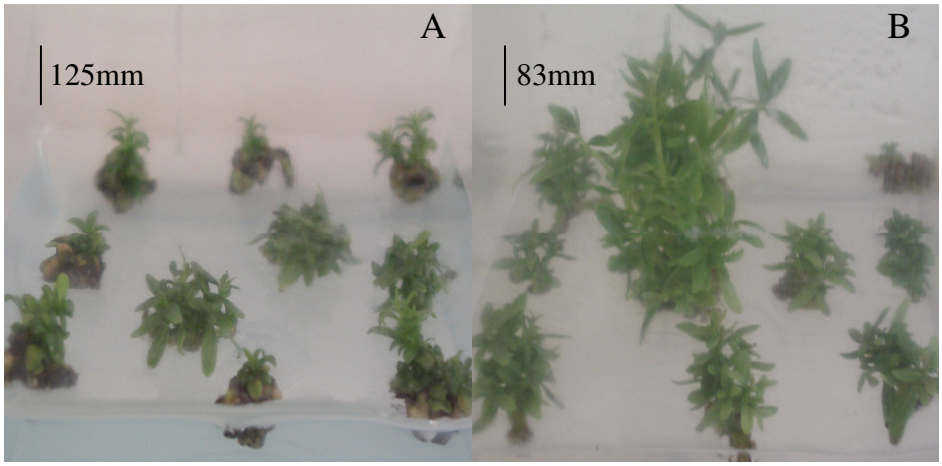



Figure 7A: Four-week old *E. dunnii* clusters grown on *E.grandis* x *E. nitens* medium. 7B: Four-week old *E. dunnii* clusters grown on *E. grandis* and *E. camaldulensis* medium.

All factors (culture conditions, culture vessels and plant material) were kept constant throughout this experiment, so that only the effects of the different media on plant regeneration could be observed. It is highly plausible that the differing media may have had an effect on plant nutrient uptake and growth (Cheng *et al.*, 1992b), as well as the resulting hyperhydricity (Asareh and Hennerty, 2002).

The *E. grandis* x *E. nitens* medium contained 5g.l$^{-1}$ more sucrose than the *E. grandis* and *E. camaldulensis* medium. An increased concentration of sucrose in culture media has been proven to inhibit photosynthesis of *in vitro* plantlets (Nakayama et al.,

1991), as well as decrease leaf and shoot proliferation (Ziv *et al*., 1983). This may explain the slightly lower growth rate on the *E. grandis* x *E. nitens* medium and the stunted appearance of plant clusters. An increase of sugar to the medium can also reduce the relative humidity in culture vessels to a degree (George, 1996) and reduce hyperhydricity in plant material (Ziv *et al*., 1981, 1983). The lower production of hyperhydric plants on this medium could therefore be due to the higher concentration of sucrose.

Benzyl aminopurine (BAP) is a cytokinin and is essential for cell division and initiation of shoots in culture (George, 1996). Benzyl aminopurine has also been found to encourage shoot elongation (Brondani *et al*., 2011). Cytokinins, and BAP in particular, have also been found to induce hyperhydricity when used at high concentrations in culture (George, 1996). In intact plants, cytokinins delay or reduce senescence, by slowing down the breakdown of chlorophyll and cellular proteins in general (Hartmann *et al*., 2002). Hyperhydric material has been found to be lower in concentrations of protein and chlorophyll (Kevers *et al*., 2004). The concentration of BAP varied between the media ($0.1 mg.l^{-1}$ more BAP in *E. grandis* x *E. nitens* medium). The hyperhydricity exhibited by the material on the *E. grandis* and *E. camaldulensis* medium could therefore possibly be linked to the concentration of BAP in the medium.

The *E. grandis* x *E. nitens* medium had $1 g.l^{-1}$ more Gelrite® than that of the *E. grandis* and *E. camaldulensis*. Through measurement of media water potential and conductivity, Spomer and Smith (1996) found that *in vitro* plants could potentially be sensitive to physiochemical changes in gelling agent concentration which could impact their growth and development. The increased concentration of gelling agent in the present study may then possibly explain the slightly lower production of buds in *E. dunnii* shoots on *E. grandis* x *E. nitens* medium as well as the “tightly-packed” appearance of plant clusters. The increased gelling agent, coupled with the increased sucrose concentration on this medium may also explain the low percentage of hyperhydricity.

Despite causing hyperhydricity *E. grandis* and *E. camaldulensis* medium was chosen for the multiplication of *E. dunnii*, as non-hyperhydric shoots appeared visibly much healthier and were longer and would be easier to sub-culture, maintain and root. Various tests were subsequently undertaken to reduce the formation of hyperhydric material (section 4.2.1.6.1) to increase plant numbers *in vitro*.

### 4.1.2 Preparation of Plant Clusters for Rooting

#### 4.1.2.1 Elongation of *In Vitro* Plant Clusters

Before *in vitro* rooting is attempted, plant clusters are usually elongated to obtain shoots of a manageable size (George, 1993). Since the multiplication medium employed for the *E. grandis* and *E. camaldulensis* species was successful with *E. dunnii*, the same elongation medium and protocol was used for all tested clones. Shoots (with approximately 4 leaves) were excised from clusters and placed onto the elongation medium for a four-week period. During this time, shoot development was monitored.

By the end of the second week, it was apparent that shoot growth had ceased and callus began to develop from the base of the stem in 95% of the shoots/clusters. At the end of the fourth week, very little of the original shoot structure remained (a few leaves were present) and callus dominated (Figure 8). Superficial roots had also developed from the callus. Studies by Brondani *et al*. (2012b) on *E. grandis* showed that mineral nutrition affected organogenesis, For example shoot growth was influenced by the concentration of boron and calcium, with reduced quantities causing callus development. Since the difference in multiplication and elongation medium in this study was the increased concentration of the auxin NAA in the elongation medium, callus formation may be attributed to this plant growth regulator. Other studies involving *Eucalyptus grandis* and *Eucalyptus grandis* x *urophylla* found that IBA, NAA and IAA encouraged callus proliferation within the *in vitro* environment (Wachira, 1997; Mycock and Watt, 2012). The elongation stage was

repeated three times with the same results. With the probability of occurrence, the quantity of callus produced over time, and the degeneration of the shoot system, further exploration of this medium for "rooting" was not attempted. Since healthy elongated shoots were produced on the multiplication medium, such material was used directly for various subsequent rooting experiments.

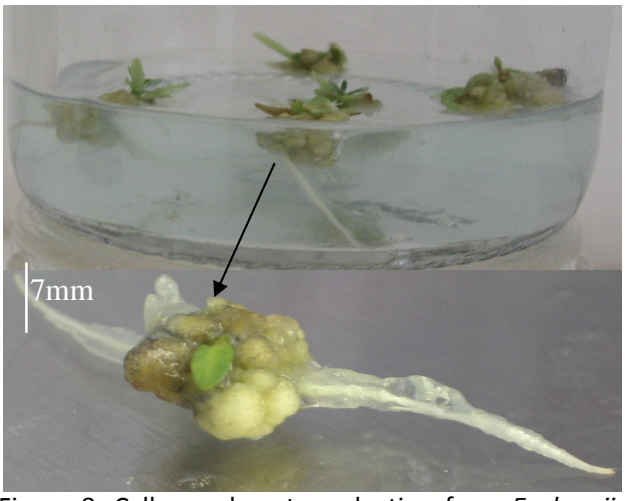



Figure 8: Callus and root production from *E. dunnii* shoots on elongation medium after four-weeks.

## 4.2 APPROACHES TO ROOT PRODUCTION

### 4.2.1 Production of Roots *In Vitro*

#### 4.2.1.1 Effect of pH of the Semi-Solid Medium on *In Vitro* Rooting

Studies have shown that a change in pH of the medium has been successful with triggering rooting of *in vitro* plant material (Williams *et al.,* 1985; George, 1993). The induction of rooting was attempted by growing *E. dunnii* shoots for 4 weeks on *E. grandis* and *E. camaldulensis* multiplication medium at varying pH levels (4.5, 5, 5.5, 5.6-5.8) (Figure 9). The pH was not lowered further as decreasing the pH of plant tissue culture media to below 4.5 was also shown to adversely affect morphogenesis of plant tissue cultures and to prevent solidification of the Gelrite® (Smith and Krikorian, 1990).

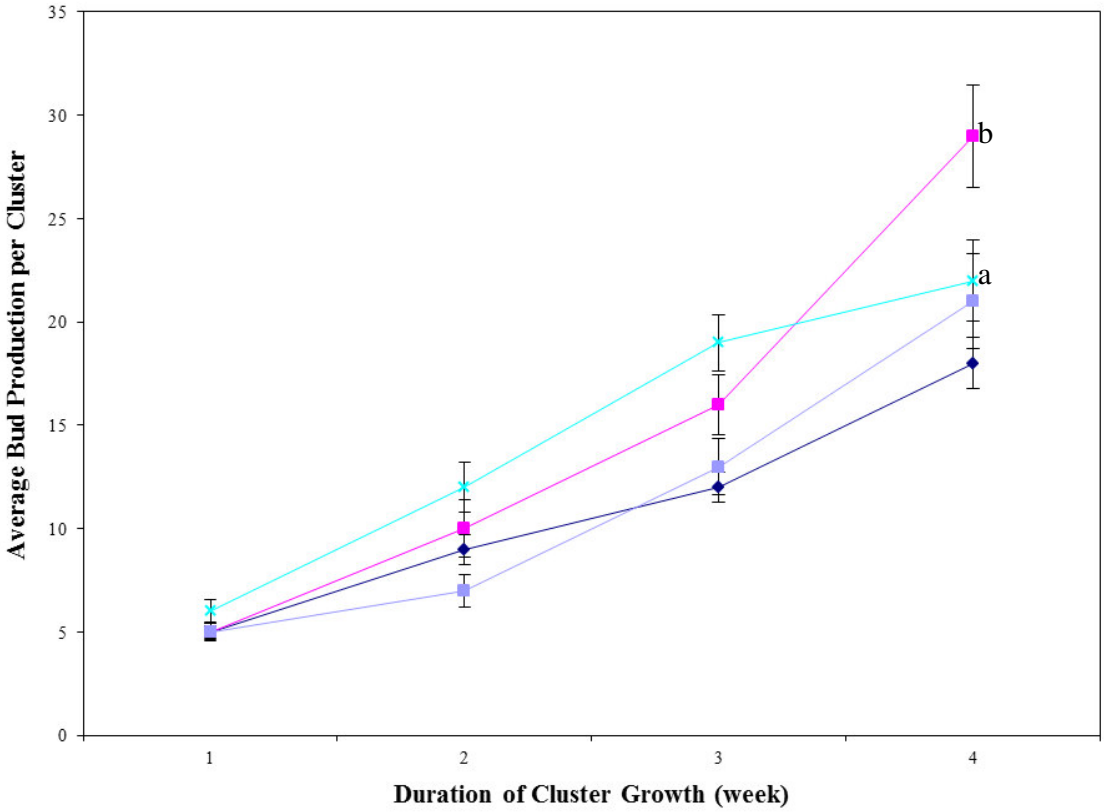



Figure 9: The growth of *E. dunnii* clusters on pH-modified *E. grandis* and *E. camaldulensis* multiplication medium was monitored for shoot and root development over 4 weeks. Shoots were grown at pH 4.5 (dark blue), pH 5 (pink), pH 5.5 (lilac) and pH 5.6-5.8 (turquoise blue). Shoot growth was measured by bud production. Bars represent the average ±SE of 3 replicates (*n*=20). At week 4, clusters on pH 5 medium were found to be significantly different (b) using the Bonferroni ANOVA ($P < 0.05$).

No roots were produced in all cases, although minor callus formation occurred from shoot bases of material cultured at pH 4.5.While cluster development on all media was statistically similar over the test period (excluding those clusters on pH 5 medium at week 4); as the pH of the medium decreased, the internodes of shoots were much shorter. The shoots at the lower pHs (pH 4.5 and 5) were darker than at those closer to the pH of standard *Eucalyptus* media (5.6-5.8), which is the pH range generally used for eucalypt *in vitro* culture (Watt *et al.,* 1991, Mokotedi *et al.,* 2000; Hajari, 2004; McAlister *et al*., 2005).

