Influence of stockplant management on yield and subsequent rooting of cuttings of cold-tolerant *Eucalyptus grandis* x *E. nitens* clones

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PREFACE

The experimental work described in this thesis was carried out at the University of KwaZulu-Natal, Pietermaritzburg and Sunshine Seedlings Home Nursery from March 2010 to September 2011, under the supervision of Dr. Isa Berling (University of KwaZulu-Natal), Ms Felicity Blakeway and Dr. Oscar Mokotedi (co-supervisor, Council for Scientific and Industrial Research).

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

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Matabaro Ziganira

I certify that the above statements are correct:

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Dr I. Bertling (Supervisor)

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Date

RESEARCH DAY AND CONFERENCE CONTRIBUTION

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ABSTRACT

Clones of the *Eucalyptus grandis* x *Eucayptus nitens* (GN) hybrids were produced and selected through the CSIR's breeding programmes for colder plantation sites in South Africa. Some GN clones consistently exhibit high and superior pulp properties, which makes them valuable for commercial plantations in South Africa. In nurseries, stockplants are usually seven cm in length and maintained at high (100 x1.5 m^{-2}) planting density. However, rooting frequency varies with season and little is known about the impact of position of cuttings on overall rooting frequency of a clone. The aim of this study was to investigate the effect of size and planting density of stockplants in mini-hedges, on the yield and subsequent rooting of cuttings from various positions of GN clones of known rooting potential (i.e. GN 018B: difficult-to-root and PP 2107: easy-to-root clones).

Stockplants (10 cm vs. 20 cm) were established at high (100 x 1.5 m^{-2}) and at low (25 x 1.5 m^{-2}) densities for GN 018B and PP 2107 under commercial nursery conditions in a polyethylene tunnel. Cuttings were harvested every two to three weeks in September-October 2010 (spring), December 2010-January 2011 (summer), April-May 2011 (autumn) and June-July 2011 (winter). The harvested material was 5 – 7 cm in length and the light intensity received by individual stockplants at the two planting density levels was recorded. Harvested cuttings from the three positions (apical, middle and basal shoots) were used for: (i) rooting experiments under nursery conditions, (ii) bio-stimulant analysis using the mung bean bioassay, and (iii) analysis of soluble sugars.

Between spring and summer 2010, the two GN clones established at low density yielded a similar number of cuttings, but differences in the rooting frequencies were significant in favour of PP 2107 clone. Similar observations were made at high density in terms of production of cuttings, but the significant differences in the rooting observations were reversed between the clones. The GN 018B clone had low rooting rates in summer under nursery conditions but its tissue extracts promoted higher rooting in the bioassay during that time, when compared to spring. Spring and summer had similar effects on rooting responses of PP 2107 cuttings in nursery and bioassay experiments. For both clones, short stockplants produced fewer cuttings but had a higher rooting frequency than cuttings from tall stockplants, with a high rooting frequency recorded from basal cuttings. Similar results

were observed in the bioassay experiments which showed high rooting potential of mung bean hypocotyls cuttings using tissue extracts of PP 2107 cuttings maintained at high planting density. Although apical cutting tissues had high concentration of sugars (i.e. sucrose, glucose and fructose), their rooting rates were usually lower at high and low planting density compared to middle and basal cuttings. Sucrose concentration was the highest sugar present in stockplants grown under low planting density. A higher and lower rooting frequency was also observed in autumn although the two clones responded differently to *Quambalaria eucalypti* (*Sporothrix eucalypti*) disease infestations. Position, size and genotype had a significant impact on type and concentration of sugar (i.e. sucrose, glucose and fructose), particularly in PP 2107 clone, although rooting rates in the bioassay did not correlate with sugar contents of *Eucalyptus* cuttings.

High carbohydrate (i.e. soluble sugar) content and auxin concentration increased production and subsequent rooting of cuttings across both clones, particularly in spring. Furthermore, rooting was enhanced by relatively higher light intensity intercepted by individual stockplants and in particular the GN 018B clone. Light intensity in the high and low planting densities caused variation in the rooting frequencies thereby increasing or decreasing soluble sugar and auxin concentrations of the two clones. Light intensity and fertiliser concentration received by tall and short stockplants impacted on endogenous hormone levels thereby increasing or decreasing rooting. High sugar concentration levels of PP 2107 clone increased its susceptibility to fungal infection thereby decreasing its rooting frequency in autumn, as its rooting rates increased in winter.

Overall results of the investigation revealed that PP 2107clone has higher rooting potential than GN 018B clone, in particular at high planting density and if stockplants are not infected by fungal diseases. Higher sugar levels were recorded in spring for PP 2107, although rooting rates of mung bean hypocotyl cuttings were higher in summer for GN 018B, suggesting that sugars have nothing to do with rooting of GN cuttings. Season, planting density and size of stockplants affect the rooting frequency of GN clone. Thus, short stockplants maintained at low and high planting densities are recommended for GN 018B and PP 2107 respectively, although the impacts of fertilisers and pathogen resistance on rooting rates still need to be investigated under similar conditions.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. Economic use of *Eucalyptus* in plantation forestry

Globally, most trees in the plantation forestry industry are members of the genus *Eucalyptus* Maiden (family Myrtaceae), and many originate from Australia (Westoby, 1989). Approximately 17.8 million ha are covered by *Eucalyptus* (Turnbull, 1999; FAO, 2000). Key features of eucalypts in exotic plantations include fast growth, short rotation periods, superior wood quality, and more uniform stands than most indigenous trees (Zacharin, 1978; Blake, 1983; Gupta and Mascarenhas, 1987; Turnbull, 1991; Bouillet *et al*., 2004; Pallett and Sale, 2004). *Eucalyptus* is used for several economic purposes, such as production of charcoal, furniture, poles and is a major source of pulp and cellulose for the paper industry (Eldridge *et al.*, 1993) thereby reducing the pressure on indigenous forests and associated biodiversity (Gattapaglia and Kirst, 2008). In addition, eucalypt trees produce coppice readily, while many are relatively easy to clone and pure species can be crossed to produce hybrids with desirable characteristics (Denison, 1999). Further, the global success of *Eucalyptus* can also be attributed to its ability to adapt quickly and grow well in nutritionally poor soils that may even be acidic (Pallet and Sale, 2004).

Eucalyptus plantations were first established in South Africa primarily for the production of mining timber (Le Roux and van Staden, 1991; Denison and Kietzka, 1993; Smit and Pitcher, 2003; Pallett and Sale, 2004; Anon, 2006). In South Africa, the forestry and forest products industry is an important part of the economy and one of the most globalised industrial sectors (Pallett and Sale, 2004). Forestry plantations in South Africa cover 1 266 197ha (Godsmark, 2008).International forestry exports contribute to valuable foreign exchange (Cellier, 1993; Edwards, 2000; Smit and Pitcher, 2003). In South Africa, pulpwood has become the most prominent and profitable among end-products of eucalypts (Anon, 2006). *Eucalyptus* makes excellent pulp suitable for printing, writing and tissue paper due to its wood that produces uniform material with high brightness, good density and bulk (Turnbull, 1991).

In South Africa, the pulp and paper industry forms a large and important part of the forestry industry (Forestry South Africa, 2008). The industry is likely to benefit from the development and introduction of new *Eucalyptus* hybrid material with improved fibre quality and pulping properties. However, the planting of such high yielding species is limited by water availability since South Africa is a water-limited country and good patches of rainfall are predicted to slowly dry up (Denison and Quaile, 1987; Olbrich *et al.*, 1993; Pallet and Sale, 2004). The South African Department of Water Affairs and Forestry (DWAF) has imposed strict laws governing the use of land and water, to minimise competition for productive land with the agricultural sector (www.dwaf.gov.za). Thus, the challenge for the South African Forestry sector is to maximise and increase production of forestry products from existing plantation sites. In this regards, hybrid forestry has been intensified and continuous efforts are made to match clones to sites that maximise their growth and productivity (Denison and Kietzka, 1993). Since the production of hybrids is an ongoing process, it is important that new hybrid clones are assessed in nurseries to ascertain if they meet expectations with respect to propagation ability in South Africa.

1.2. Distribution of *Eucalyptus* plantations in South Africa

Eucalyptus species and hybrids are grown along the eastern seaboard of the country, particularly in KwaZulu-Natal and Mpumalanga between latitudes 23 °S and 32 °S (Pallett and Sale, 2004). Rainfall in those regions varies from 700 mm to over 1200 mm per annum and the mean annual temperature ranges between 14 to 22 °C (Schulze, 1997). Plantations have also been established in the Eastern and Western Cape and the Northern Province (Denison and Quaile, 1987; Godsmark, 2003; Smit and Pitcher, 2003) (Figure 1.1). The wide and scattered distribution of plantations in South Africa covers a considerable variation in climate and temperature (Herbert, 1996). Thus, site diversity is the major reason for working with a range of species in the timber industry (Webb *et al.*, 1980; Schönau, 1991).

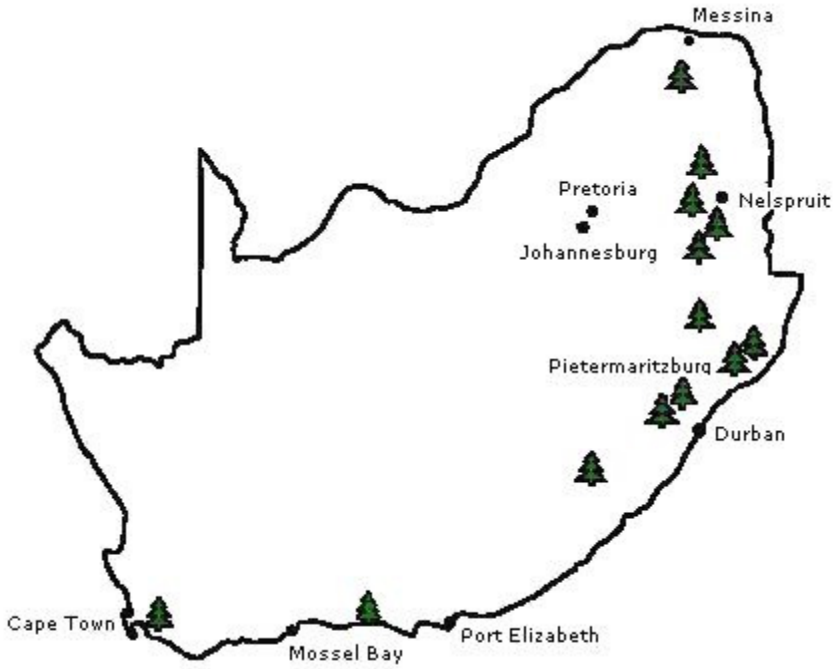

Figure 1.1: Geographical distribution of commercial eucalypt plantations in South Africa (Adapted from Anon, 2004).

1.3. The importance of *Eucalyptus* hybrids in South Africa

Tree improvement by breeding is slow because there is a long interval between generations, and trees are very heterozygous (Libby *et al.,* 1969). These constraints can be overcome in the shorter term by selecting superior hybrids and propagating them vegetatively. Non-additive genetic gain is transferred in full in vegetative propagation, which is particularly useful in rapidly improving those attributes which are poorly heritable, such as volume growth and cellulose yield (Zobel and Ikemori, 1983). Frequently, *Eucalyptus* hybrids have emerged in exotic environments where they have shown an advantage over either one or both of the parent species (Ferreira and dos Santos, 1997). Hybrids play an important role in the production of fast-growing and large-scale commercial plantations. In particular, hybrids can provide greater genetic variation through combinations that are not found in nature (Zobel and Talbert, 1984).