It has previously been noted that eucalypts exhibit a substrate preference for acidic soils (Turnbull, 1999). In a study by Symonds *et al.* (2001) of 35 *Eucalyptus* species, which were grown on an experimental pH range of 5.1 to 8.9 in a peat-based growing medium, it was also found that growth was favoured under acidic (5.1 to 5.6) conditions. Under higher pHs, results ranged from no change in development to reduced growth of *Eucalyptus* species. This could also be related to the vigour noted in plant material grown on the pH of 5.5 to 5.8, with a decrease in pH below 5.5 having a similar effect as a high pH on eucalypt material (Symonds *et al.,* 2001). However, there needs to be consideration of the soil pH of the natural habitat of the eucalypt species as pH response of plant material has been shown to correlate to this (Symonds *et al.,* 2001). Since the natural preference in soil pH of *E. dunnii* has been found to range between 4 to 8 (McMahon *et al*., 2010), it is understandable that growth on semi-solid media appeared to be optimal for *E. dunnii* at a pH of 5 to 5.5.

Nutrient deficiency could be the explanation for the observed decreased vigour of *E. dunnii*, as the uptake of nutrients such as ammonium has been observed to decrease with decreasing pH (Martin and Rose, 1975), and certain mineral nutrients are known to become immobilized in soil at low pH values (Mengel, 1984). Since the quality of the plant clusters in this experiment was affected, it was apparent that a decrease in pH (< 5.0) did not have positive effects on *E. dunnii* growth or rooting *in vitro.*

#### 4.2.1.2. Effect of Indole-3-butyric acid (IBA) Concentration Range in Semi-Solid Medium on *In Vitro* Rooting

The plant growth regulator auxin has the greatest influence over root formation and development (George, 1993). The auxin idole-3-acetic acid, IAA, is produced in young shoots, and promotes root and vascular development and possibly the development of graviresponse in *Eucalyptus* (Aloni *et al.,* 2005; Nakhooda *et al*., 2011). The concentrations and types of the auxin present in the medium therefore impacts the morphology and quality of the roots produced; generally higher concentrations produce callus (Wachira, 1997) while lower concentrations may not

promote root induction or gravitropism (Nakhooda *et al*., 2011). A recent study by Nakhooda *et al.* (2013) suggests that the inclusion of IAA in micropropagation of *Eucalyptus* is necessary for production of quality roots and that the type and concentration of auxin used earlier in the micropropagation process affects rooting later in the process.

In general, studies show that *Eucalyptus* root growth appears to be optimal with treatments of the auxin, Indole-3-Butyric Acid (IBA); and concentrations vary between species, from 0.5mg.$l^{-1}$ employed with *E. grandis* x *urophylla* by Hajari (2004), to 1.0mg.$l^{-1}$ used with *E.* grandis, *E grandis* x *nitens*, *E. tereticornis* and *E. camaldulensis* (Wachira, 1997; Mokotedi *et al.,* 2000; Sharma and Ramamurthy 2000; Girijashankar, 2012).

The general rooting medium used in this study, and employed by the Mountain Home, Mondi Group South Africa, has a quarter strength MS and half the quantity of sucrose as multiplication medium and 0.5mg.$l^{-1}$ IBA. Reducing the concentration of MS and sucrose in media has been found to support root growth (Nourissier and Monteuuis, 2008; Girijashankar, 2012). *E. dunnii* shoots (and later, clusters) were therefore placed upon the general rooting medium, and exposed to a concentration range (0.1-1.0mg.l) of IBA. After approximately 3 days, bacterial contamination developed, and destroyed the explants on all media shortly thereafter. The bacteria appeared to be endogenous. Repeated executions of the experiment as well as eight antibiotic treatments (Table 1) were applied with no success.

In an attempt to overcome the contamination problem, a less humid environment was then considered to reduce the humidity and minimize bacterial contamination (Leifert *et* al., 1991; Reed and Tanprasert, 1995).

#### 4.2.1.3. Effect of Rooting Substrate

While most clusters that were placed on a coir:vermiculite (1:2) mixture in culture bottles and watered with rooting medium remained green and healthy over the test period, no roots were formed in 96% of material after 8 weeks. In 61% of shoots, callus had formed, 34% of the callus was white while the remainder (66%) was black. The production of callus *in vitro* as a result of plant growth regulators/nutrients has been noted in the micropropagation of other *Eucalyptus* species, namely *E. polybractea* (Goodger *et al*., 2008), *E. benthamii* x *E. dunnii* (Brondani *et al*., 2011), *E. macarthurii, E. smithii, E. macarthurii* x *E. grandis* and *E. saligna* (Le Roux and Van Staden, 1991). In callus that develops at the base of stems, vascular connections between shoots and roots produced are often incomplete, and this may cause problems during acclimatization of plantlets (Gregory, 2006). Only 4% of plant clusters were callus-free and exhibited root production directly from the cluster and subsequently developed into healthy *in vitro* plantlets.

The initiation of roots may be attributed to the type of rooting medium, with coarse substrates having been found to promote improved aeration of the substrate surrounding the material and decrease microbial activity (Hartmann and Kester, 1975; Agbo and Omaliko, 2006). This appeared to be the case in this study. Bacterial contamination was prevalent (100%) in semi-solid media used for rooting, while it was observed that a change to a coarser medium *in vitro* resulted in decreased microbial activity but still with a small percentage of rooting success (4%).

Apart from culture conditions, the low rooting success of *E. dunnii* may be linked to the duration spent under *in vitro* conditions as well as endogenous rhythms of the plant (Mankessi *et al*., 2009). These need to be considered before forming conclusions on the rooting capacities of *E. dunnii in vitro.* In this regard the *E. dunnii* plant material was introduced to *in vitro* conditions approximately 6 months prior to commencement of rooting experiments, and this may have influenced rooting success.

#### 4.2.1.4 Effect of Dimensions of *In Vitro* Plantlet Roots and Seedling Roots (Height Range: 2cm-5cm)

In studies conducted by Bell *et al*. (1993) no architectural differences either above- or below-ground could be found between *in vitro* plantlets and seedlings. However, the recent findings of Mokotedi (2010) differ from those of Bell *et al*. (1993). That author found that root architecture and characteristics of *Eucalyptus nitens* x *grandis* trees produced from seedlings proved to have better anchorage when compared with vegetatively propagated trees (Mokotedi, 2010).

Therefore, in this study, *in vitro E. dunnii* plantlets (unhardened) were compared with seedlings (root structure specifically) within the same height range to determine if any correlations could be drawn with regard to the root structure. Since the main roots of seed-propagated *Eucalpytus nitens* and *E. grandis* and *E. camaldulensis* were shown to penetrate deeply into soils (Bell., *et al.,* 1993; Mokotedi, 2010), including clay soils, they were of importance to anchorage. It is therefore important that an *in vitro* method produces not only a high percentage of rooting, but also long and well established roots. Therefore, the average length of the longest taproot or "main root" (if there was more than one), along with the length of the major lateral or "side" roots and number of lateral roots of *in vitro* plantlets were compared with those of seedlings within the same height range (Figure 10).

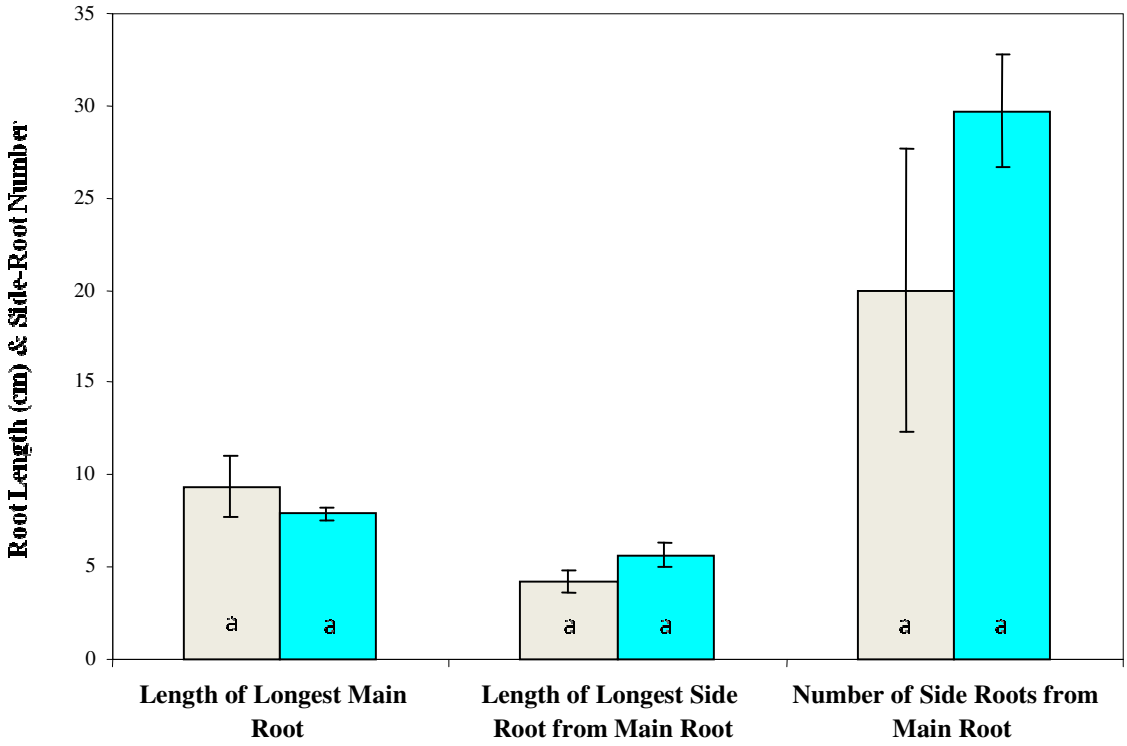



Figure 10: Comparison of root dimensions of *in vitro* plantlets (blue) and seedlings (grey) within the same shoot-height range (2-5cm). The extension of the longest main root produced by the *in vitro* plantlet was compared with that produced by the seedling. The extension of the longest lateral root from the main root was also represented along with number of lateral roots. Bars represent the average ±SE of cuttings ($n$=10) and seedlings ($n$=5). It was concluded that no significant difference (a) existed between the *in vitro* plantlets and seedlings of the same height range using the Kruskal-Wallis ANOVA ($P > 0.05$).

Statistically, it was determined that no differences existed between all the parameters that were compared. The dimensions of the root structure of an *in vitro* plantlet appeared to mirror that of a seedling of the same shoot height. When making the comparison of root systems, one must also consider the shoot system in order to gain a comprehensive picture, as each cannot naturally exist independent of the other. Dry shoot and root masses were therefore compared to determine if the similarity of *in vitro* plantlets and seedlings surpassed the proportions of the root structure. While the shoot:root dimensions of the *in vitro* plantlet (Figure 11) did not visually match that of the seedling (possibly due to the low sample size of *in vitro* plantlets and propagation method), the masses were proven to be statistically similar (Figure 11: *P*

= 0.0532 for shoots, and $P$ = 0.1319 for roots). This implied that both plant-types had approximately the same quantitative morphology, belowground and aboveground.

It should be noted that the quantitative data did not match qualitative observations for each plant type. While statistically, the root morphology was similar (i.e. number and length of main and lateral or "side" roots), the lateral roots of the *in vitro* plantlets were less developed than seedlings (Figure 12). Age of the plant material may also influence rooting. *E. saligna* and *E. globulus* cuttings of differing ages (cuttings taken from 2 and 3 month-old seedlings) responded differently to auxin applications and light exposures (Fett-Neto *et al*., 2000). This raises questions as to the influence of propagation methods and root development. Studies by Warang *et al*. (1989) and Bell *et al.* (1993), working with *E. grandis*, found that over time, any occurrence of differences in architecture and morphology of micropropagated plantlets and seedlings early in development disappeared as the trees matured. Mokotedi *et al*. (2003) compared the the roots and shoots of micropropagated and macropropagated *E. grandis* x *nitens* and found that differences between the two were not significant. Other studies showed the contrary, for example with *E. marginata* and *E. grandis* x *E. nitens*, micropropagated plants lacked a taproot and had shallower, more fibrous roots than seedlings and had less resistance to uprooting (Bennet *et al*., 1986; Mokotedi *et al*., 2009; Mokotedi *et al*., 2010). These results therefore differ with species of *Eucalyptus* and micropropagation conditions. Therefore, further studies may be necessary to fully understand the influence of micropropagation of *E. dunnii* and root system development.

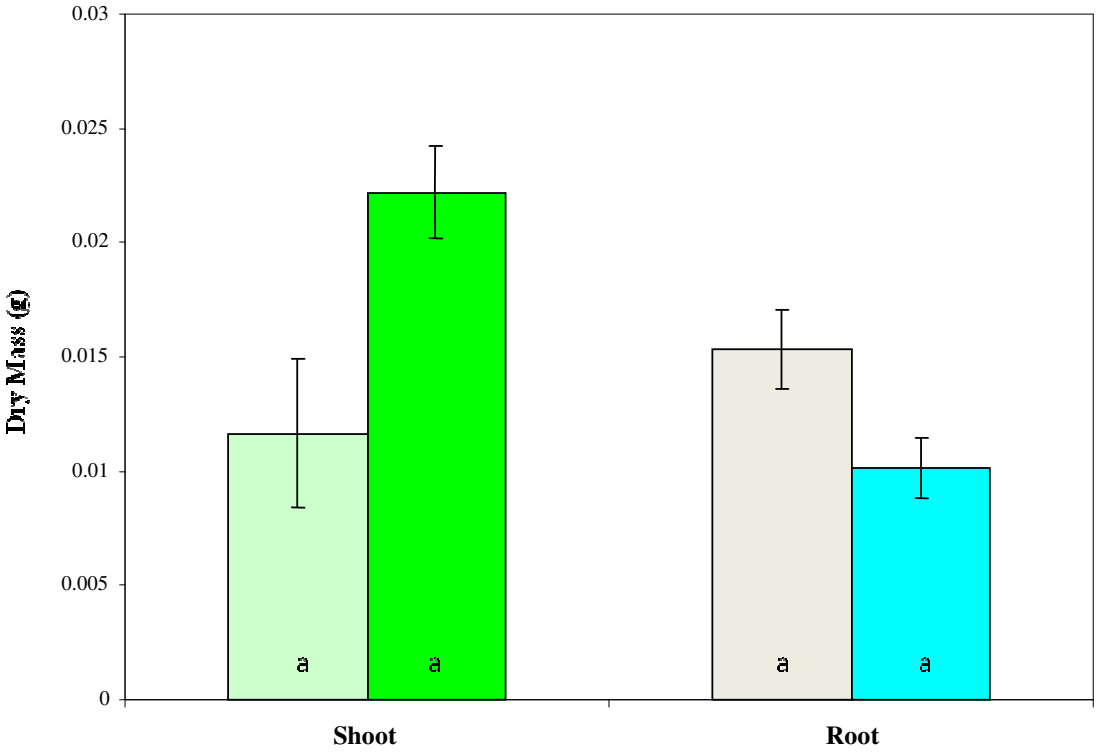



Figure 11: Shoot mass of *in vitro* plantlets (light green) and seedlings (seed-derived) (bright green) were compared, along with root mass of *in vitro* plantlets (grey) and seedlings (seed-derived) (bright blue). Both were not significantly different (a) using a Mann-Whitney Signed Rank Test ($P > 0.05$; for shoots $P = 0.0532$ and roots: $P = 0.1319$).


Figure 12: *In vitro* plantlet (A) structure compared with seedling (B) structure of the same size height range (2-5cm).

#### 4.2.1.5. General Trends Observed with *In Vitro* Plantlet Root Growth and Comparison with Seedlings

Visually, *in vitro* plantlets and seedlings of the same height range differed (Figure 12A, 12B), although some common trends were still observed within and between each propagation type.

One main stem developed and elongated from each *in vitro* cluster (in 75% of *in vitro* plantlets) and new shoots formed from the base of this stem. The leaves of clusters on rooting medium were larger than those found in material on multiplication medium. Seedlings exhibited the same morphology, that is, in all cases, one main stem was observed, and new shoots were found growing from the basal nodes of these stems in 50% of the test material. A study of *E. polybractea* indicated that after a year of growth in the natural environment, the leaves of micropropagated-derived plants and seed-derived plants displayed no morphological differences (Goodger and Woodrow, 2009). The same may occur with micropropagated *E. dunnii* plantlets once acclimatised. That is, the statistically similar above-ground biomass of *E. dunnii* seedlings and *in vitro* plantlets (Figure 11) may remain the same once *in vitro* plantlets are transferred to the field.

The roots of *in vitro* plantlets formed from the base of the original cluster-stem. In 100% of *in vitro* plantlets, some callus formation occurred from the base of the stem (possibly due to damage inflicted during excision); however, in all cases root development was not initiated from callus but directly from the stem (Figure 12A). No callus was observed at any stage of seed germination and seedling development.

In 75% of *in vitro* plantlets all main roots were “fleshy” and white in appearance toward their ends, while the remaining 25% had blackened, dried dead ends. Twenty percent of seedlings were found to have the same white “fleshy” ends and 90% of these seedlings had dried dead ends from the main root.

At the Mountain Home, Mondi Group South Africa, bottomless propagation trays with vertical grooves along the sides are used for propagation. This design allows for air pruning (exposure of the root to air) of roots. Air pruning has been found to promote root growth (both accelerated development and density) (Maguire and Harun, 2007). This could have explained the appearance of the seedlings but not that of the *in vitro* plantlets, which may suggest that this trait may be linked to *Eucalyptus* root growth.

All (100%) seedlings were found to have a "kink" at the interface of the shoot stem and root (Figure 12B) as well as in the middle region of the main root, some cases were more pronounced than others. Kinks were also observed in *E. grandis* x *E. nitens* vegetatively propagated plant material by Mokotedi (2010). All (100%) main roots of *in vitro* plantlets grew out horizontally; either at approximately 90° to the stem, or at approximately 135° to the stem (for approximately 0.5cm in both cases) before responding to gravity and growing downward. Bell *et al*. (1993) also observed that older seedlings and micropropagated plantlets had several major lateral roots that grew at a shallow angle from the base of the plant before then turning vertically down. With *E. globulus* seedlings, multiple secondary roots grew laterally out from the main tap root, close to the surface and formed a dense network just below the surface of the soil (Sasse and Sands, 1997). External stimuli, such as nutrient availability has been found to act with aerial tissue of the plant in the development and architecture of lateral roots within the substrate (MacGregor *et al*., 2008; Graciano *et al*., 2009). This may have been the case with *E. dunnii.* However, Mokotedi *et al*. (2010; 2011) observed similar trends (kinks, lateral root growth etc), and after 16 months of field growth, *E. grandis* x *E. nitens,* micropropagated plants yielded an inferior root system when compared with their seedling-derived counterparts. Further, the work by Nakhooda *et al*. (2012) proposed that rooting of "poor rooters" *in vitro* could possibly be attributed to depletion of exogenous cytokinin supply from shoots. Therefore while above-ground biomass was statistically similar between seedlings and *in vitro* material, nutrient and plant growth

regulator supply must be considered to provide a comprehensive view on *in vitro* rooting and their architecture.

#### 4.2.1.6. Difficulties Experienced with *In Vitro* Cultures

In 1977, Murashige identified the inability to completely disinfect explants and tissue deterioration during the initial stages of plant-media introduction, as major problems that needed attention. Hyperhydricity or vitrification of plant material has also been noted as a common problem along with callus formation (George, 1993). These problems were also encountered while establishing an *E. dunnii* culture *in vitro*, and tests were undertaken in an attempt to eliminate or control their manifestation.

##### 4.2.1.6.1 Vitrification of Plant Cultures

Hyperhydricity or vitrification in plant tissue is a physiological disorder, characterized by distortion of regenerating tissue (Ziv *et al*., 1983). Plant material, such as *Eucalyptus grandis*, has been found to exhibit diminished lignificaton, be lower in protein and chlorophyll, have a higher cell vacuolation and water content and water potential than healthy regenerating tissue (Kevers *et al*., 2004; Picoli *et al*., 2008). This results in brittle plant material with a translucent, water-soaked, succulent appearance and can result in cultures that deteriorate, fail to proliferate, acclimatize and sometimes lead to tissue death (Warren 1991; Hartmann *et al*., 2002). It has been suggested that all external causes of vitrification refer exclusively to the culture conditions, medium and atmosphere within the culture vessel (Gaspar *et al.,* 1987). With *Eucalyptus* vitrification, supplements are usually applied to the culture medium to reduce its occurrence (Sharma and Ramamurthy, 2000; Whitehouse *et al*., 2002).

Hyperhydricity was found to be the most prevalent in the experiment which compared the range of semi-solid media compositions (3.1.1.1.). Since plant material was precious and a high percentage (68%) was affected, three tests were undertaken to reduce/reverse the hyperhydric state of the explants. The high humidity present in most culture vessels has been found to promote hyperhydricity of plant material (Ziv,

1986; George, 1996; Benson 2000). The reduction of humidity in *E. dunnii* culture vessels was attained by the introduction of plastic boats containing activated silica gel to the vessels (Figure 13). It was hoped that the drier environment would possibly reverse the hyperhydric state of plant material.

The reduction in humidity of the vessel had an immediate impact on plant clusters. Within the first week (Figure 14A), the lower leaves of 60% of the clusters began to dry and turned brown. This increased in the second week and was coupled with wilting in 64% of clusters. Those plants affected began to appear less glassy. 83% percent of stems and leaves of plant clusters with these symptoms (80% browning and 87% wilting by week 3) lost the glassy, fleshy appearance associated with vitrification. While clusters still retained the elongated stems, these were no longer brittle and succulent by the fourth week, and 87% of plant clusters reverted to their original "normal" appearance. The clusters did still retain the dried lower leaves produced over the test period and while stems remained upright, there was a slight inclination, possibly due to the observed reduction in stem girth.

Once the test period ended, the clusters were sub-cultured onto multiplication medium and placed into vessels devoid of activated silica. Within a week all material showed the characteristic signs of vitrification, indicating that the material had been irreversibly altered.

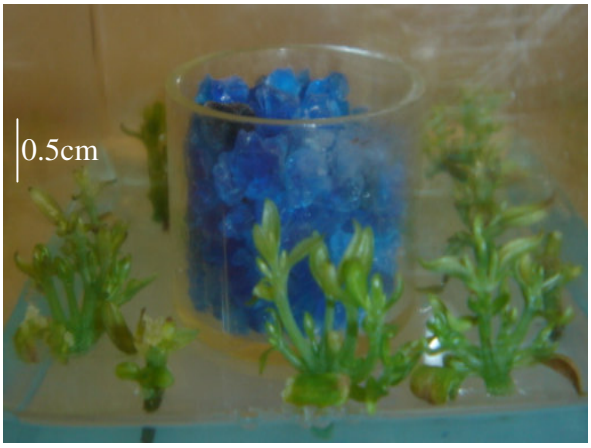
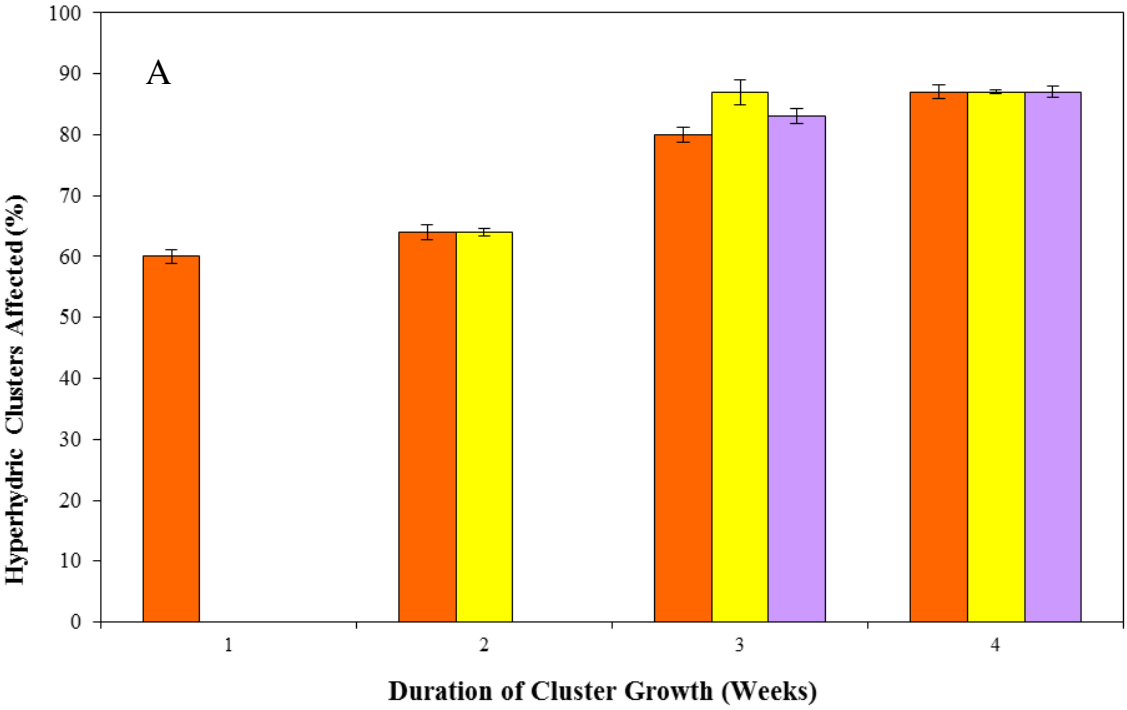



Figure 13: Plastic boat containing activated silica gel, surrounded by hyperhydric plant clusters.