Most eucalypt hybrids are successful because they either have great heterosis or complementary traits allowing them to grow in areas outside the normal productive range of the two parent species (Nikles, 1991; Falconer and Mackay, 1996). Hybrids of subtropical *Eucalypti* are presently the most prominent in the South African forest industry, with thousands of hectares of selected clones already established (da Costa, 2002). Common hybrid combinations in subtropical areas are *Eucalyptus grandis* crossed with *E. urophyla*, *E. camaldulensis*, or *E. tereticornis*. For temperate areas, *E. grandis* is usually crossed with *E. nitens* or *E. macarthurii* (Denison and Kietzka, 1993). The genetic improvement of the five commercial eucalypt species in South Africa began with *E. grandis* in the early 1960s (Schönau, 1991) and has continued with other species due to expansion of *Eucalyptus* afforestation programmes into new areas not suited to *E. grandis* (Pallet and Sale, 2004).

The selection of the two *Eucalyptus grandis* x *E. nitens* clones used in the present study (i.e. PP 2107 and GN 018B) was based on known seasonal and annual rooting performance at Sunshine Seedling Services nursery. The GN 018B clone is regarded as being unimproved because it is difficult-to-root, whereas PP 2107 is considered an improved clone because it is easy-to-root (Pollard, *pers. comm.*[1]). Easy-to-root plants have all the essential endogenous substances including auxin, so that rapid root formation occurs under favourable environmental conditions. Difficult-to-root plants are those plants which lack essential rooting substances or cell sensitivity to respond to endogenous rooting stimuli (Hartman *et al.*, 2002). Usually, the external application of auxin may give little or no response to rooting of difficult-to-root plants (Hartman *et al.*, 2002).

Eucalyptus grandis is frequently favoured in the cross for its excellent growth potential, good form, desirable pulp and superior timber properties. Unfortunately, this species is susceptible to cold temperatures and fungal diseases in South Africa which pose a threat to its survival as a pure species in both the subtropical and temperate regions (Wex and Denison, 1997). In the mid 1980s, *E. dunnii* and *E. smithii* were identified as species of good growth potential over a range of sites in the mid altitudes (Wex and Denison, 1997).

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Eucalyptus nitens was confirmed superior on high altitude cold sites of South Africa characterised by frosts throughout winter months and moderate rainfall (800 to 1000 mm) (Wex and Denison, 1997). In addition to rooting with difficulty, the planting of *E. nitens* is limited by its susceptibility to leaf spot *Mycosphaerella mulleriana*, the incidence of which worsens with increasing ambient temperature (Wex and Denison, 1997). *Eucalyptus grandis* x *E. nitens* (GN) is one of the best hybrids for planting in cold and dry "marginal" areas previously considered unsuitable for commercial forestry (Denison and Kietzka, 1993). The GN clones can adapt to sites more readily than pure species (Denison and Kietzka, 1993), and use water more efficiently for the production of equal amount of carbon than do pure species (February *et al.,* 1995). *Eucalyptus grandis* x *E. nitens* (GN) clones are planted in KwaZulu-Natal midlands and other cooler parts of South Africa, and have shown excellent wood properties ideal for the pulp and paper industry. These clones are also gaining popularity by replacing planting stock for plantation sites previously occupied by *E. grandis* (Denison and Kietzka, 1993).

1.4. Vegetative propagation of *Eucalyptus* hybrids

Vegetative propagation is used by agriculturalists, horticulturalists and foresters to capture and multiply individual genotypes by producing cultivars and clones (Leakey, 1985; Mudge and Brennan, 1999). Mass vegetative propagation is a better and quicker way to commercially exploit the vigour (heterosis) found in several *Eucalyptus* crosses compared to seed orchards (de Assis*et al.,* 2004). This technique allows for maximum benefits of wood properties and productivity, as well as production of more uniform raw material, which is highly beneficial to industrial processes and product quality (de Assis*et al.,* 2004). Thus, mass vegetative propagation of improved genetic material is the foundation of the commercial forestry industry and an important tool for increasing the competitiveness of the forestry-based paper and pulp industry. The main principles of this technique are the multiplication of selected improved genotypes followed by rooting cuttings obtained from stockplants.

As trees grow, a gradient of reproductive maturity (ontogenetic ageing) develops mature shoots which have the ability to fruit and flower, while those below this level are still juvenile (Leakey, 2004). Cuttings from mature shoots are usually difficult-to-root than

those from juvenile (seedling or coppice) shoots (Schneck, 1997). Further, there are age variations within shoots from the basal to apical which affect leaf size and growth, leaf water potential, leaf carbon balance, stem lignifications and carbohydrate content (Leakey and Mohammed, 1985). It is possible to use these gradients node by node as a diagnostic tool for how physiological factors affect rooting (Leakey, 2004). Paton *et al.* (1970) demonstrated that loss of rooting ability in *Eucalyptus grandis* was correlated with the position cuttings were taken from, i.e. the number of nodes from the base of the plant. These authors suggested that rooting was highly unlikely above node 15 in *E. grandis* (Paton *et al.*, 1970). The suggestion would provide some indication about the differences in rooting ability of mini-cuttings derived from either apical, middle or basal part of the (mini-) stockplant.

Adventitious roots arise from any plant part other than by the normal development and ontogeny (Hartman *et al.*, 2002). Adventitious root formation is an essential step in the vegetative propagation of economically important woody species (Fett-neto *et al.,* 2001). Yet, many economically important woody plants have a low genetic and morphological capacity to form adventitious roots, which limits their commercial production (Hartman *et al.*, 2002). The formation of adventitious roots depend on plant cells to dedifferentiate, a process which is the capability of previously developed, differentiated cells to initiate cell division and form a new meristematic growing point. Since this characteristic is only pronounced in some cells and plant parts than in others, it is up to the propagator to provide and manipulate growing conditions of the stockplants and cuttings to be propagated (Hartman *et al.*, 2002).

Frequently, roots emerge through callus formation. Callus is an irregular mass of parenchyma cells in various stages of lignification that develops at the basal end of a cutting placed under environmental conditions favourable for rooting (Taiz and Zeiger, 2002). In easy-to-root species, the formation of callus and the formation of roots are independent of each other, even though both involve cell division. However, in some species, the formation of callus is a sign of adventitious root formation, while in other species excess callusing may delay rooting (Hartman *et al.,* 2002). Origin of adventitious roots from callus tissue has usually been associated with difficult-to-root species (Hiller, 1951). Normally, cuttings are naturally wounded when taken from stockplants. Following wounding, callus production and root development are frequently heavier along the margins of the wound. Wounded tissues are stimulated into cell division and production of

root primordia (MacKenzie *et al.,* 1986). This is due to a natural accumulation of auxins and carbohydrates in the wounded area (MacKenzie *et al.,* 1986).

The cheapest and easiest technique for propagating forest trees vegetatively is by cuttings, but traditionally only a few very easy-to-root genera, such as *Populus* L. have been propagated in this way (Zobel and Ikemori, 1983). In eucalypts, the popular method of rooting cuttings has limitations, such as the rapid loss of rooting competence, due to ontogenetic ageing, intra-clonal variation resulting from topophysis (the effect on growth and differentiation of position of axillary buds along the shoot) (Hartman *et al.,* 1990) and poor quality root systems that negatively affect the genetic expression of superior clones (de Assis, 2001). Several vegetative propagation methods exist, and these include:

1.4.1. Macropropagation system

Macropropagation is the vegetative propagation from cuttings, air-layering, grafting or other plant part. In South Africa, macropropagation was the primary production tool in commercial forestry (Denison and Quale, 1987). The system uses clonal hedges which are complicated to manage and poorly controlled because it requires large areas, intensive labour management and large quantities of fertiliser and water (Carvalho *et al.,* 1991; Higashi *et al.,* 2000). Furthermore, the system is outdoors and driven by ambient environmental conditions (Carvalho *et al.,* 1991; Higashi *et al.,* 2000). In addition, the major problem encountered in the macropropagation of cold-tolerant hybrids such as *E. grandis* x *E. nitens* is poor and variable rooting (Mokotedi *et al.,* 2000). Factors influencing rooting of such cold-tolerant hybrids are poorly understood that more than double the numbers of cuttings are set in order to reach a target number of plantable units (da Costa, *pers. comm.*[2]). Also, there is high mortality of cuttings during rooting due to fungal attack in nurseries of the KwaZulu-Natal midlands (Mokotedi, 2006). Due to such limitations of rooting cutting, alternative methods were developed for commercial cloning of *Eucalyptus* species (Campinhos and Ikemori, 1983).

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1.4.2. Micropropagation system

In the early 1990s, micropropagation technology for *Eucalyptus* clones accelerated the concept of a 'super-intensive' management system of producing vegetative propagules on a commercial scale in Brazil (de Assis*et al.*, 1992, Xavier and Comério, 1996). De Assis *et al.* (1992) observed that the *ex vitro* rooting rates of micropropagated cuttings decreased faster due to ontogenetic aging (developmental phase that a seedling undergoes from embryonic to juvenile to intermediate to mature adult plant). De Assis (2001) noted that, in theory, the *ex vitro* rooting rates of *Eucalyptus* clones should increase if the 'physiological distance' between the maximum juvenility stage (obtained *in vitro*) and the propagule collecting stage is reduced. To test this, de Assis *et al.* (1992) used the shoot apices (micro-cuttings) of very juvenile, micro-propagated plants of an *E. saligna* clone as propagules. These micro-cuttings had a 30% higher rooting rate than the stem cuttings (*ex vitro*). These findings were re-tested on seven clones of *E. saligna* and five clones of *E. grandis*. On average, the rooting rates of micro-cuttings were 18% higher than cuttings (*ex vitro*). Further research has shown that rooting of recalcitrant cold-tolerant can be improved *in vitro* (eg. *E. grandis* x *nitens*, MacAllister, *pers. comm.*[3], Mokotedi *et al.*, 2000; *E. grandis* x *E. marchathurii*, Jones and van Staden, 1994). Micropropagation in forestry is also useful for bulking up during the stage of selection of superior genotypes in clonal programmes (Yasodha *et al.*, 1995).

Protocols using micropropagation through tissue culture, and rooting of nodal cuttings, have been established for some commercially important *Eucalyptus* trees (see reviews by Le Roux and van Staden, 1991, Watt *et al.*, 2003). However, plantlets produced in tissue culture were usually criticized as 'fragile' because of anatomically deformed roots (Grout and Aston, 1978). Similarly, xylem tissues of microcuttings were reported to be less efficient in term of water transport (Fila *et al.*, 1998) than cuttings produced *ex vitro*(Wilson, 1996). In addition, 'fragile' roots produced *in vitro* are easily damaged during plantlet transfer to *ex vitro* conditions thereby producing deformed plants (Wilson, 1998).

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Most studies on micropropagation of eucalypts and their hybrid clones have focused on improving rooting and survival rates during acclimatisation (Watt *et al.,* 2003). However, few studies have focused on improving rooting *(ex vitro*) by manipulating stockplant environmental conditions.