Increasing the concentration of the gelling agent in culture media has been successful in alleviating the problem of hyperhydricity (Ziv *et* al., 1983; Ziv, 1986; Brand 1993; George, 1996; Majada *et al*., 1997; Hartmann *et al*., 2002). Therefore, the concentration of gelling agent (Gelrite®) in the multiplication medium of *E. dunnii* was increased, and hyperhydric shoots (approximately 7 buds per shoot) were sub-cultured onto this modified medium.

Three concentrations of Gelrite® were tested, 3, 3.5 and 4g.$l^{-1}$. Results (Figure 14A&B) showed that over the test period, shoots on the media displayed the same growth pattern and there were no statistical differences in the number of buds produced each week. The hyperhydric status of shoots was monitored weekly and did not change over the test period, i.e. 100% of the shoots remained hyperhydric.

Visual inspection showed that while plant material was fleshy and transparent, the newly developing buds at the tops of clusters appeared healthy and normal. It was therefore hypothesized that isolation and growth of these buds could possibly result in healthy plant material. They were therefore isolated and plated onto multiplication medium, made using fresh constituents (including MS with vitamins prepared in-house according to the protocol of Murashige and Skoog, 1962, see appendix). Within 5 days, 100% of test-buds developed the characteristic fleshy, glassy state of vitrified material. According to Picoli *et al*. (2008), the ultrastructure of *Eucalyptus* material has shown that the presence of intercellular spaces on its surface may hinder recovery of hyperhydric plants. According to George (1996), the most extreme cases of hyperhydricity are usually non-reversible.



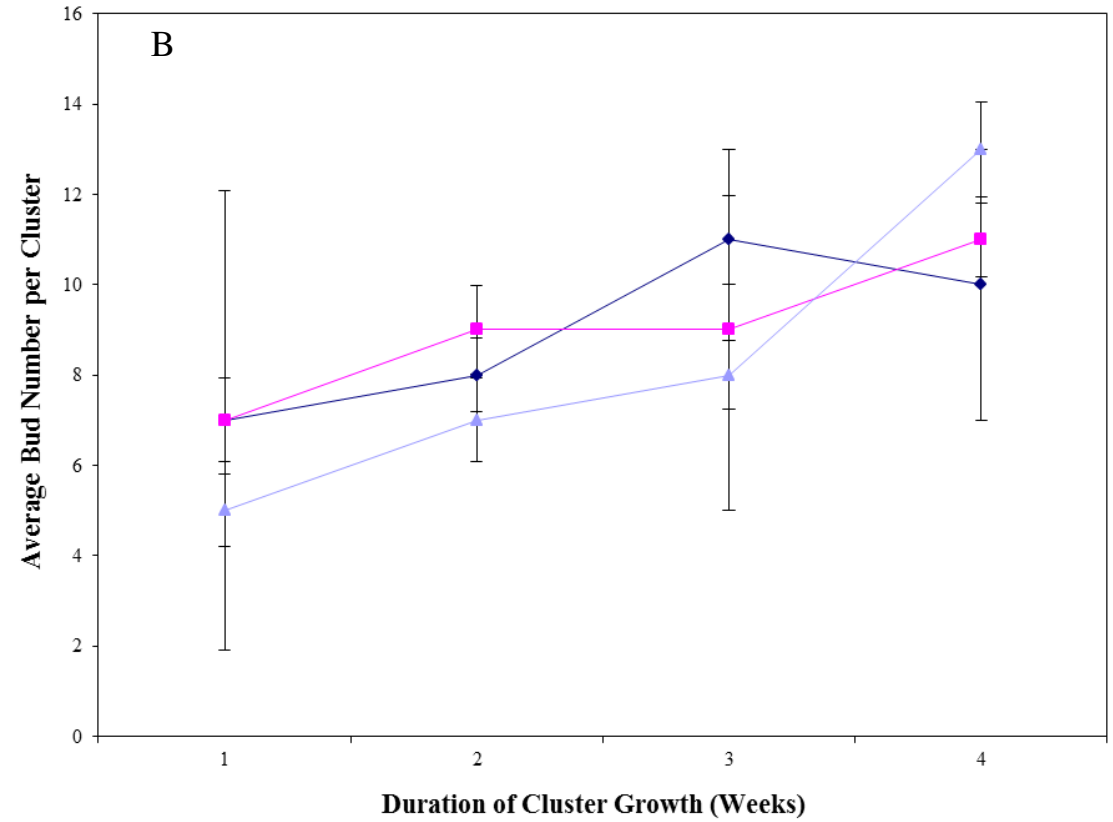




Figure 14: The effect of reduction in humidity (14A) and increase in gelling agent (14B) on the status of hyperhydric plant clusters over 4 weeks. In A, silica gel-containing boats were introduced to the culture vessel to reduce the internal humidity. The orange bar represents leaf browning; yellow - wilting and purple, a reversal of the hyperhydric appearance of clusters. With regards to increasing the gelling agent (14B), plant clusters were placed onto multiplication medium containing increasing quantities of gelling agent. The dark blue line represents 3g.l$^{-1}$ Gelrite®, pink - 3.5g.l$^{-1}$ Gelrite®, and lilac - 4g.l$^{-1}$ Gelrite®. Bars represent the average ±SE of 3 replicates (*n*=20). At week 4, clusters on the differing medium were found not to be significant using the Bonferroni ANOVA ($P > 0.05$).

#### 4.2.1.6.2. Contamination *In Vitro* and Antibiotic Treatments

Use of the RITA® Temporary Immersion Bioreactor System for the multiplication of semi-solid established *E. dunnii* material was attempted. The use of such a scaled-up vessel has been described to have many benefits (Watt, 2012) for example, the multiplication of *Eucalyptus camaldulensis* resulted in enhanced growth, higher plant densities and uniformity of material (Zobayed *et al*., 2000). Use of this system in this study however was discontinued due to bacterial contamination which was rife and could not be controlled. Bacterial contamination was also observed with *in vitro* plant material placed on semi-solid rooting medium (3.2.3.2). Since the rooting experiment could not be successfully completed as all plant material was destroyed by microbes on this medium, a combination of antibiotics (Table 6) was tested to control the contamination.

The type of explant (shoots/clusters) that was used had no effect on the success of the treatment as shoots were destroyed faster than clusters by microbes but both did not survive. Treatments 4, 7 and 8 were partially successful as the placement of antibiotic solution into the medium suppressed bacterial growth for approximately 10 days. Thereafter, bacteria possibly grew resistant to the solution or the solution lost its efficacy and explants were destroyed. As rooting was being attempted it would not have been practical to transfer the material to fresh medium containing antibiotic solution over the test period. Antibiotic applications for endogenous contamination are also expensive for prolonged treatments, and require optimal growth conditions for success (Watt, 2012). In this case, once material became infected, it was impossible to eliminate or even suppress the contamination and all plant material was destroyed. The use of the RITA® system was therefore abandoned. The problem of bacterial contamination in the RITA® system was also identified by Watt *et al.* (2006), who were able to circumvent it by screening axillary buds on a microbial medium before placement into the system.

#### 4.2.1.6.3. Callus Production from Plant Clusters

In this study, callus production was most prevalent when elongation of plant clusters was attempted (4.1.2.1). While excising the callus from plant material was a short-term solution, plant growth regulators in the medium possibly promoted re-growth of callus (including production of roots from the callus) (Goodger *et al*., 2008; Brondani *et al*., 2011) and resulted in continual callus development and subsequent destruction of the plant material.

While the production of callus after excision is a natural wounding response, with rooting, callus formation between roots and shoots can result in incomplete vascular connections between the organs (de Fossard *et al.,* 1977; George, 1993). With the alternate rooting substrate (4.2.1.3), large masses of callus formed at the base of 61% of clusters. Two colours (white and black) and possibly "types" ("soft" and "hard") of callus existed (Figure 15), even though all clusters were subjected to the same conditions.

Unlike the callus on elongation medium which produced roots, no roots were produced from callus in the alternate rooting (peat:vermiculite) substrate. A similar observation was noted by Brondani *et al*., (2011) with *E. benthamii* x *E. dunnii*, where high concentrations of IBA (greater than $1mg.l^{-1}$) promoted callus production. With *E. dunnii* in this study, the callus did not grow and dominate the explant. Callus also formed from the base of clusters that did root. These calli were much smaller than in clusters that did not root however, and did not impact on root development, as all roots were produced just above or below the balls of callus.

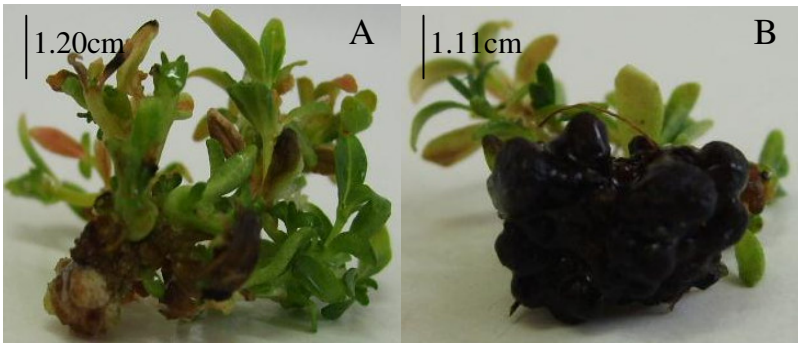



Figure 15: A - White callus of a "soft"/spongy texture produced on the peat:vermiculite substrate. B - Black callus, hard in texture, produced on the same substrate.

### 4.2.2 PRODUCTION OF ROOTS FROM MINI-CUTTINGS

#### 4.2.2.1 Effect of IBA Treatments on Rooting

The South African forestry industry currently relies on cuttings for mass-propagation of trees. The Mountain Home, Mondi South Africa, in particular, produces cutting-derived-plantlets by dipping mini-cuttings into a rooting powder. In keeping with this procedure, cuttings from *E. dunnii* clones A and B were subjected to the same treatment (dipping into rooting powder) along with IBA solution applications and rooting success of both was recorded (Figure 16).

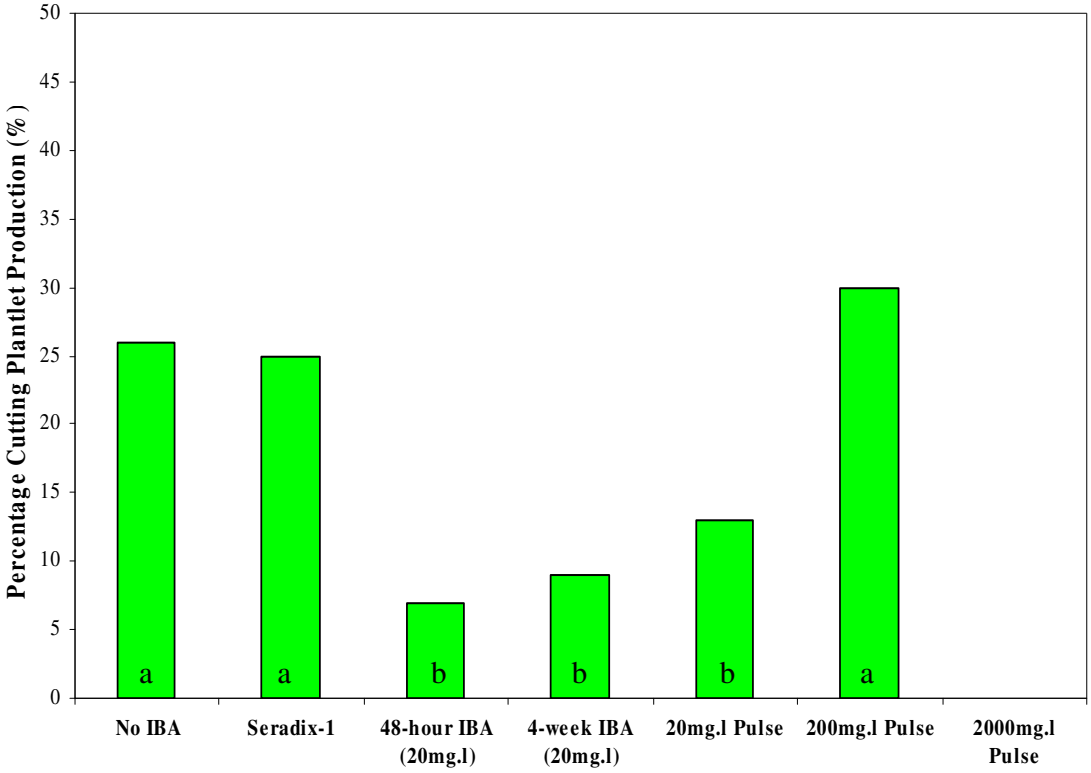



Figure 16: Effect of IBA administration on plantlet production from mini-cuttings. Prepared mini-cuttings were exposed to no IBA (control) (3 replicates, *n*=33); dipped in seradix-1 (3 replicates, *n*=80); 20mg.l$^{-1}$ 48-hour and 4-week IBA applications (3 replicates, *n*=66); and 20, 200 and 2000mg.l$^{-1}$ pulse treatments (3 replicates, *n*=10). No significant difference existed between the treatments that had moderate rooting success, as well as those that had a low rooting success using the Kruskal-Wallis ANOVA ($P > 0.05$)

Since both of the tested clones showed similar growth trends and morphology, all results were pooled. Mini-cuttings devoid of any treatment (no IBA) were found to have approximately the same percentage success (26%) with rooting as those treated with Seradix1 (25%). The 20mg.l$^{-1}$, 48-hour and 4-week IBA applications along with 20mg.l$^{-1}$ pulse treatment showed a very low percentage (7, 9 and 13% respectively) of root production at the end of the test period. Cuttings that underwent the 200mg.l$^{-1}$ pulse treatment showed a 30% success, but this was found not to be significantly different from the other two treatments, while the 2000mg.l$^{-1}$ pulse IBA treatment produced no roots.

The endogenous level of auxins measured in stem tissues during root initiation is known to vary between the different stages of root initiation (Hartmann *et al*., 1990). It is, therefore, likely that the optimum level of auxin needed within tissues to maximize root initiation varies substantially at different stages of the root initiation process of *Eucalyptus nitens* (Luckman and Menary, 2002). Other experiments with delayed auxin applications have proven more successful than the standard practice of applying auxin at the time of cutting collection (Luckman and Menary, 2002). The results of this study reinforce that the success of root initiation is significantly influenced by the timing of the application of auxin. However, other factors may also need to be considered.

The success of mini-cutting survival, development and rooting of *Eucalyptus camaldulensis, Eucalyptus pauciflora*, *Eucalyptus regnans* and *Eucalyptus nitens* have been found to be linked to the seasonal conditions and temperature of the substrate. Chaudhry (1994) found that rooted mini-cuttings of *Eucalyptus camaldulensis* were significantly more successful when planted in spring while growth rates of *Eucalyptus regnans* and *Eucalyptus cloeziana* were found to correlate strongly with the higher day/night temperatures as those experienced in spring and early summer (Ashton, 1975; Trueman *et al*., 2013). Trueman *et al.* (2013) also found that increased day/night temperatures resulted in greater root production from cuttings. In the development of *Eucalyptus urophylla* x *Eucalyptus grandis* mini-cuttings (Mankessi *et al*., 2011), seasonal variations played a noticeable role in survival and rooting rates along with the type of mini-cutting taken.

Mankessi *et al*. (2011) observed that the initial position of the mini-cuttings within the shoot from which mini-cuttings originated impacted the success of plantlet formation. Length of mini-cuttings may also impact rooting success. The length of *Eucalyptus grandis* x *Eucalyptus urophylla* mini-cuttings were tested for their impact on rooting (Naidu and Jones, 2009). The smallest mini-cutting lengths (5cm) had the lowest rooting success, lowest number of roots per mini-cutting and root dry mass (Naidu and Jones, 2009).

While the mini-cutting experiments in this study were conducted at the onset of winter (late May), which may have impacted on the vigour of the plant material, the mini-cuttings were grown under consistent greenhouse conditions thereby minimizing the likelihood of external seasonal influences. The position of the mini-cuttings within the shoot was not noted during mini-cutting preparation. This and the effect of length may have had an influence on rooting success.

#### 4.2.2.2 Root Architecture of *E. dunnii* Mini-cutting Plantlets and Seedlings (Height Range: 5-7cm and 7.1-9cm)

Mini-cutting plantlets were compared with seedlings of the same height range to investigate if any similarities existed with the root architecture. With both height ranges, the length of the living main roots (Figure 17A&B) of mini-cutting plantlets was found to be longer than seedlings (mini-cutting plantlet main roots of both height ranges were 11cm on average while seedlings of both height ranges were 8cm). The same results were noted by Sasse and Sands (1997) in their comparison of root length of *E. globulus* seedlings and mini-cutting plantlets. The fundamental difference however, between mini-cuttings and seedlings, was the structure of their root systems. In both this experiment and that by Sasse and Sands (1997), seedlings developed taproots that were strongly gravitrophic while mini-cuttings had no main taproot. The main structural components of their root systems were the primary adventitious roots which had formed during propagation. Secondary and tertiary roots then developed from the primary roots. While seedlings did display uniform distribution (observed but not quantified) of secondary and tertiary roots throughout the length of the primary root, in plantations over time, the root density of mature *Eucalyptus* roots can decrease with depth and increase with distance from the primary root (Laclau *et al*., 2001). Age of the seedlings, as well as resource availability (Laclau *et* al., 2001) must therefore also be considered when assessing root architecture. The same was observed with lateral-root length in both height ranges. In the 5-7cm-height range, lateral-roots of mini-cutting plantlets (11cm) were 5.5cm longer on average than lateral-roots of seedlings (5.5cm). With the 7.1-9cm-height range (Figure 17B), lateral-roots of mini-

cutting plantlets (10cm) were on average 4cm longer than those of seedlings (6cm). Other similar studies have reported the opposite results, with seedlings having a greater number and total length of secondary roots (Sasse and Sands, 1997).

The design of the growth trays kill exposed roots through air-pruning and prevent horizontal spiralling of the root system (Hartmann *et* al., 2002; Maguire and Harun, 2007). The trays were used for propagation of mini-cuttings in this study. This may have limited the length of growing roots and may possibly explain the slight reduction of lateral root length in mini-cutting plantlets as shoot height increased.

The number of lateral roots of both seedlings and mini-cutting plantlets in each height range was found to be statistically similar (Figure 17). Between height ranges, though main and side root lengths did not differ, mini-cutting plantlets and seedlings in the 5-7cm-height range appeared to have more lateral roots on average than those in the 7.1-9cm-height range (approximately 5 and 8 more lateral roots respectively). For the 7.1-9cm-height range, the decrease in side root number could possibly mean that while shoot growth was underway, root growth was possibly in stasis between those shoot heights. Shoot and root dry masses were therefore examined to gain a better perspective.

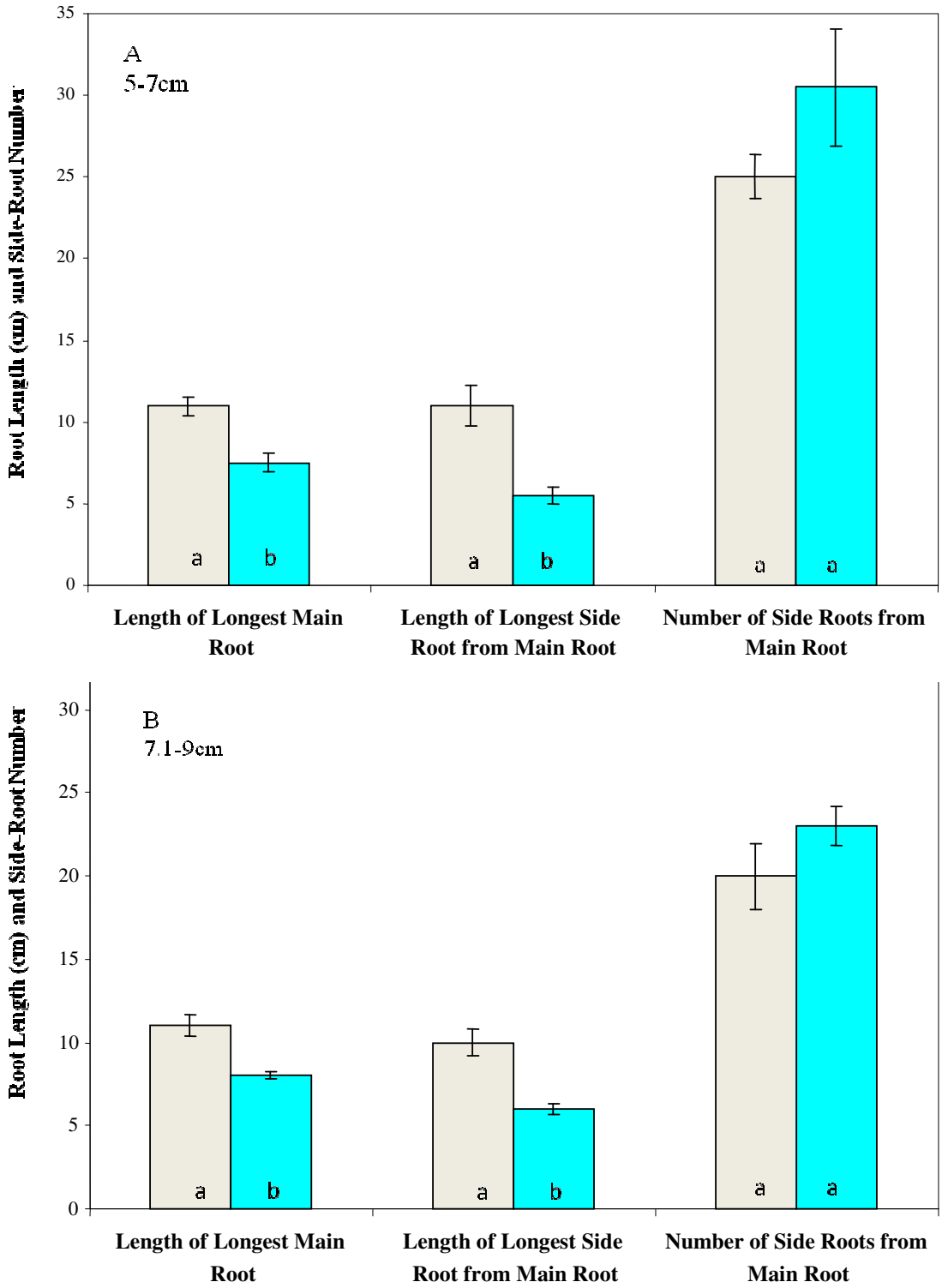



Figure 17: Comparisons of root dimensions of mini-cutting plantlets (grey) and seedlings (bright blue) within the same height range (A=5-7cm, B=7.1-9cm). The extension of the longest main root produced by the mini-cutting plantlets was compared with that produced by the seedlings. The extension of the longest lateral root from the main root was also represented along with number of side roots. Bars represent the average ±SE of mini-cuttings ($n$=20) and seedlings ($n$=15). Using the Kruskal-Wallis ANOVA, ($P < 0.05$), it was concluded that in both height ranges, significant differences (b) existed between the lengths of the longest main and side roots of mini-cutting plantlets and seedlings, while no significant difference (a) was found in the number of side roots of both.