1.4.3. Minipropagation and mini-hedge systems

Mini-cuttings are shorter cuttings as compared to macro-cuttings or stem-cuttings which are derived from macropropagation. The mini-cuttings are harvested from mini-hedge garden, hydroponics usually under polyethylene tunnel (de Assis *et al.,* 2004). The mini-cutting system was developed in commercial scale from 'cascade propagation' system previously used in France (de Assis*et al.,* 2004). This system has less operational and technical challenges when compared to themacropropagation system. Furthermore, intensive activities in this system are operated in a shorter area and practices such as soil preparation, fertilisation, irrigation, cultivation, weeding, pest control are significantly reduced (de Assis, 2001; de Assis*et al.,* 2004). The rooting rates of mini-cuttings are usually higher than those of macropropagated cuttings although results can vary with clone and species(de Assis*et al.,* 2004). Furthermore, the saplings produced in this system develop roots which are more responsive to fertilization, tolerant to environmental factors thereby reducing intra-clonal variation (de Assis*et al.,* 2004).

Commercial cloning of *Eucalyptus* mini-hedge system under polyethylene covered tunnels produces more cuttings, lower labour demand and lower water consumption (through mist system) than macropropagation system (de Assis, 2001). This system allows for CO_2 enrichment, control of temperature, light intensity and photoperiod manipulation (de Assis, 2001). These factors, along with optimum nutrition, are of fundamental importance to enhance rooting predisposition of clonal eucalypts. Additionally, the system allows for easy mechanical foliar spray of plant growth substances to stockplants to improve the yield of cuttings and their subsequent rooting potential (de Assis, 2001).

1.5. "Project Pulp" the creation of new hybrids for cold and dry (marginal) sites

Project Pulp was initiated in 2002 and developed by the Council for Scientific and Industrial Research (CSIR), the Department of Science and Technology's (DST) Innovation Fund and Natal Co-operative Timber Company, Limited (NCT Forestry) in an effort to improve wood quality, pulp yields and *Eucalyptus* hybrids. *Eucalyptus grandis* and *E. nitens* parent species were used and compared to commercial GN clones namely GN 107 and GN 075 (Naidoo *et al.*, 2008). The process of species screening, analysis, assessment and selection of the most suitable clones with favourable wood properties was identified, followed by the new material's release to the nurseries for vegetative propagation (Eatwell, *pers. comm.*[4]). Project Pulp has yielded hybrids such as PP 2107, which are considered genetically improved GN hybrids expected to fast adapt to sites more readily and have improved wood properties and pulp yields (http://www.engineeringnews.co.za). The PP 2107 germplasm is vegetatively propagated at Sunshine Seedlings Services nursery (29º 31.709'S; 30º 28.583'E), KwaZulu-Natal, South Africa and planted in the KwaZulu-Natal midlands (Naidoo *et al.*, 2008). *Eucalyptus grandis* x *E. nitens* GN 018B was created from *E. grandis* 'pollen recipient' (G0036) and *E. nitens* pollen donor (N0010) obtained from the Institute for Commercial Forestry Research (ICFR). However, PP 2107 clone, which is also a *Eucalyptus grandis* x *E. nitens* hybrid, was also created from an *E. grandis* 'pollen recipient' (AG0049) and an *E. nitens* 'pollen donor' (JN013) obtained from the ICFR's Jessievale seed orchard. The seed from the hybrid crosses were then germinated and plants sent to nurseries for growing hedges and making cuttings that went into the trial (1010806 EA0027) at Enon S29° 18.529' E30° 12.686') (Eatwell, *pers. comm.*[4]).

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1.6. Plant growth substances

Plants produce a variety of plant growth regulators, which include abscisic acid (ABA), gibberellins (GA), ethylene (ET), salicylic acid (SA), jasmonates (JA), brassinosteroids (BR), auxins, cytokinins (CK) and peptide hormones (Bari and Jones, 2009). Adventitious root formation is a developmental process marked by a series of histological events, which are characterised by different requirements for growth substances such as auxins, cytokinins and gibberellic acid. Thus, the time intervals of adventitious root initiation and development has made possible the correlation of sequential physiological and histological events in rooting (Eriksen and Mohammed, 1974; Davies and Joiner, 1980; Bollmark and Eliasson, 1986; Geneve and Kester, 1991).

1.6.1. Auxins

Auxins are thought to regulate different responses on a whole-plant level, such as tropisms, apical dominance and root initiation, and responses on a cellular level, such as cell extension, division and differentiation (Hagen and Guilfoyle, 2002). Endogenous auxin, indole-3-acetic acid (IAA), plays a central role in adventitious rooting (de Klerk *et al.*, 1999) andlight conditions are known to affect auxin metabolism and tissue sensitivity (Reid *et al.* 1991). Endogenous IAA is believed to be synthesised in shoot apices and young leaves, and transported basipetally via the polar transport pathway, considered to occur in living tissue (Moore, 1989). Auxins can also be conjugated, usually with amino acids, sugars, and most conjugates can revert to free auxin creating a useful mode of regulation of auxin activity (Crozier *et al.* 2000). Auxin action involves binding to a receptor protein and triggering of a signal transduction cascade that probably involves gene de-repression by proteolysis of transcriptional regulators (AUX/IAA) via the ubiquitin proteasome pathway (Dharmasiri and Estelle, 2004).

Indole butyric acid (IBA) is usually used for rooting during commercial propagation of plants; however other auxins such as IAA and naphthalene acetic acid (NAA) are also used (de Klerk *et al.,* 1999). Auxin type efficacy depends on (i) the affinity for the auxin receptor protein involved in rooting, (ii) concentration of free auxin that reaches target

competent cells, (iii) amount of endogenous auxin, and (iv) metabolic stability (lower in IAA, intermediate in IBA and higher in NAA) (de Klerk *et al.,*1999). IBA synthetically occurs in plants and has been discovered to have the same function as IAA even though it does not disintegrate easily when applied to living plant tissues (Hartman *et al.,* 2002). In mung bean hypocotyls cuttings, IBA applied to the cutting base was found transported quicker to the upper part of the cuttings than IAA, and rapidly metabolised into IBA conjugates (Hartman *et al.,* 2002). However, the interaction of endogenous IAA and exogenous auxins requires clarification (Ford *et al.,* 2001). Furthermore, basal application of auxins may not lead to an increase in IAA concentration in the specific cells that would give rise to adventitious root primordia (Ford *et al.,* 2001).

1.6.2. Ethylene and cytokinins

Ethylene is induced by high auxin concentrations through the promotion of transcription 1-aminocyclopropane-1-carboxylic acid (ACC) synthase gene and may affect rooting responses (Brock and Kauffman 1991). Ethylene may also enhance the sensitivity to auxins (Visser *et al*., 1996). Ethylene induces acidic peroxidases involved in lignin biosynthesis and cellulases and pectinases that facilitate root emergence through stem tissues (González *et al.,* 1991; Faivre-Rampant *et al.,* 1998). Ethylene may also promote rooting by stimulating cytokinin catabolism (Bollmark and Eliasson, 1990).

Cytokinins are a group of phytohormones which are active throughout the plant life cycle and involved in cell division, control of bud development and differentiation, shoot initiation and growth (Roitsch and Ehneß, 2000). There is a possible link between cytokinins and carbohydrates, since all cytokinin responses are associated with active growth or the activation of biological processes (Kuiper, 1993). An increased uptake of sugars in response to cytokinin treatment has been experimentally demonstrated in cells of *C. rubrum* in suspension culture (Ehne ß and Roitsch, 1997). It was also shown that D-type cyclins are regulated both by sugars and cytokinins (Murray *et al.,* 1998) indicating a direct link between cytokinins, sugar status and cell division. Cytokinins are also involved in axillary bud stimulation thereby inhibiting apical dominance (Ehneß and Roitsch, 1997).

Cytokinins interact synergistically or antagonistically with different phytohormones such as auxins (Crowell *et al.,* 1990), ethylene (Simmons *et al.,* 1992), and abscisic acid (Izhaki *et al.,* 1996). A high auxin/low cytokinin ratio usually favours adventitious root formation and a lower auxin/high cytokinin ratio favours adventitious bud formation (Bousa *et al.,* 1994). Further interactions exist between cytokinin and light (Lerbs *et al.,* 1984), sugars (Sheen *et al.,* 1999) and pathogen infection (Memelink *et al.,* 1987). Low nitrogen supply to plants leads to a reduced cytokinin production and a decreased rate of export from the roots to the shoot resulting in changes in carbon allocation (van der Werf and Nagel, 1996).

1.7. Plant-pathogen interactions

Plant inducible defence mechanisms against pathogens include oxidative burst, expression of defence-related genes, production of antimicrobial compounds, and programmed cell death (van Loon *et al.,* 2006). Infection of plants with diverse pathogens cause changes in various phytohormone concentration levels (Adie *et al.,* 2007; Robert-Seilaniantz *et al.,* 2007). Many phytopathogenic bacteria and fungi have been shown to produce cytokinin and/or auxin levels in the infected tissue thereby changing endogenous cytokinin and/or auxin biosynthesis and/or metabolism (Clarke *et al.,* 1999). Fungi associated with leaf and flower malformation, such as *Fusarium moniliforme,* have been shown to produce cytokinin *in vitro* (van staden and Nicholson, 1989) and to metabolise cytokinins (van Staden *et al.,* 1989). Angra and Mandahar (1991) found that extracts from culture filtrates of the fungus contained cytokinin-like activity, and cytokinin, starch and sugar were higher at infection sites. Reports have shown that *Quambalaria eucalypti* threaten a wide range of *Eucalyptus* species and clones in KwaZulu-Natal nurseries, and in young subtropical plantations in Queensland and New South Wales (Pegg *et al.,* 2005). Further, *Pantoea ananatis* causes shoot wilt and die-back (Coutinho *et al.,* 2002) and in particular this pathogen is able to infect a wide range of *Eucalyptus* species, hybrids and clones in nurseries and in the field (Coutinho and Venter, 2009). Environmental factors influence the severity of the diseases caused by *P. ananatis* on its different hosts (Coutinho and Venter, 2009). Although there are reports addressing how pathogens impact stockplants

morphology (Coutinho and Venter, 2009), understanding how pathogens impact the rooting ability of hybrids and clones would add great value to nursery production and management.

1.8. Environmental factors

Seasonal changes in temperature, rainfall and photoperiod influence growth, flowering and a stockplant's ability to provide cuttings which form adventitious roots (Moe and Anderson, 1988; Day and Loveys, 1998). Rooting ability of *E. globulus* can vary unpredictably from 100% to 14% after 51 harvests of cuttings and due to environmental variation (Wilson, 1999). Cuttingsof *E. saligna* took longer to root under constant low temperatures and the process was faster at higher temperatures (da Rocha Corrêa and Fett-Netto, 2004). Murugan (2007) reported that cuttings of a GN clone set in June (winter) developed high rooting rates than cuttings set in November (spring) or April (autumn). Similarly, *E. nitens* cuttings set in winter developed higher rooting percentage than cuttings set at other times of the year (Tibbits *et al.*, 1997). The quality of stockplants influenced rooting and spring favoured high yielding shoot development of GN stockplants. Therefore, season may influence the yield of cuttings and rooting rates of GN clones (Murugan, 2007), suggesting that the physiological status of the stockplant is also relevant for the rooting response (da Rocha Corrêa and Fett-Netto, 2004).

Environmental conditions such as light and temperature have been shown to act through various plant regulatory compounds (ABA, GA, and phytohormone), and to influence production of sugars (Anderson and Chao, 2001). For example, auxin and cytokinins have been implicated in dormancy control of adventitious and axillary buds (Nooden and Weber, 1978; Nissen and Foley, 1987; Cline, 1991). Light and dark conditions are also known to influence the rate of entry of exogenous IAA, endogenous auxins and the processes of conjugation and oxidative breakdown of IAA (Tam *et al.*, 1998). There are a number of different pre-severance techniques resulting in a variation in rooting ability of cuttings (Newton *et al.*, 1996). In particular, rooting is influenced by the position of the cutting on the shoot, the position of the shoot on the stockplant canopy and the number of shoots on the stockplant (Newton *et al.*, 1996). Many of these positional effects may be attributed to the pre-severance light level experienced by the cutting. The light intensity

under which stockplants and cuttings are exposed to influences aspects of cutting morphology, such as stem length and specific leaf area (Newton *et al.,* 1996). For instance, a reduction in the ratio of red (660 nm) to far-red (730 nm) wavelengths (R: FR) has been shown to increase stem elongation rate and leaf area. Additionally, a low R: FR ratio strengthens apical dominance by reducing lateral branching (Newton *et al.,* 1996). Hence, an understanding on how light and temperature on stockplants influence rooting of cuttings can add value to commercial forestry.

1.9. Influence of water

Separation of the cutting from its water-supplying root system would influence volume, turgor pressure, and water content of cells, and therefore upset physiological integrity (Jackson, 1986). The transport of assimilates and nutrients from the leaf to the base of the stem, and of water from the base of the stem to the leaf, are also important (Leakey, 2004). Water uptake in cuttings usually declines after cuttings are initially inserted into propagation media (Hartman *et al.,* 2002). Furthermore, a factor determining the rate at which cuttings lose water is the difference in water vapour pressure between the leaves and surrounding air (Hartman *et al.,* 2002). Commercial nurseries can minimise this difference either by decreasing vapour pressure of the leaf through reducing leaf temperature like in intermittent mist (Hartman *et al.,* 2002). Hence, taking cuttings early in the morning when the plant material is in a turgid condition is highly recommended by plant propagators. The management of stockplant hedges and the environment (light, water and nutrients) can have both short-term and long-term impacts on the rooting ability of cuttings (Leakey, 2004).

2.0. Nutritional effects

One of the factors determining the rooting rates of hardwood cuttings is the hydrolysis and availability of carbohydrates stored within the stem tissues (Leakey, 2004). Leakey and Storeton-West (1992) suggested that rooting is promoted by the production of specific sugars (i.e. sucrose, glucose, and fructose) during propagation. The concentration of

carbohydrates and nutrients in tissues of cuttings also vary with position of cuttings on stockplant (Leakey, 2004). High growth under elevated CO_2 concentrations is related to increased sugar concentrations in the plant and sugar-sensing systems of meristem cells and may activate increased cell division rates (Smeekens, 1998). The yield of cuttings from stockplants usually increases with increased photosynthesis, higher relative growth rate, and greater lateral branching of stockplants (Moe and Andersen, 1988). Carbon dioxide enrichment on stockplant environment has increased the yield of cuttings, but there is considerable variation of rooting response among species (Hartman *et al.,* 2002). The influence of nitrogen on root initiation and development relates to carbohydrate availability (C/N ratio) and to hormonal interactions (Hartman *et al.,* 2002). Nitrogen has been negatively correlated to rooting (Hambrick *et al.,* 1991) which suggests that the correlation between high C/N ratios and rooting may be due to low nitrogen levels (Hartman *et al.,* 2002). Maintaining stockplants under a high carbohydrate/high nitrogen level is optimal for rooting cuttings under mist, and high carbohydrate/low-to-moderate nitrogen ratio is optimal for rooting dormant hardwood cuttings (Hartman *et al.,* 2002).

2.1. Aims and objectives

Clones of the *E. grandis* x *E. nitens* hybrids (PP 2107) that have been created and propagated at Sunshine Seedlings nursery have consistently been shown to be suitable to sites in KwaZulu-Natal. These clones exhibit high and superior pulp properties, which makes them valuable for commercial plantation in South Africa. In commercial nurseries such as Sunshine Seedlings Services, the rooting frequency of *E. grandis* x *E. nitens* clones varies seasonally and little is known about the impact of the position of cuttings on the rooting ability of a clone. Further, the impact of morphological and developmental changes of stockplants over time as a result of frequent successive harvests on GN rooting rates has not been adequately addressed. In addition, many studies on adventitious rooting have focused on cutting performance (in *vitro*/*ex-vitro*) during the root formation period. The aim of this study was to understand whether there is a link between the rooting efficiency of PP 2107 and the management practices/manipulations of stockplants towards improving the rooting efficiency of GN 018B in line with the best management practices learned from PP 2107. The specific objectives were: to determine whether size of stockplants and

planting density and season affect coppice yield and rooting frequency; to determine whether rooting differences between GN 018B and PP 2107 are dependent on the position of cuttings on stockplants and seasonal variations and to understand the impact of stockplant management on endogenous factors through chemical analysis of soluble sugars and root initiation in the mung bean rooting bioassay.

CHAPTER 2: VEGETATIVE PROPAGATION OF *E. GRANDIS* X *E. NITENS* CLONES UNDER COMMERCIAL SETTINGS

2.1. INTRODUCTION

Vegetative propagation of selected *Eucalyptus* genotypes is highly beneficial to the clonal forestry industry because of rapid genetic gain and reduced variability among planted individuals (Yang *et al.*, 1995; de Assis *et al.*, 2004). Some eucalypt species do not sprout readily (e.g. *E. nitens* and *E. regnans*), and propagation through cuttings have been proven operationally useful in clonal eucalypt forestry than other vegetative propagation methods (Hartney, 1980; Zobel, 1993). Commercial propagation of clonal *Eucalyptus* using the mini-hedge system has many advantages over outdoor traditional propagation systems (de Assis *et al.*, 2004). In that system, ecological variables such as the competition for mineral nutrition, temperature, moisture, light, water are usually considered. Intra-clonal competition for light, affecting the sprouting performance of *Eucalyptus* stockplants (Blake, 1979) is the driving factor in polyethylene tunnels because water and nutrients are usually balanced in these managed systems. Under high light intensity, a large number of cuttings can be expected from vigorous plants which enhance the multiplication rate of shoots, from which cuttings are obtained (Pellicer *et al.*, 1998). However, experiments have demonstrated that changes in the irradiance or photoperiod of stockplants may affect the subsequent rooting of cuttings (Hansen, 1987; Moe and Andersen, 1988; Maynard, 1993). Rooting cuttings from selected hybrids plays an essential role in developing operational and sustainable hybrid forestry (Yang *et al.*, 1995). The ability of many woody species to form adventitious roots from cuttings is related to the stockplants growing conditions (Kibbler *et al.*, 2004).

The spacing and size of stockplants influence coppice production and the rooting ability of mini-cuttings (de Assis*et al.*, 2004). Some plant species become sensitive to auxins during specific periods of the year or auxins become limiting during seasons of high root formation (Davies, 1984). In order to enhance rooting, plant propagators have drawn

attention to the application of exogenous auxins (De Klerk *et al.,* 1999; Ford *et al.,* 2001), alternate nutrition, different type of fertiliser, and control of temperature and water status (Hartman *et al.,* 2002; de Assis *et al.,* 2004). The availability and concentration of endogenous auxins, as well as the ability of cuttings to respond to exogenous auxins have been used to explain the rooting ability between difficult-and easy-to-root plants (Epstein *et al.,* 1993; Baraldi *et al.,* 1993; Kibbler *et al.,* 2004).

Plant-pathogen interactions suggest that *Eucalyptus* species and clones are susceptible to diseases which change various phytohormone concentration levels and cause shoots to wilt and die-back and the severity is increased by environmental factors such as temperature and humidity (Coutinho and Venter, 2009). As part of general nursery protocol, stockplants are usually maintained under a 5 x 5 cm planting density. Mini-cuttings are harvested from any part of the stockplants and rooting rates vary with season (Pollard *pers. com.*[5]) (Fig. 2.1). It is not known whether maintaining a particular spacing or size of stockplants is a precise practice as the rooting frequency of cuttings differs based on their position (Hartman *et al.,* 2002) on stockplants. Furthermore, under successive harvests, stockplant morphology changes over time affecting the number of cuttings which can be yields (de Assis *et al.,* 2004; Fig. 2.2). By understanding variations in rooting ability due to position (Paton *et al*., 1970; Wilson, 1993; Hartman *et al.,* 2002), season (Maile and Nieuwenhuis, 1996; Wilson, 1999), and optimal planting density, the overall rooting rates of a hybrid can be maximised and efficiently managed throughout the year as well as the duration and maintenance of stockplants can be elaborated. The aim of this study was to investigate stockplants management factors affecting coppice yields and rooting rates of *E. grandis* x *E. nitens* cuttings under commercial settings. The specific objectives were: (a) to determine whether size of stockplant and planting density and season affect coppice yield and subsequent rooting frequency; (b) to determine whether rooting differences between GN 018B and PP 2107 clones is dependent on position of cuttings on stockplants and seasonal variations (Fig. 2.1), (c) to ascertain whether stockplant-pathogen interactions affect the rooting rates of GN clones.

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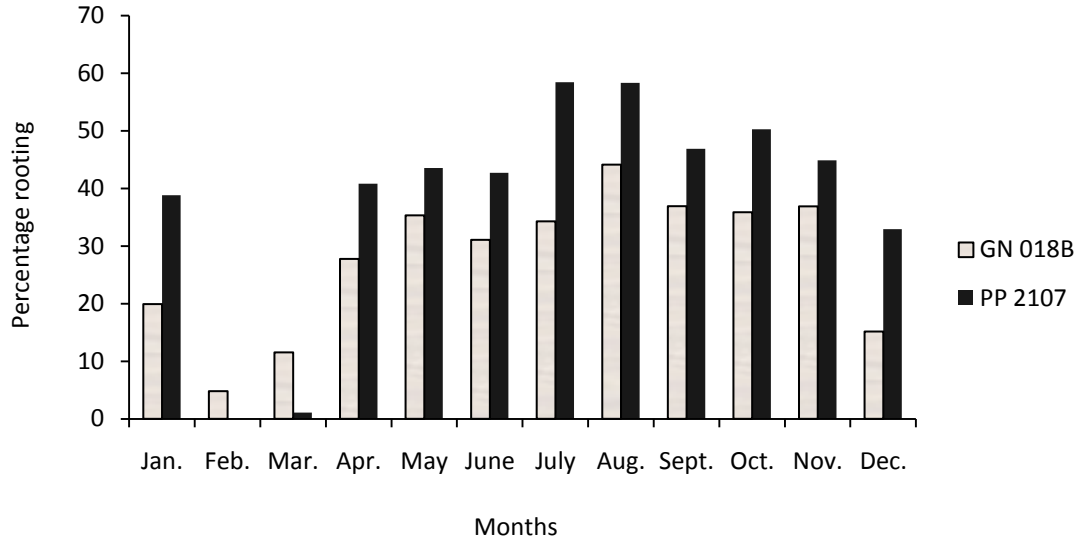


Fig. 2.1. Seasonal variations on overall rooting rates of *E. grandis* x *E. nitens* clones at Sunshine Seedlings Nursery from 2005 to 2010. (Pollard, unpublished data).