While it was evident (and statistically shown) that mini-cutting plantlets had a much greater dry shoot and root mass (Figure 18) than seedlings within both height ranges, both plant-types exhibited the same growth pattern. Shoot:Root ratios for mini-cutting plantlets were 2.2:1, and seedlings, 2.8:1 in the 5-7cm height range. In the 7.1-9cm height range, mini-cutting plantlets had a shoot:root ratio of 2.5:1, and seedlings, 2.4:1. This would imply that both plant-types had approximately the same mass ratio in relation to their height range. Alcorn *et al*. (2013) found that defoliation had no effect on stem growth in *Eucalyptus pilularis* and *E. cloeziana* saplings, and this may also be true for *E. dunnii* mini-cuttings, as even though leaves were excised during preparation, the dry shoot weights seem unaffected.

As height range increased from between 5-7cm to 7.1-9.0cm, shoot mass was found to increase by 15% in mini-cutting plantlets and 7% in seedlings. This was expected as the shoot increased in size. The difference in root lengths may be due to the age of the mini-cutting plantlets when compared with seedlings or their initial investment of mini-cuttings into their root system rather than shoot system (Gregory, 2006). The root mass data supported this suggestion, as there was only a 4% increase in mini-cutting-plantlet root mass as shoot height increased; while with seedlings, the root mass was 22% higher between height ranges although the length remained constant.

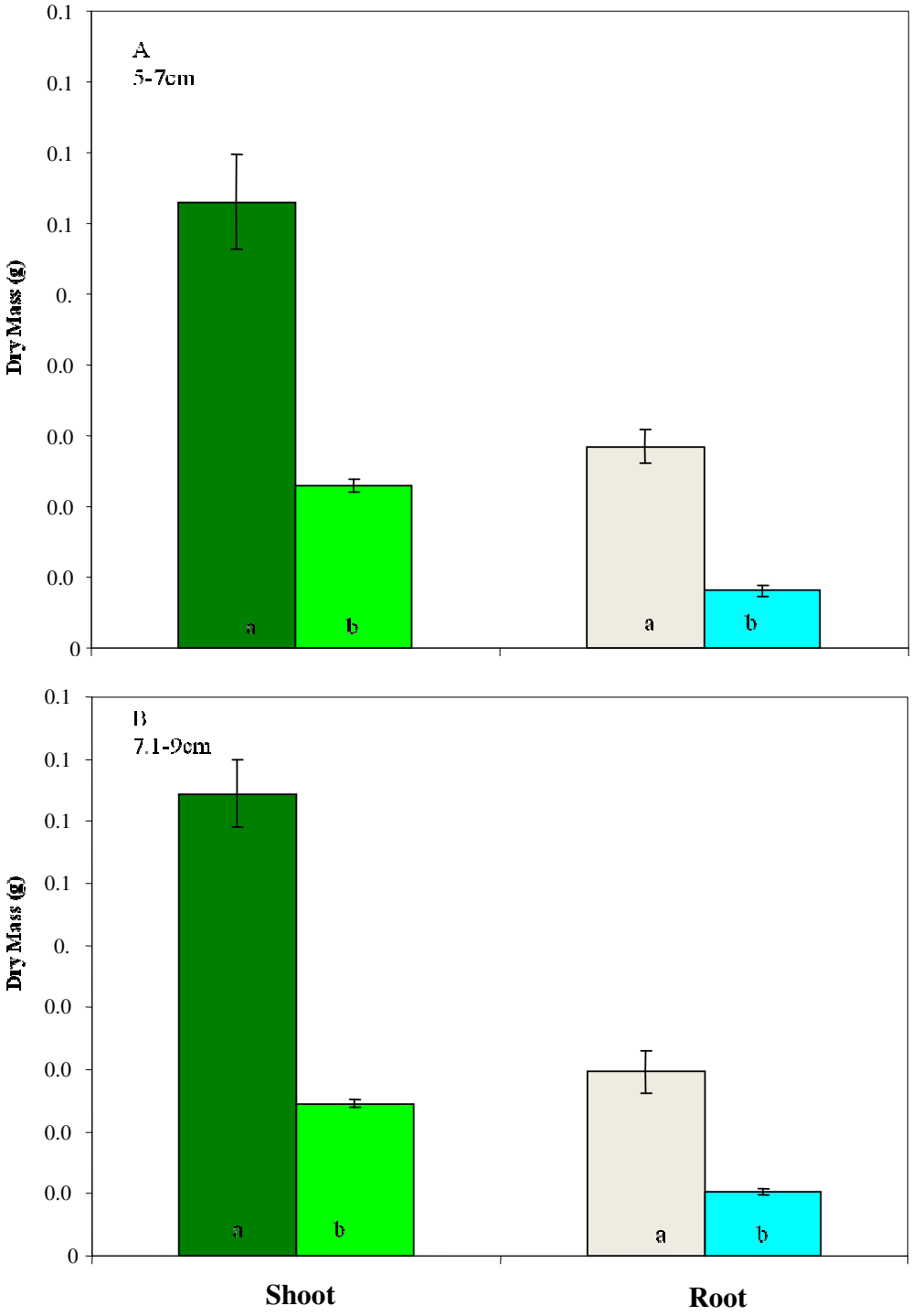



Figure 18: Shoot mass of mini-cutting plantlets (dark green) and seedlings (bright green) of the same height range (A=5-7cm, B=7.1-9cm), were compared, along with root mass of mini-cutting plantlets (grey) and seedlings (bright blue). With height ranges (A & B), shoot and root mass were found to be significantly different (b) using a Mann Whitney Signed Rank Test ($P < 0.05$).

#### 4.2.2.3 General Trends Observed with Mini-cutting Root Growth and Comparison with Seedlings

As with the *in vitro* plantlets, mini-cutting plantlet morphology (Figure 19A) differed from seedlings (Figure 19B) of the same height range.


Figure 19: Mini-cutting plantlet (A) structure compared with seedling (B) structure of the same size height range (7-9cm).

During the study, general trends were observed. Like *E. dunnii* mini-cutting-derived plantlets, the topmost lateral-roots of the seedling root system also showed horizontal growth for approximately 0.5cm before growing downward, while the main root kinked at the top and/or in the middle in all (100%) seedlings (Figure 19). Mokotedi *et al*. (2003; 2010) noticed the same trend with vegetatively propagated *E. grandis* x *E. nitens*, where root growth was asymmetrical.

Both kinking and root knots have been noted in commercially-grown seedlings and saplings, as the root system may be contained in a single container for an extended period (Mokotedi, 2010). In the past, kinking of roots was also linked to the use of shallow trays for seedling germination. The result was deflection of the tap root and even circling of the root system within the container (Gordon, 2007). Kinking was observed in older *E. dunnii* plant material.

Gordon (2007) suggested that air pruning could possibly prevent distortions such as kinking and bending from occurring. According to Gordon (2007), the exposure to the zone of dry air causes the root tips to "burn" or "shrivel" as they emerge from the container. This then stimulates lateral root growth from further back in the root system. The inserts used for propagation of *E. dunnii* mini-cuttings were designed for this purpose and the "burning" effect was observed in test specimens. Most (93%) mini-cutting plantlet roots displayed the same "fleshy" and white appearance as all seedlings within this height-range, although most were from their lateral-roots rather than the main root as was observed in seedlings (97% of seedlings had fleshy ends). As previously mentioned, a large percentage (75%) of cutting plantlets also had a significant number of dead root tips as they increased in length and were probably restricted by the insert; 71% of seedlings also displayed this trend.

While shoots of seedlings exhibited straight bole growth, due to the structure of mini-cuttings, shoots were formed from axillary buds on either side of the stem and displayed a branched structure. In both cases, new growth was observed from the basal nodes of 46% of the mini-cuttings and seedlings.

In most cases (77%), the excision of the shoot during preparation of the cuttings resulted in callus production at the cutting base. No callus was observed in 23% of material. Callus production rated as one (1-3mm thick), was found in 21% of material. Moderate (4-6mm thick) callus production and was present in 34% of material. Prolific (greater than 6mm thick) callus formation was observed in 21% of cutting plantlets.

Roots either formed above the callus (in 55% of cutting plantlets) or below it (18%), with a very low percentage of rooting (7%) occurring directly from the callus. As expected, no callus production was present in seedlings.

In mini-cutting plantlets, two types of root formation were observed: a single root would form from the side of the stem, or two roots would be produced from the stem, symmetrically and in one plane. All roots that developed grew out horizontally, either at 90° or 135° to the stem, before growing downwards. In some cases, these roots continued to develop horizontally and crossed over into a neighbouring insert. The same root architecture was noted by Mokotedi *et al.* (2010) in *E. grandis x nitens* macropropagated saplings. Mokotedi *et al.* (2010) noted that a characteristic of *E. grandis x nitens* root architecture was that roots grew out on one side of the stem, or two roots were produced. When two dominant roots were produced, they grew approximately 180˚ apart. Under 16 months of growth under field conditions, Mokotedi *et al.* (2010) identified two types of roots from macropropagated *Eucalyptus grandis x nitens* trees; those that grew out perpendicular from the stem (type 1), and those that exhibited perpendicular root growth along with one or more roots that grew out directly under the root-shoot connection (type 2). The root architecture noted in this study conforms to the type 1 roots observed by Mokotedi *et al.* (2010).

Mokotedi *et al.* (2010) also found that roots of macropropagated *Eucalyptus grandis x nitens* were shallower than seedlings in 16-month old field grown specimens as a result of an inadequate root system which resulted in toppling over in 10% of trees. The number of roots and root cross sectional area were both found to have the greatest significant effect on anchorage (Mokotedi *et al*., 2010). Rooting depth close to the tree trunk has also been found to be a major factor in root anchorage (Ghani *et al*., 2009).

The type 1 root architecture exhibited by *E. dunnii* cuttings may hinder the field growth of *E. dunnii* as research has shown that the anchorage efficiency of this type of root was less when compared with type 2 and seeding-derived trees and seeding-derived trees (Mokotedi *et al*., 2010). Although not statistically significant, visually, *E. dunnii* seedlings in this study appeared to produce more lateral roots than cuttings (Figures 17 and 19), possibly also giving an indication of the anchorage success of the propagation types when planted in the field. However, root biomass has been found to increase with age/size of *E. globulus* and *E. nitens* trees (Resh *et al*., 2003) and both macro- (45%) and micropropagated (99%) trees sampled have been found to develop into the type 2 morphology in *Eucalyptus grandis x nitens* field-grown species from 16 to 24 months (Mokotedi *et al*., 2010). Time, specifically duration of growth, may therefore be an essential factor in the root development and anchorage in *E. dunnii* trees.

### 4.2.3 PRODUCTION OF SEEDLINGS

#### 4.2.3.1 General Root Architecture of *E. dunnii* Seedlings (Height Range: 20-70cm)

A closer consideration of seedling root growth in relation to shoot growth would provide insight on the pattern of natural root development, its contribution to, and concurrence with shoot growth (Gregory, 2006). Such data could help make limited predictions of early *in vitro* and mini-cutting plantlet development. This may be possible as results of this study have shown shoot:root masses of *E. dunnii in vitro* plantlets and mini-cutting-produced plantlets to be similar to those of seedlings of the same shoot height (Figures 11 and 18).

*Eucalyptus dunnii* seedling shoot and root growth was compared by measuring the main root and lateral root lengths, along with number of lateral roots in seedlings ranging between shoot heights of 20 to 70cm (subdivided into height ranges: 21-30cm, 31-40cm, 41-50cm, 51-60cm, 61-70cm) (Figure 20).

The length of the main roots of seedlings within all height ranges were found to be similar statistically and was 12cm in the 21-30cm shoot-height group, and 10cm in the 61-70cm shoot-height group. This implied that as shoot height increased by approximately 50cm, the main root length remained relatively the same. The same trend was noted in lateral-root length. The lengths of all lateral roots regardless of the height group were found to be statistically similar and were 9cm in the first two height-groups and 10cm in the next three height groups. The number of lateral-roots did not differ statistically between the various height-ranges. Seedlings, with a height-range of 20-30cm had on average 10 lateral-roots per main root, while seedlings of the 31-40cm and 41-50cm height-range averaged 11 lateral-roots per main root, and finally the last two height-groups (51-60cm and 61-70cm) had 12 lateral-roots on average.

All the external parameters examined showed no statistical differences in roots of all shoot-height ranges, and it can therefore be concluded that the external root systems of seedlings ranging from a height of 21cm to 70cm remained the same, as shoot growth increased.

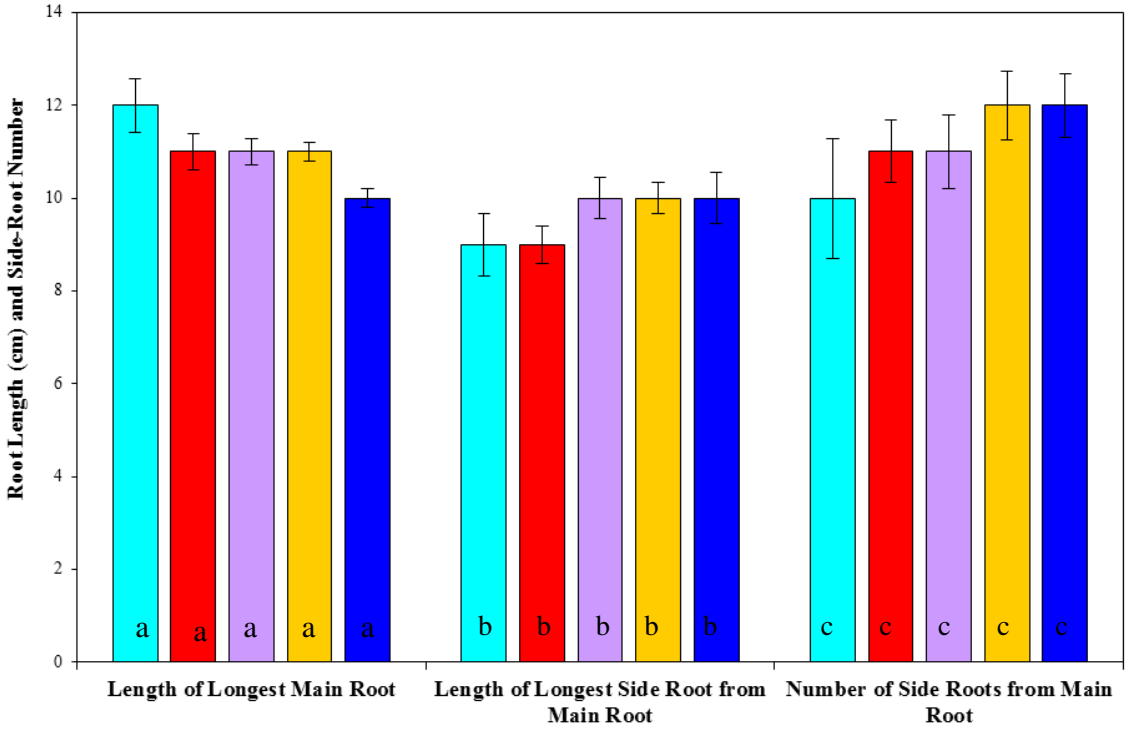



Figure 20: Comparison of root dimensions of seedlings of height ranges 21-30cm (bright blue, *n*=10), 31-40cm (red, *n*=20), 41-50cm (purple, *n*=20), 51-60cm (gold, *n*=20) and 61-70cm (dark blue, *n*=20). The Kruskal-Wallis ANOVA ($P < 0.05$) was used to compare extensions of the main roots within each height range, along with the extension of the longest side root from the main root, and the number of side roots produced from the main root. No significant differences (a, b & c) were found in main root length, length of longest side roots, or number of side roots within all height ranges.

As found in mini-cutting plantlets, even though root structures may appear to be similar externally (Figure 17), internal variances may have existed between height-ranges (Figure 18), and this was examined by analysis of the dry masses. It was expected that as the size of a seedling increased, so would its mass. While some shoot dry masses were not significantly different from others, a general growth trend was observed. Shoot dry mass of seedlings gradually increased in accordance with an increase in the height of shoots (Figure 21).

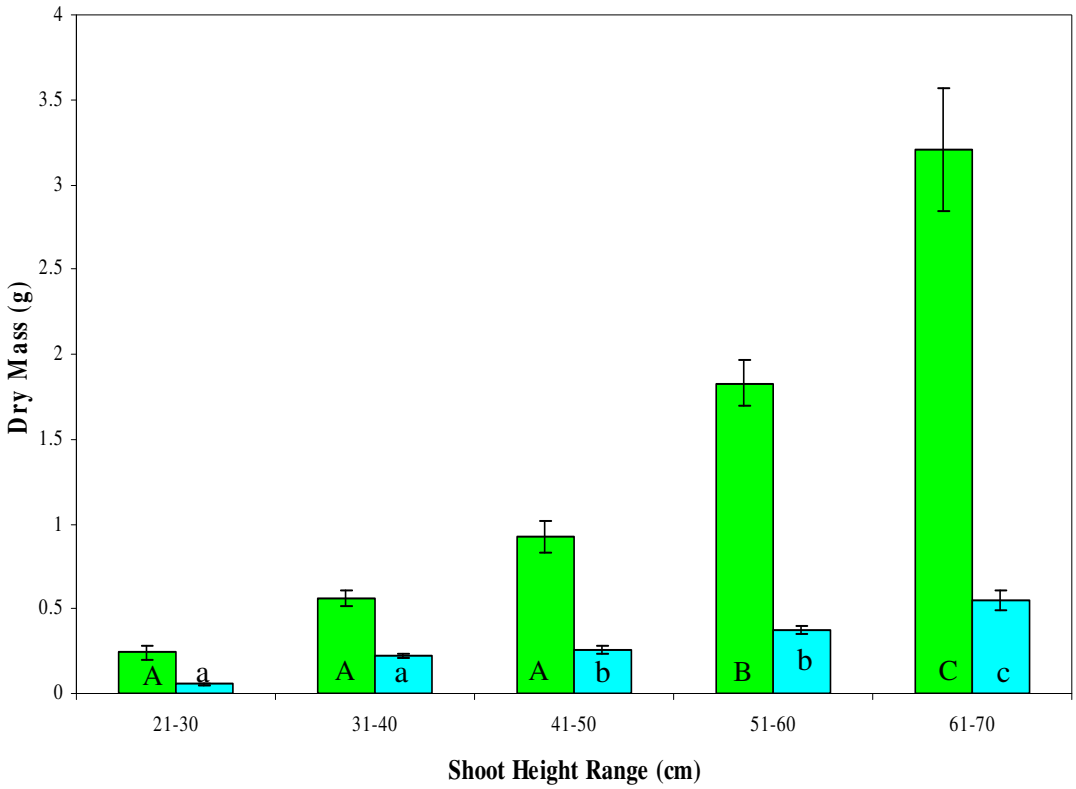



Figure 21: Shoot dry mass (green) and root dry mass (blue) of seedlings of height ranges 21-30cm, 31-40cm, 41-50cm, 51-60cm and 61-70cm were compared using the Kruskal Wallis ANOVA ($P < 0.05$). Similar letters signify no significant difference between shoot/root masses of those groups, while different letters represent a statistical difference.

Percentages were calculated from the average mass for each height-range, and shoot mass increased by 57% from height-group 20-30cm to 31-40cm and thereafter by 39%, 50% and 43% between increasing height groups, respectively. These percentages indicated that with every 10cm increase in shoot height, shoot mass increased by roughly 50%; i.e. half its weight.

As there appeared to be no difference in root systems externally (Figure 20), the root masses were analysed. As shoot heights increased, the average root masses increased in accordance with these heights. Average root mass increased by 75%, 14%, 30%, and 33% respectively between increasing height groups (Figure 21). While increase in root mass seemed more erratic than shoot mass, the highest increase in mass was noted between 20-30cm and 31-40cm, where root mass increased by three quarters.

However, despite these results, root masses of most groups were not significantly different. With the exception of the highest increase in root mass (75%), it appeared that on average, shoot mass was highly favoured over root mass.

Various factors could contribute to the above-mentioned results. Halter *et al*., (1996) noted that root elongation in juvenile *Eucalyptus pauciflora* seedlings was much greater than that of older material, which grew at a much slower rate. With *E. camaldulensis* it was observed that below-ground biomass in plantation trees grew faster and larger when spaced further apart (Barton and Montagu, 2006). The explanation for this trend may therefore be simple. For example, all *E. dunnii* material sampled for this study could have been older, or the limited space within seedling trays could have resulted in the stasis in root development of *E. dunnii*. However, the root:shoot biomass could also indicate a complex relationship between *E. dunnii* roots and shoots. Brouwer (1963) suggested a theory of equilibrium between above- and under-ground plant material. The author proposed that plants can "shift" their resource allocation to compensate for environmental challenges (Brouwer, 1963; Reich, 2002). In this case, limited resources aboveground, such as low levels of light may have resulted in increased shoot biomass to optimize the acquisition of those limiting resources (Reich, 2002).

Equally, however, the decline in root growth in relation to shoot growth in other studies has been considered as plants receiving adequate resources so that the needs of the shoot are met despite restriction of the root system (Troughton, 1960; Brouwer, 1963; Gregory, 2006; Al-Zalzaleh, 2009). The study by Al-Zalzaleh (2009) of *E. viminalis* rooting showed that once roots reached the container depth, root growth then declined; likewise, it was confirmed that deep pots encouraged root extension. With the results observed from this study, both findings may be supported. *E. dunnii* shoot growth continued due to the seedlings receiving the required resources even though root growth stayed relatively static.

It would not be unreasonable to consider that *in vitro* plantlets and mini-cutting-produced plantlets would respond similarly to the observed growth patterns of seedlings in the event that their root systems are restricted and other resources are readily available or not.

#### 4.2.3.2. General Trends Observed with Seedling Root Growth

The trends observed in seedlings of a much smaller shoot height (2-5cm – 4.2.1.5), 5-7cm and 7.1-9cm (4.2.2.3) were also common in seedlings within 20-70cm shoot-height ranges.

Excessive bending, or kinking of the root at or above the transition from root to stem was prevalent in 69% of material (Figure 22A). These kinks were also present in the middle of the main root. In some cases, the root would form a knot at this point (Figure 22B). This phenomenon could possibly occur due to the physical pressure placed on the root system by the much heavier shoots in the taller plants (shoots of 61-70cm plants were approximately 6 times heavier than the root system – Figure 21). However, early signs of kinking were also observed in young seedlings (2-5cm – 4.2.1.5). Kinking causes complications when the plant is transferred to the field, as the root system continues to develop in the same manner, and this impairs subsequent growth and can induce swivelling of trees.

It was observed that lateral-roots were much more sparsely distributed in older plants than in young seedlings (Figure 22C). Further, lateral roots at the uppermost portion of the main root exhibited a trend of growing out to the side (for approximately 2cm) and then down (Figure 22D). This architecture was also noticed in both field-grown *E. grandis* and *E. grandis x nitens* seedlings and *E. camaldulensis* (Bell *et al*., 1993; Mokotedi *et al*., 2010). The vertical growth of the main root with lateral roots radiating equally in all directions from the stem is characteristic of trees grown from seed and is believed to contribute to their superior uprooting resistance (when compared with its micropropagated and macropropagated counterparts) (Mokotedi *et*

*al*., 2010). This type of root architecture would therefore give seedlings an advantage over propagated trees in the field.

While the relationship between air pruning and lateral root development was not considered in this study, the exposed roots of both young and older seedlings had the fleshy appearance (74% of seedlings) and dead root tips characteristic of air pruning (Gordon, 1997) (Figure 22E).

Basal nodes of the stem were usually devoid of any leaves or side-branches, and new shoot growth would originate from these lower nodes (Figure 22F). This trend was noted in 33% of seedlings. This rejuvenation is characteristic of the species, and is useful when producing hedges for micro- and macropropagation (Watt *et al.,* 1991). The growth environment and care of young trees appears to contribute to successful anchorage and even resource exploitation in the field (Gregory, 2006; Mokotedi *et al*., 2010).