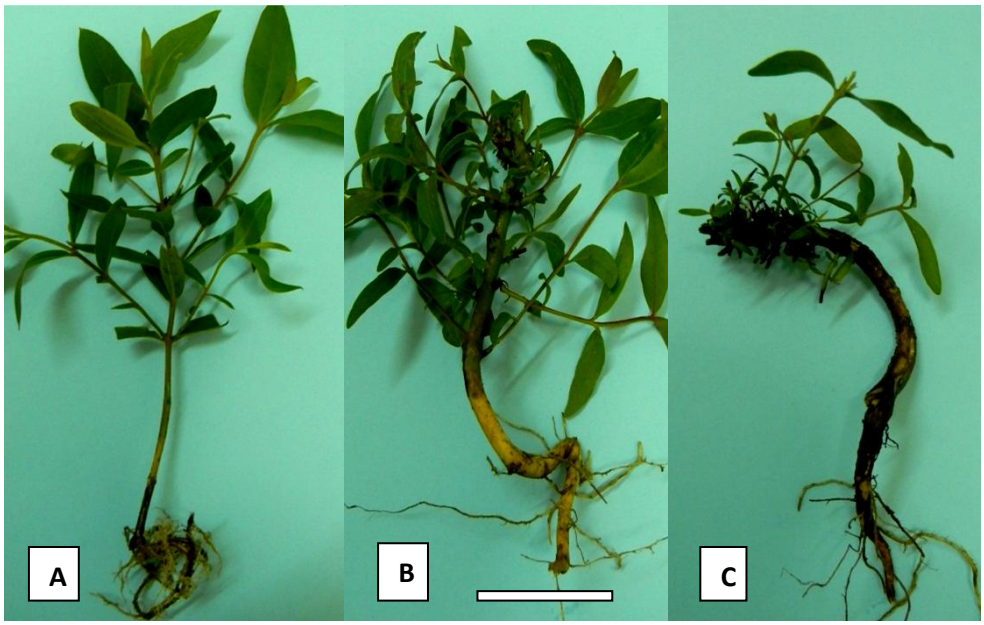


Fig. 2.2. Morphological and developmental changes of short stockplants before and after successive harvests (A: stockplant not yet been harvested after two weeks under the polyethylene tunnel, B: stockplant after four-six months of 2 – 3 week harvesting regime, C: stockplant after more than eight months of 2 – 3 week harvesting of cuttings), bar = 5 cm.

2.2. MATERIALS AND METHODS

2.2.1. Operational maintenance of stockplants and vegetative propagation practices at Sunshine Seedling Nursery

Cuttings from two *E. grandis* x *E. nitens* (GN) clones were prepared from clonal mini-hedges at Sunshine Seedling Services nursery (29º 31.709'S; 30º 28.583'E), KwaZulu-Natal, South Africa. A difficult-to-root GN 018B and an easy-to-root PP 2107 were both GN clones. Coppice of 5 - 7 cm in length was collected from these hedges and placed in buckets of clean tap water (no additives were added) and sectioned into cuttings of approximately 2.5 to 3 cm in length. All cuttings contained an internodal tissue and one pair of leaves on the apical tip, and leaf size was reduced to 1/3 of its original length in order to minimize transpiration. Unigro 98® trays (65 cm x 33 cm x 10.5 cm) with 128 inserts (3.5 cm^2/insert) per tray were filled with growth medium comprised of coir: vermiculite: perlite, (5:3:2). The trays were labelled and placed randomly in a polyethylene rooting tunnel. The air temperature within the tunnel was maintained between 28°C and 38°C by thermostatically activated fans and the root zone temperature was maintained at 25°C by bed heaters. An automatic above-head misting system maintained the air humidity inside the tunnel close to 100%, and the system was automated to mist every four minutes for ten seconds throughout the day and night. The mist contained a mixture of water and chlorine (tablets chlorine for swimming pool uses) at a concentration of 0.4 g/l to reduce algal and fungal growth on trays and plants. No artificial light was provided to the plants. Cuttings were then washed into NU-FILM® and the basal ends dipped in to Seradix 2, 4-(indol-3-yl)-butyric acid (3 g kg^{-1}IBA) (Bayer Crop Science, Leverkusen, Germany) and planted vertically and centrally at ± 1cm deep into the supporting/growing medium on 11 March 2010. NU-FILM® (poly-1-p-methene Miller Chemical and Fertiliser Corporation, Pennsylvania, USA) is a 'sticker-spreader' agent designed to control the effective lifespan of insecticides, fungicides, herbicides and foliar fertilizers. Seradix 2, is a commercial rooting powder for the stimulation of rapid and prolific rooting of semi-hardwood cuttings and it is used in the commercial operations at Sunshine Seedlings Service.

Cuttings were maintained inside a rooting tunnel for four weeks, thereafter transferred to a polyethylene tunnel for pre-hardening-off for another four weeks and finally moved to a greenhouse for a further week (hardening-off) prior to being transplanted into the

stockplant polyethylene tunnel. The pre-hardening-off tunnel, greenhouse and the stockplant polyethylene tunnel were under natural ambient temperature and humidity (Table 2.1). The beds of the pre-hardening-off tunnel were kept moist by an automatic mist sprinkler system. The misting frequency was every 45 minutes for a misting duration of one minute. However, in the stockplants polyethylene tunnel, a sprinkler irrigation system was set to keep the beds and the tunnel moist and consisted of 150-200 mL of water irrigated once a week for 45 minutes. Equal amount of foliar fertilizer was sprayed in rotation once a month and this was the standard practice used at Sunshine Seedling nursery (Table 2.2).

Table 2.1 Maximum temperature and relative humidity for the day during the trial (spring, summer and autumn) at the experimental site. Pre-and hardening off tunnels were under similar temperatures but experienced different light intensity and humidity.

Season	Harvest Date	Temperature (°C)		Light intensity (µmol s^{-2} m^{-1})		Relative humidity (%)	
		Ambient	Pre-& Hardening Tunnel	Ambient	Pre-& Hardening Tunnel	Ambient	Pre-& Hardening Tunnel
Winter	21/06/2010	26	27	400	320	55	65
Spring	18/09/2010	24	26	1100	500	50	65
	05/10/2010	30	33	1400	700	75	80
	25/10/2010	34	37	1500	900	80	90
	18/11/2010	38	42	2100	1200	90	100
Summer	08/12/2010	36	40	2200	1300	85	100
	27/12/2010	33	36	2000	1200	85	90
	15/01/2011	35	39	2200	1300	90	95
	31/01/2011	37	39	2105	1200	90	95
	14/02/2011	35	38	1800	1300	85	90
Autumn	28/02/2011	33	36	1800	1200	85	90
	14/03/2011	34	36	1800	1100	80	80
	28/03/2011	35	37	1750	1100	85	80
	11/04/2011	32	35	1700	1100	80	85

Four misting beds (8 m x 6 m) filled with re-cycled growing medium (coir: vermiculite: perlite, 5:3:2), were covered with plastic sheets and clearly separated and labelled into blocks. Each block was (1.5m x 1m) in dimension and 40 mm (diameter) perforations

were created with a gas gauge to allow the three months old saplings to exchange gases with the atmosphere upon transplanting into beds (Fig. 2.3; 2.4). After transplanting saplings (stockplants) these future stockplants were allowed to grow for two months before the rooting experiments were initiated. After that period, the length of shoots was reduced to tall (20 cm) and short (10 cm), and shoots were allowed to re-sprout for one month. Simultaneously, two planting densities of stockplant hedges were tested: high planting density (HD) comprised of 100 stockplants and low planting density (LD) comprised of 25 stockplants per treatment (Fig. 2.3; 2.4). Stockplants were kept at daily ambient light. The ambient maximum temperatures, relative humidity and light intensity received by the stockplants were recorded with Hansatech® light meter/thermometer at mid-day (Table 2.1).

Table 2.2 Types of fertiliser used as standard protocol at Sunshine Seedlings nursery

	Nitrosol®		Fleuron®	Microplex®	Hortichem®
Description	Natural Organic Plant Food contents Micro elements	growth stimulant	Liquid suspension, concentrate, readily absorbed and plant nutrient	Soluble chelated trace element mixture	Water soluble fertilizers 3:1:3
Composition	80g/kg N, 20g/kg P, 58g/kg K, 7g/kg Mg, 6g/kg Ca, 4g/kg S;	60 mg/kg Fe, 1mg/kg Cu, 1mg/kg Zn, 40mg/kg Mn, 23mg/kg B, 15mg/kg Mo	0.003g/kg	83g/kg Fe, 20g/kg Mn, 10g/kg Zn, 1.4g/kg Cu, 25g/kg B, 2.5g/kg Mo	164g/kg TOT-N, 84 g/kg NO_3-N, 55g/kg K, 10g/kg Mg, 56g/kg S
City/ Country	Pty(Ltd), Braamfontein, South Africa	Pty(Ltd), Braamfontein, South Africa	Pty(Ltd), Braamfontein, South Africa	Ocean Agriculture Pty(Ltd), Muldersdrift, South Africa	Ocean Agriculture Pty(Ltd), Muldersdrift, South Africa

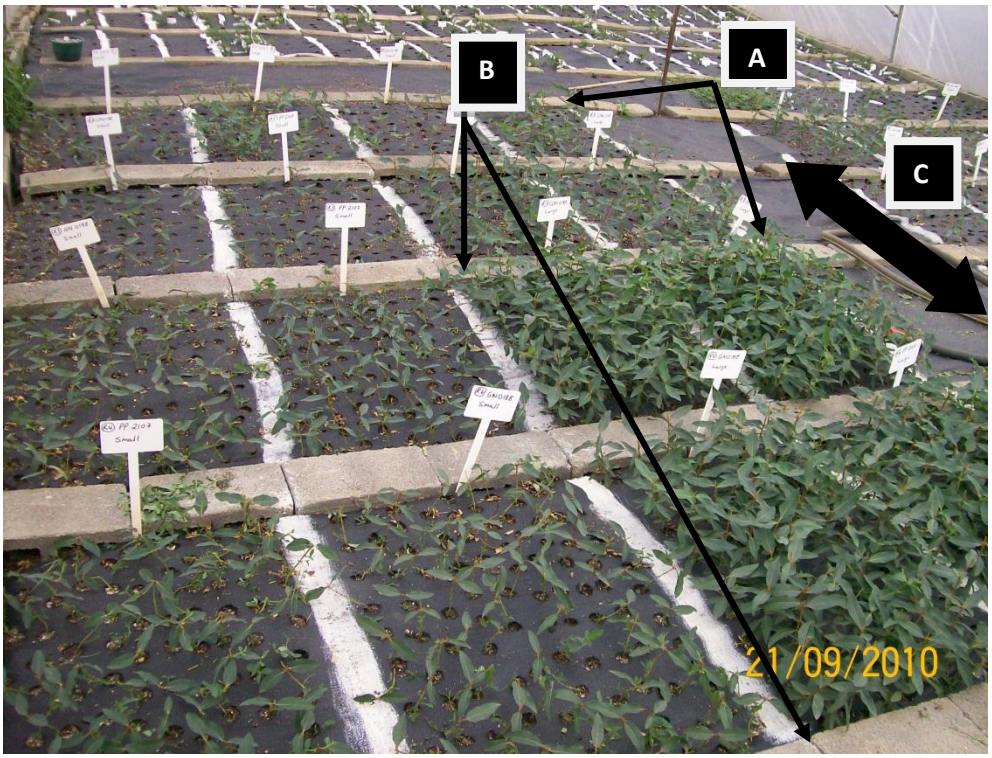


Fig. 2.3. Stockplant planting map showing the four treatments (GN 018B tall/short and PP 2107 tall/short) and two replicates per planting density in the tunnel: (A) Low planting density on top, (B) High planting density at the bottom. In total, four replicates per density separated by the stone walk-way (C). The right hand side shows similar set-up.

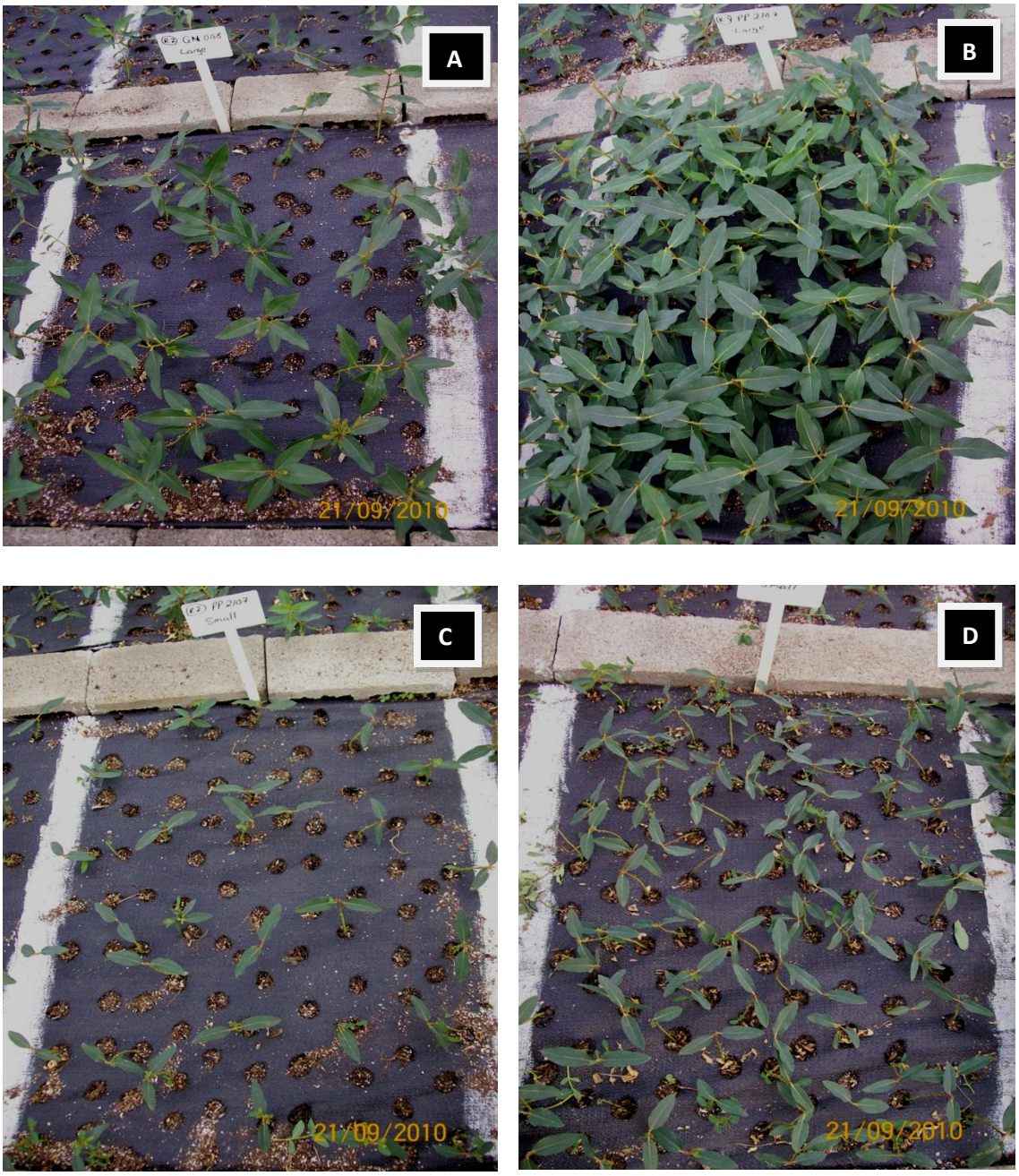


Fig. 2.4. A representation of tall and short stockplants on 1.5cm x 1cm mini-clonal hedges after transplating: (A) tall stockplants at low planting density (LD), (B) tall stockplants at high planting density (HD), (C) short stockplants at low planting density (LD) and (D) short stockplants at high planting density (HD).

2.2.2 Experimental design and techniques

2.2.2.1. Production of cuttings

After one month of re-sprout, cuttings were harvested from stockplants every two/three weeks in spring and summer and the number of cuttings per stockplant was counted. Furthermore, light intensity was measured between and within individual stockplants on the plant's apical, middle and basal part using a Hansatech® light meter/thermometer. Environmental parameters were recorded at each harvest date at mid-day (Table 2.1). The total number of cuttings produced per stockplant was recorded for each treatment and used to establish the rooting experiments while stockplants were kept at their experimental heights throughout this experiment. The yield of cuttings was not recorded in autumn as stockplants were infested with fungal diseases in late summer. Harvested cuttings were also not recorded in winter because spring and summer results showed no significant differences on the number of cuttings produced per GN stockplants. Similarly, although environmental parameters were not recorded in winter, rooting experiments were conducted in autumn and winter.

2.2.2.2. Rooting of cuttings from stockplants

Cuttings from PP 2107 and GN 018B harvested from the apical shoots were termed "apical cuttings", while "basal cuttings" were those harvested from the first/ and second most basal nodes on the stockplants, and "mid cuttings" were those cuttings that were taken "in between" apical and basal nodes of the shoot. Cuttings harvested from high and low planting density, as well as tall and short stockplants were treated according to the standard root induction protocol used at Sunshine Seedling nursery (see section 2.2.1). Rooting experiments were repeated in September and October (spring 2010), December 2010, and January 2011 (summer), April, and May (autumn) and June and July 2011 (winter). Since stockplants developed diseases related to fungal infestations in summer, no rooting experiments were set in February and March until the fungus was identified by FABI laboratory in Pretoria. Thus, in the rooting experiments set in April 2011 highly infected cuttings with fungi were used compared to experiments set in May 2011 which used less infected cuttings, as the fungal problem was over. At the end of the hardening-off period (nine weeks),the following parameters were measured: i.e. rooting frequency (%), callus formation (%) and root architecture (Fig. 2.5). 'Type one' root was described as root system which had a single primary root, 'type two' roots was the presence of two roots and

‘type three’ root was described as saplings with more than two lateral roots (Fig. 2.5). The same assessment was applied for all rooting experiments.

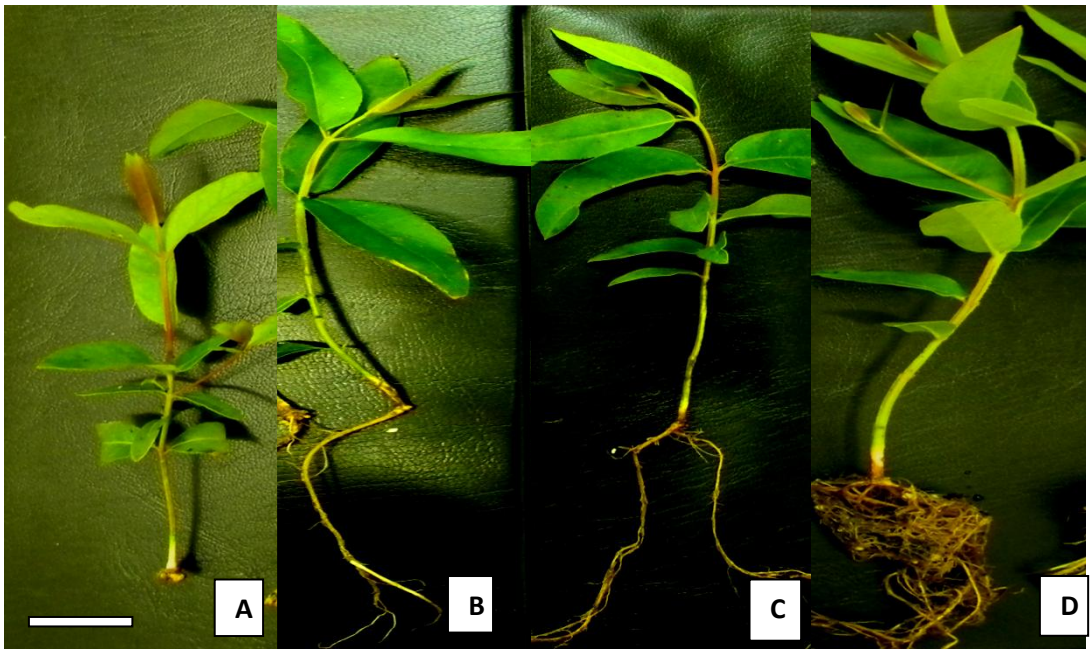


Fig. 2.5. Root architecture of evaluated saplings at the end of nine weeks of the root formation period. Evaluated saplings had the same age, and cuttings were from different positions and size of stockplants. Callus (A), type one (B), type two (C) and type three root systems (D). (Bar = 3 cm).

At high or low planting density, cuttings that were harvested from the same position of tall and short stockplants between GN 018B and PP 2107 were set into three trays (apical, middle, basal) in a random block design. Each tray constituted a block which was divided into four treatments of 24 cuttings each. There were two blocks per planting density which made a total of 48 cuttings per treatment. The four treatments were: GN 018B tall/short and PP 2107 tall/short repeated for apical, middle and basal shoots separately and also repeated twice for the two planting densities. Therefore, twelve trays were set per planting density and randomly arranged in the polyethylene rooting tunnel. In overall, a total of 1152 cuttings were separately evaluated at high and low planting densities. These experiments were set in September, October 2010 (spring) and December 2010 and

January 2011 (summer), April and May 2011 (autumn), and June and July 2011 (winter). Stockplants developed diseases from fungal infestations in early February, and the disease was over in May. Thus, experiments set in April and May 2011 also investigated the impact of fungal infections on rooting as well as GN clones resistance/susceptibility to pathogen infestations. All the cuttings were treated exactly the same under nursery protocol as described in section 2.2.1.

2.2.3. Statistical analysis

After the cuttings were harvested, light intensity within individual stockplants, number of cuttings produced, survival of stockplants, and dry mass of shoots and roots before and after the experiment were recorded during the experimental period. To determine plant material dry mass, fresh material was dried for 48 hours in the oven and the dried material was weighed and the mean calculated.