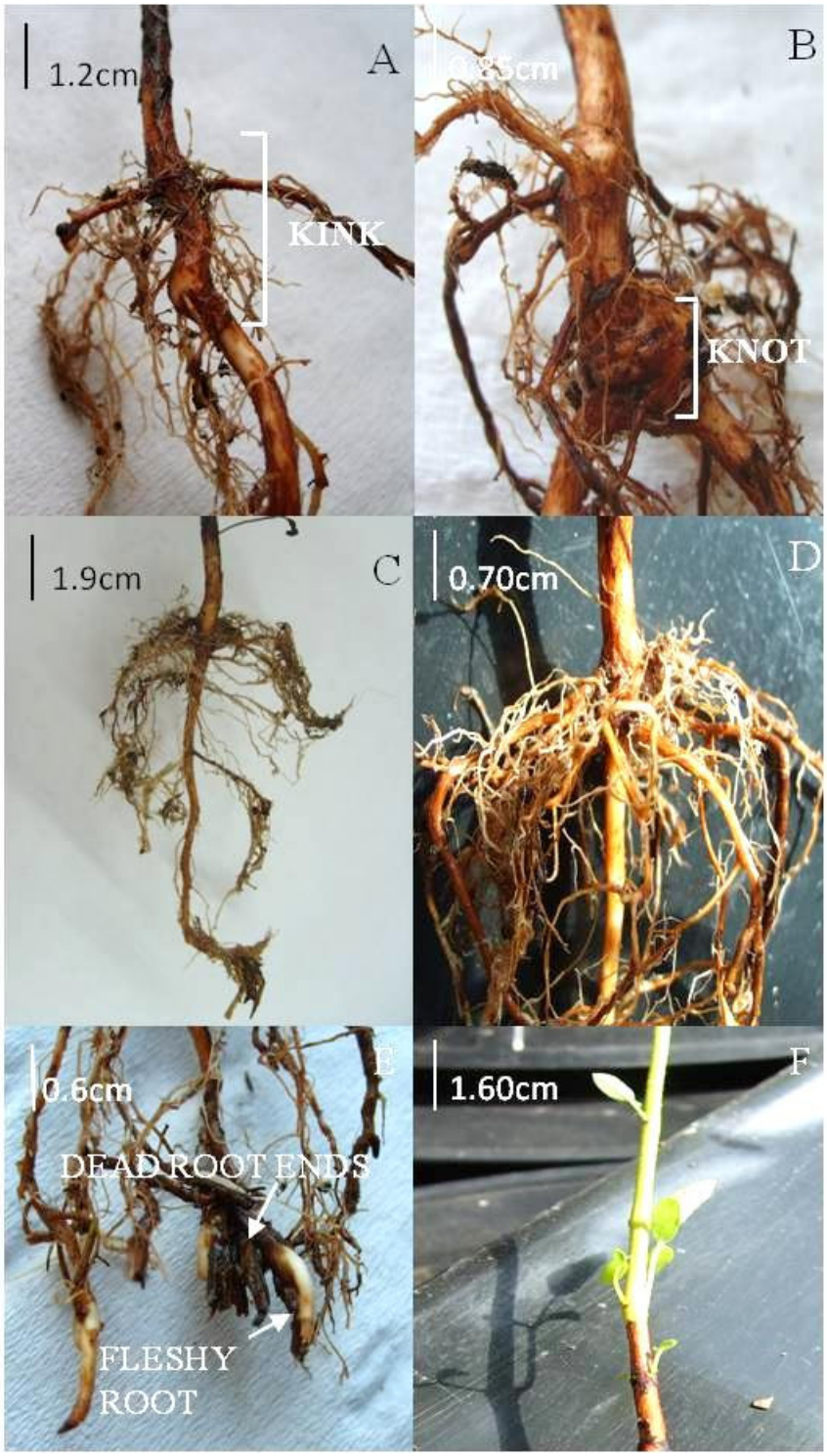



Figure 22: Kink in seedling-stem (A), along with knot (B). Sparse arrangement of (C) and horizontal growth of upper side-roots (D). Dead root, and fleshy root ends (E) and new shoot production from basal nodes of stem (F).

## SUMMARY AND CONCLUSIONS

Micropropagation of *E. dunnii* posed a challenge initially. Although both temporary immersion and semi-solid culture was attempted using healthy *in vitro* material, success was achieved only with the *E. grandis* and *E. camaldulensis* medium.

The failure of the elongation medium to lengthen the stems of plant clusters, as well as the quality of the multiplied plant material (elongated stems and healthy, large leaves) motivated the use of multiplied shoots directly in rooting experiments *in vitro.* It may also be possible to use this approach with other *Eucalyptus* species.

Rooting success was poor in culture. The attempt to induce rooting by reduction of the medium's pH, and IBA application in semi-solid culture was unsuccessful. However, the shoot growth of plant material on pH 5 was significantly higher than that on the standard of 5.6-5.8, which has been found to be optimal for most *Eucalyptus* species (Le Roux and Van Staden, 1991; Bennet *et* al., 1994; Wachira, 1997; Brondani *et al*., 2011; Neto *et al*., 2013). The increased vigour of plant material suggested that improved multiplication of *E. dunnii* may be possible by using this modification.

While exogenous auxin application has been found to promote the rooting response in other *Eucalyptus* species (Fogaça and Fett-Netto, 2005), *in vitro E. dunnii* plant material did not appear to respond well to increased concentrations of auxin. This, combined with the concentration of Gelrite® in the medium may have adversely impacted rooting success. In this regard, the increase in NAA concentration in the elongation medium presumably resulted in the observed prolific callus formation (de Klerk *et al*., 1999; Gomes and Canhoto, 2003).

The lack of rooting following IBA application in the rooting experiments could possibly have promoted the endogenous bacterial infestation due to the prolonged exposure of micro-cuttings to the substrate (de Klerk *et al*., 1999) and possibly the latent infections of cuttings in the substrate (Makhado, 2006).

The use of Gelrite® and its concentration may have further impaired rooting by providing a hypoxic, watery environment, which encouraged bacterial growth (Whitehouse *et al*., 2002). Rooting on semi-solid media was therefore not considered in this study. The results obtained from the change in substrate supports this theory. Plant clusters placed in the coir:vermiculite substrate did not succumb to any bacterial contamination. However, even with the change in substrate, only 4% of shoots produced roots. The low rooting success may be attributed to more than one factor. While shoots did not seem to respond to auxin applications, further manipulation of the substrate, as well as growth room conditions may have yielded different results. The plant itself may have also affected the outcome. Endogenous rhythms of the plant and genetic make-up may have contributed the "poor rooting' that *Eucalyptus dunnii* has been found to exhibit (Billard and Lallana, 2005; Mankessi *et al*., 2009). It is encouraging to note that where roots were produced, those roots grew directly from the plant cluster (Figure 12A). This may imply that continuous vascular connections between the two were formed during rooting.

Comparison of plantlets and seedlings of the same height range showed that root architecture of main roots, main side roots, and the number of side roots were statistically similar (Figure 10). Root architecture defines the manner in which the substrate is exploited (Thaler and Pagès, 1998). Similar architecture would suggest that both propagation types would have similar success if all external factors were constant. Internally, plantlets and seedlings seemed to be matched as root and shoot masses were also statistically similar. This would imply that there was very little difference between the micropropagated plantlet and seedling. However, visually, minor side roots of the seedling were more developed than that of the plantlet. The main root of the plantlet was also fleshier and appeared less resilient than that of the seedling. Other studies show conflicting results, with some indicating that differences in morphology disappear as trees mature (Warang *et al*., 1989; Bell *et al.,* 1993; Mokotedi *et al*., 2003). While this may be the case externally, some studies show that initial differences continue to influence tree growth in the field. For example, micropropagated saplings and even mini-cuttings have been found to have less

resistance to uprooting than saplings propagated from seed (Mokotedi, 2010). With *E. dunnii*, these comparisons may need to be investigated as trees mature.

Production of roots from mini-cuttings was more successful than *in vitro* rooting. Mini-cuttings that did not undergo any treatment had statistically similar rooting success as those that were treated with concentrated IBA (Seradix1) (Figure 16), which is currently the practice used by the Mountain Home (Mondi South Africa). The dip applications of auxin were relatively unsuccessful while the 200mg.$l^{-1}$ pulse treatment proved to be effective with 30% producing roots (Figure 16). It may be possible that the quantity and timing of auxin applied to this species may be optimal for rooting (de Klerk *et al*., 1999).

In the comparison of seedlings of the same height range as the mini-cuttings sampled, it was observed that the root architecture differed in some aspects. Roots of mini-cuttings extended out horizontally before going downward into the soil (Figure 19A). Plantlets that were produced *in vitro* also displayed initial horizontal angling of the root to an extent. This may be a common response to propagation from mini-cuttings possibly due to the manner that the root is created from the stem vascular tissues (both micropropagated and macropropagated) (Lynch and Brown, 2001). The shoot:root ratios of seedlings and mini-cutting plantlets of the same height (Figure 18) showed that both propagation types exhibited similar growth patterns, including patterns of investment in shoot versus root growth.

As with the comparison of *in vitro* plantlets with seedlings, mini-cutting plantlets appeared to have less lateral roots than seedlings of their height range (this number was not significant). Lateral roots play an integral role not only in acquisition of nutrients, but also anchorage (Bell *et al*., 1993; Mokotedi *et al*., 2010; Ghani *et al*., 2009). This trend may continue with age, and therefore give the seed-propagated trees an advantage e.g. anchorage, over their micro- and macro-propagated counterparts.

With *E. urophylla* x *E. globulus* and *E. grandis* x *E. globulus*, *in vitro* propagation and rooting was found to be better than macropropagation (de Oliveira *et al*., 2012). At present with *E. dunnii*, the production of plantlets via macropropagation is a better option than micropropagation. Although *E. dunnii* can be established successfully *in vitro*, however, it appears to require a different approach to other species in terms of root production. Factors such as age of *in vitro* material, environmental conditions and substrate also need to be considered.

Though *in vitro* plantlets, mini-cutting plantlets and seedlings of the same height range seem similar in shoot:root ratios (Figure 11 and 18) and root lengths (Figure 10 and 17) etc, one cannot make any direct comparisons due to the varied growth conditions of the samples before analysis. This study was conducted using material housed in containers (seeds and mini-cuttings in seed-trays, and *in vitro* material in Magenta jars). That poses restrictions on root growth and may have possibly affected the results e.g. the increase in shoot height of taller seedlings while roots remained in relative stasis (NeSmith and Duval, 1998; Gordon, 2007; George *et* al., 2008; Al-Zalzaleh, 2009). To fully understand root architecture and growth patterns, it is suggested that material be planted in larger pots or in the field to obtain an accurate representation at different stages of growth (Mokotedi *et* al., 2009; Mokotedi *et al*., 2010). With the current information provided in this study, it would not be possible to determine which propagation method would be most successful in the field.

## OPPORTUNTIES FOR FURTHER RESEARCH

Further research on *E. dunnii* at different stages of growth, from root initiation to field growth would give more insight into rooting of the species and may allow for direct comparisons to be made. The following are suggested:

- ✓ Use the screening process to eliminate bacterial contamination of the RITA® system (Watt *et al.*, 2006) for the multiplication of *in vitro E. dunnii* material using the RITA® system.

- ✓ The success of rooting with Seradix1 versus no plant growth regulator application needs to be investigated further, as do other concentrations of plant growth regulators

- ✓ Examine a range of auxin concentrations closer to the the 200mg.l$^{-1}$ pulse treatment of IBA as it proved to be most successful with mini-cutting propagation. The pulse treatment was relatively simple to execute and should be considered in further studies. The cost of execution of this method versus the profit that should be gained by the company could be considered.

- ✓ Trends, such as lateral root numbers being higher in seedlings than in macropropagated and micropropagated plantlets, and their effect on resource acquisition and anchorage may be further investigated as plantlets and seedlings grow, in order for direct comparisons to be made.

- ✓ The use of rhizobacteria may be explored to improve rooting ability of *Eucalyptus* (Díaz *et al*., 2009; Paz *et al*., 2012) and in particular, *E. dunnii* propagation material.

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