Data for the rooting experiments evaluated samplings after nine weeks i.e eight weeks of root formation period and one week of hardening-off period. The collected rooting data evaluated the impact of position of cuttings on stockplants, size of stockplants and seasonal variations on rooting rates of GN clones. Cuttings which survived were further evaluated based on callus formation through a subjective visual inspection, or presence of roots. In addition, saplings were also assessed based on their root architecture (see Fig. 2.5). The rooting morphology was evaluated nine weeks after cuttings were set for rooting experiments as per nursery protocol.

The experiment was established as a randomised complete block design with treatments randomised within a block. Statistical analyses were carried out with GenStat (version 13.2; VSN International, Hemel Hempstead, UK). All data were subjected to a Two-Way ANOVA (in randomised blocks). Data that were not normally distributed were transformed (either log or arcsine transformation was used), and the analyses were performed on transformed data, with means presented as non-transformed data plus standard error (S.E) values. Differences among treatments were separated by Tukey's Honestly Significant Difference (HSD) test ($p \leq 0.05$).

2.3. RESULTS

To test the objective of whether the number of cuttings is affected by the size of stockplants and planting density, an investigation was conducted on GN 018B and PP 2107 stockplants. All stockplants were initially grown at high planting density (HD) for four weeks. Thereafter, low plating density (LD) was initiated by removing 75 stockplants from the first two beds close to the end of the polyethylene tunnel (Fig. 2.3). Light intensity within individual stockplants (apical, middle and basal) was usually recorded at each harvest, and stockplant shoots and roots dry mass was recorded in September 2010 and in March 2011. Survival of stockplants was also compared between spring (September, October, and November) and summer (December and January). Stockplants which did not survive at either high or low planting density were not replaced because understanding how stockplant management affects their survival on clonal beds was of great interest. Stockplant dry mass recorded in March measured plants which recovered from pathogen infestations and compared fungal impacts on GN stockplants.

2.3.1. Mortality of stockplants

At the time of first harvests, dead stockplants were not found. The survival of stockplants was affected probably because of successive harvest regimes, differences in environmental conditions and pathogen infestations, which degraded the morphology and the development of stockplants. Although not significantly different, mortality of short stockplants was higher for PP 2107 as compared to GN 018B short plants at high planting density in spring. However, GN 018B short stockplants were mainly affected in summer in particular at low planting density when compared to PP 2107 stockplants. In overall, mortality was higher in summer than spring and shorter stockplants were more affected than taller stockplants (Fig. 2.6; Table 2.3).

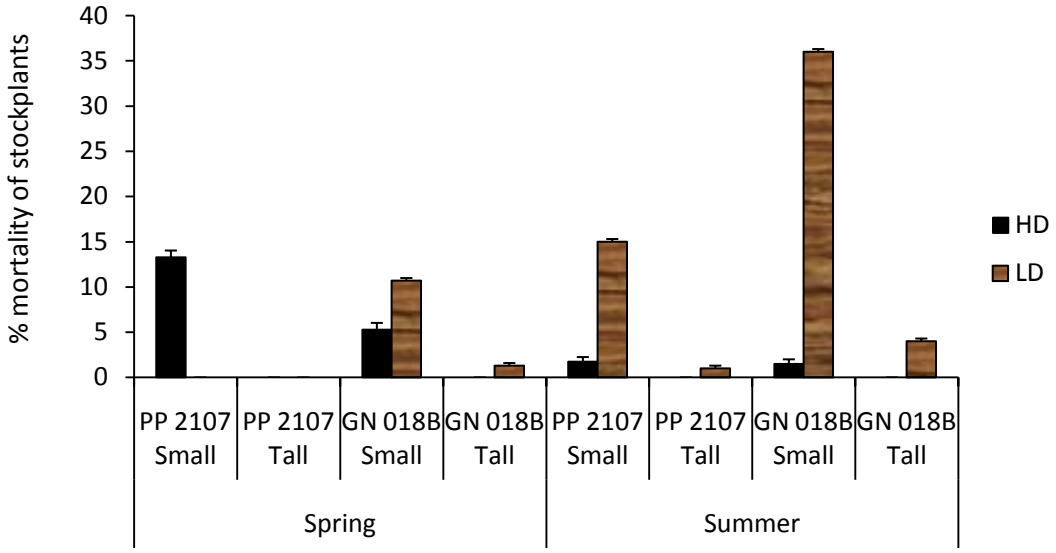


Fig. 2.6. Overall stockplant mortality over seasonat high and low planting densities (LD: low density, HD: high density). Percentage dead stockplants is shown (± S.E.) and was significantly different in summer at low planting ($p < 0.05$) but not significantly different at high planting density for both seasons.

Table 2.3: Levels of significance of stockplant mortality during spring and summer at low and high spacing densities based on size and genotype (LD = Low density; HD = High density)

Parameters	*p* - value			
	Spring		Summer	
	LD	HD	LD	HD
Size	0.400	0.164	0.001	0.029
Genotype	0.288	0.727	0.021	0.884
Size × Genotype	0.400	0.727	0.146	0.884

Analyses were performed using Two Ways ANOVA ($p \leq 0.05$, n = 1600 and 400 for high and low planting densities respectively. Data analysis performed on log-transformed data.

2.3.2. Impact of planting density and size of stockplants on yields of cuttings over season

Analysis of variance suggested that planting density had no significant effect on the number of cuttings produced between the two GN clones, except on 08 December harvest (Table 2.4; Fig. 2.7). However, a high number of cuttings was produced at lower planting density as compared to higher planting density although significant differences were not obtained (Table 2.4; Fig. 2.7). Furthermore, taller stockplants of the two GN clones produced more cuttings than shorter stockplants and significant differences were obtained at low planting density (Table 2.5). Besides variations in environmental conditions between spring and summer (Table 2.1), in overall high number of cuttings was recorded in spring as compared to summer. Four harvests were made in spring compared to three harvests in summer because the conditions of stockplants did not meet the nursery standard due to fungal infestations (Table 2.4; Fig. 2.8). As discussed later in this study, high growth vigour was observed in summer; however pathogen infestations affected the morphology and development of stockplants (Fig. 2.8). Although further harvests were not recorded following this event, the rooting experiments initiated tested the effects of fungal infestations on rooting efficiency of the two GN clones.

Table 2.4: Number of cuttings harvested from GN 018B and PP 2107 stockplants maintained at high (HD) and low (LD) planting densities

Season	Months	High Density		Low Density	
		GN 018B	PP 2107	GN 018B	PP 2107
Spring	18 Sept. 10	5 ± 0.2^{a}	5 ± 0.2^{a}	4 ± 0.3^{a}	5 ± 0.3^{b}
	05 Oct. 10	1 ± 0.1^{a}	1 ± 0.1^{a}	3 ± 0.5^{a}	4 ± 0.5^{a}
	25 Oct. 10	5 ± 0.1^{a}	5 ± 0.1^{a}	8 ± 0.9^{a}	9 ± 0.9^{a}
	18 Nov. 10	4 ± 0.4^{a}	5 ± 0.4^{b}	12 ± 0.7^{a}	12 ± 0.7^{a}
Summer	08 Dec. 10	4 ± 0.4^{a}	4 ± 0.4^{a}	6 ± 0.6^{a}	8 ± 0.6^{b}
	27 Dec. 10	3 ± 0.3^{a}	3 ± 0.3^{a}	7 ± 1.1^{a}	7 ± 1.1^{a}
	15 Jan. 11	3 ± 0.3^{a}	3 ± 0.3^{a}	7 ± 1.0^{a}	5 ± 1.0^{a}

a, b = mean separation within rows of a specified density, HSD test, ± standard error ($p \leq 0.05$, n = 1600, 400 for high and low planting densities respectively).

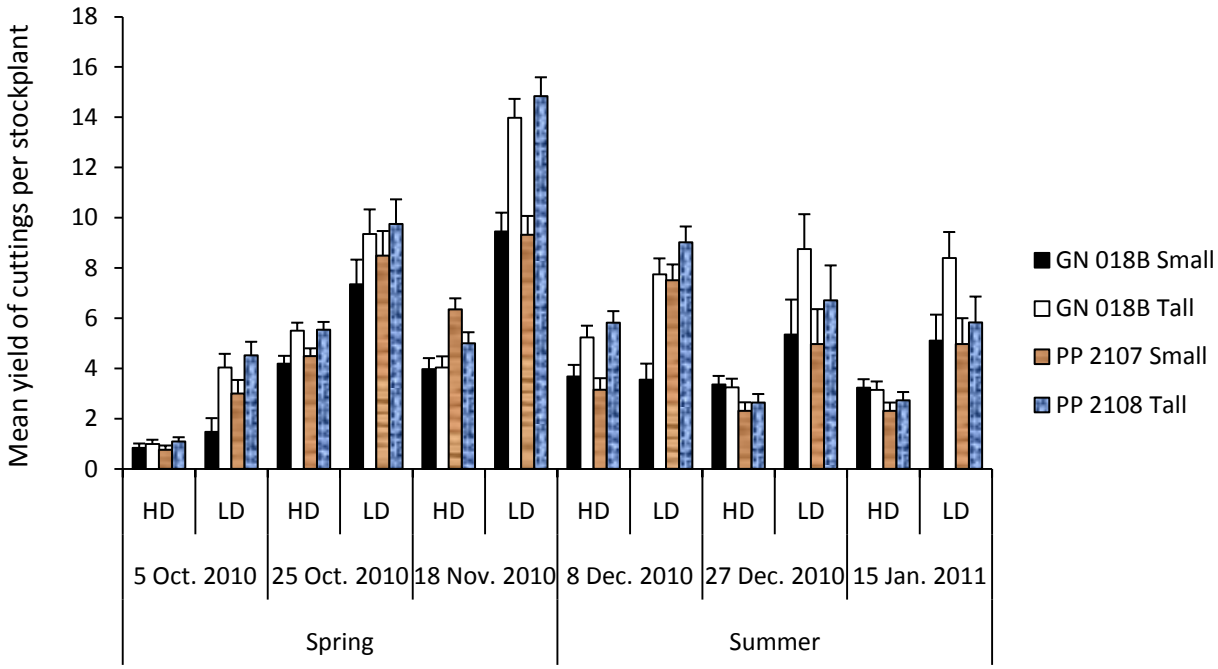


Fig. 2.7: Yield of cuttings harvested per stockplant by size and genotype interactions at high (HD) and low (LD) planting densities between spring and summer. Values are means (± S.E).

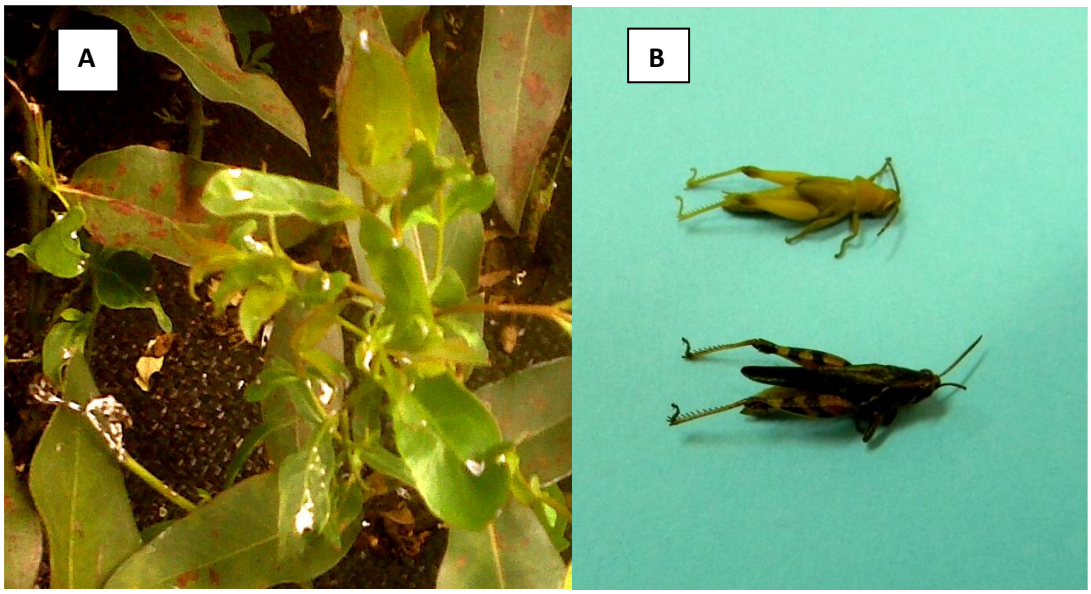


Fig. 2.8. *Quambalaria eucalypti (Sporothrix eucalypti)* infestations in the form of white powdery spore masses on leaves, shoots, stems, and branches of *E. grandis* x *E. nitens* (GN) stockplants (A). Nymph (turned yellow due to 70% alcohol storage but originally had green colour) and the adult hopper (family: Acrididae) (B), found feeding and spreading fungal spores on stockplants in the polyethylene tunnel during summer.

Table 2.5: Levels of significance on yield of cuttings produced at high and low planting density between tall and short GN stockplants

Season	Harvests	*p* - value					
		Size		Genotype		Size × Genotype	
		HD	LD	HD	LD	HD	LD
Spring	18-Sept-10	0.032	0.321	0.987	0.237	0.079	0.064
	05-Oct-10	0.234	0.011	0.964	0.141	0.624	0.446
	25-Oct-10	0.010	0.149	0.623	0.461	0.687	0.717
	18-Nov-10	0.382	< 0.001	0.222	0.630	0.462	0.549
Summer	08-Dec-10	0.004	0.004	0.965	0.006	0.273	0.081
	27-Dec-10	0.655	0.239	0.033	0.784	0.457	0.27
	15-Jan-11	0.646	0.076	0.076	0.221	0.476	0.271

Analyses were performed using Two Way ANOVA and Tukey's HSD test, where applicable ($p \leq 0.05$, n = 1600, 400 for high and low planting density respectively).

2.3.3. Stockplant shoots and roots dry mass

Because of the effects of size of stockplants and planting density differences on GN stockplants, shoot and roots dry mass was investigated before the experiment was initiated (September) and after pathogen infestation period of March 2011. Although the number of cuttings was not recorded following fungal infestations, the additional harvests which followed maintained the stockplants at their experimental sizes and experimental juvenile stages. Initially in this experiment, low planting density was not recorded as all planted stockplants were firstly grown at high planting density (Table 2.6). At low planting density, high yield of cuttings was harvested and correlated with greater shoot and root mass as compared to high planting density, possibly because more light favoured more photosynthesis, thus high growths.

Table 2.6: Shoot and root dry mass of tall and short stockplants maintained at high (HD) and low (LD) planting densities and recorded in September 2010 and in March 2011. Dry mass was not recorded at low planting density in September. Values are means (± S.E).

	Tall				Short			
	Shoot		Root		Shoot		Root	
GN 018B	HD	LD	HD	LD	HD	LD	HD	LD
Sept. 10	2.0 ± 0.4		0.5 ± 0.1		1.0 ± 0.1		0.3 ± 0.0	
Mar. 11	5.7 ± 1.5	7.5 ± 0.2	2.3 ± 1.0	2.3 ± 0.4	1.2 ± 0.0	2.3 ± 0.3	0.4 ± 0.0	0.7 ± 0.1
PP 2107								
Sept. 10	2.1 ± 0.2		0.5 ± 0.1		1.0 ± 0.2		0.3 ± 0.0	
Mar. 11	6.1 ± 0.9	7.4 ± 0.7	2.1 ± 0.1	1.6 ± 0.0	2.1 ± 0.4	1.95 ± 0.42	0.5 ± 0.2	0.5 ± 0.1

The size of stockplants had a significant effect on shoot and root dry mass possibly because tall (20 cm) stockplants had double the size of short (10 cm) stockplants (Table 2.7). However, genotype differences between the two GN clones were not significant from each other although PP 2107 had higher growths than GN 018B before the months of pest and fungal infestations (Tables 2.7; 2.8). Further, shoot and root mass of PP 2107showed greater decrease as compared to GN 018Bdry mass, in particular at low planting density (Table 2.6), possibly because PP 2107 was highly affected by pests and pathogens.

Table 2.7: Shoot and root dry mass between tall and short GN stockplants before and after harvesting regime. Data from Table 2.6

Months	Size				Genotype				Size × Genotype			
	Shoot		Root		Shoot		Root		Shoot		Root	
	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD
Sep. 10	<.001		0.01		0.59		0.66		0.81		0.59	
Mar-11	<.001	<.001	<.001	<.001	0.04	0.3	0.49	0.19	0.41	0.11	0.32	0.21

Analyses were performed using Two Ways ANOVA ($p \leq 0.05$, n = 64 at high (HD) and low (LD) planting density separately.

2.3.4. Light intensity on stockplants

Because of size differences between stockplants, position of cutting within shoots, planting density and season, light intensity measurements were recorded within stockplants (tall and short) during spring and summer. This study also investigated the impact of these factors on the sprouting behaviour and the rooting differences between the two GN clones. Further light measurements were not recorded as cuttings yielded in February and March were not used in this study because of pest and fungal infestations. Analysis of variance suggested that position of cuttings; size of stockplants and planting density had a significant effect on light intensity received by stockplants (Fig. 2.9; Table 2.8). As discussed later in this study, apical cuttings received more lights compared to middle and basal cuttings, and high light intensity was usually accumulated at low planting density. Further, cuttings from tall stockplants at high planting density experienced variations in light intensity as compared to cuttings from short stockplants. In tall stockplants, basal cuttings received lower light as compared to apical cuttings which received greater light intensity (Fig. 2.9; Table 2.8). Thus, apical and middle cuttings from tall stockplants maintained at high planting density usually developed apical dominance which was not observed on cuttings maintained at low planting density. At low planting density, short stockplants usually developed high yield of shorter mini-cuttings with shorter internodes as compared to tall stockplants which showed apical dominance.

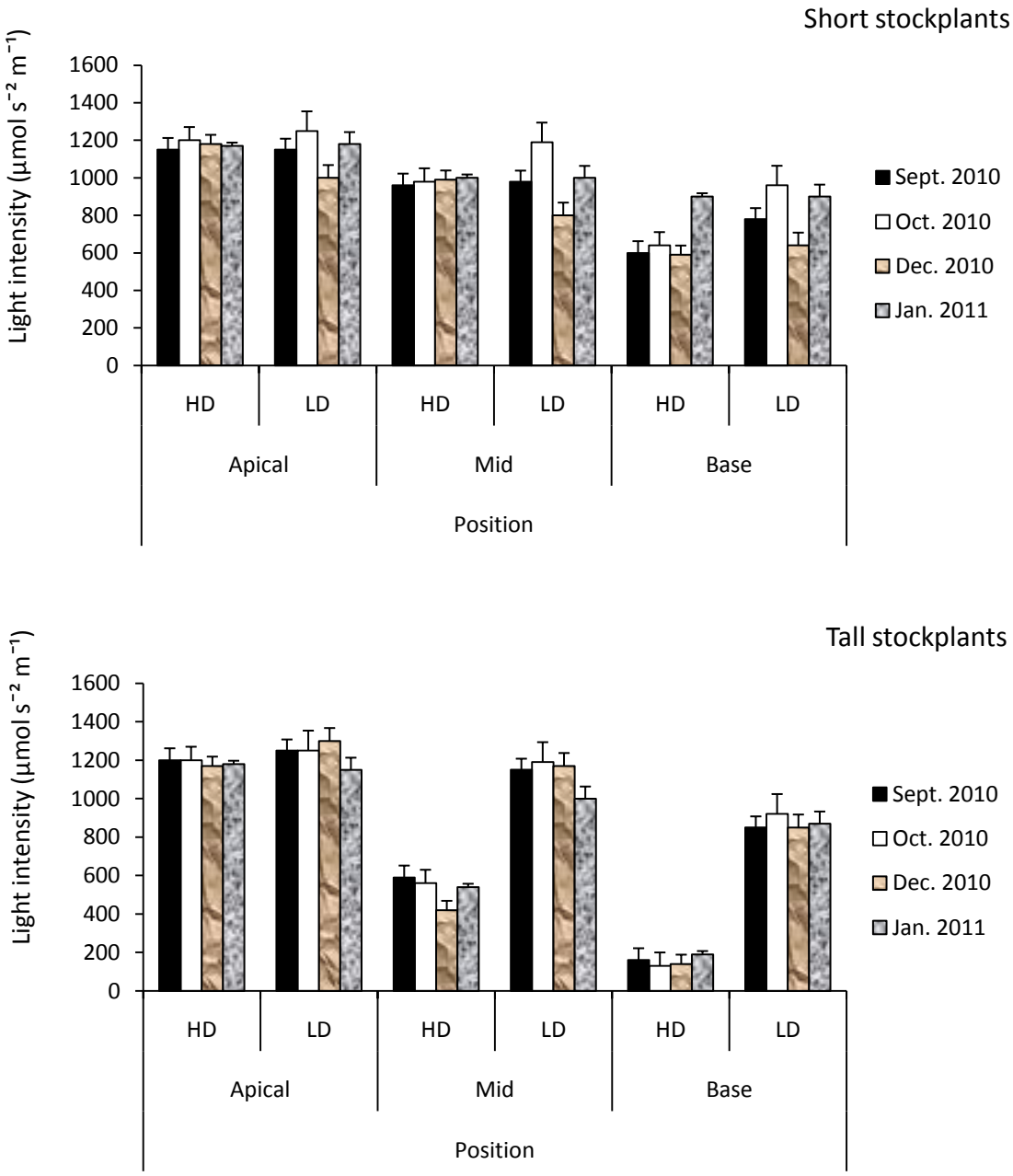


Fig. 2.9: Levels of light intensity on cuttings within stockplants maintained at high (HD) and low (LD) planting densities between spring and summer.

Table 2.8: Levels of significance on size of stockplants and position of cuttings during spring and summer at low (LD) and high planting (HD) densities.

Season	Months	*p* - values					
		Position		Size		Position x size	
		HD	LD	HD	LD	HD	LD
Spring	Sept. 2010	<.001	0.002	0.003	0.029	0.039	0.977
	Oct. 2010	<.001	0.029	0.003	0.233	0.023	0.716
Summer	Dec. 2010	<.001	0.013	<.001	0.002	0.003	0.501
	Jan. 2011	<.001	0.013	<.001	0.809	<.001	0.977

Data analyses performed by Tukey's HSD test ($p \leq 0.05$).

2.3.5. Impact of size of stockplants, planting density and season on rooting

Cuttings harvested from PP 2107 usually had higher rooting frequency and developed more roots than cuttings of GN 018B clone (Table 2.9). Cuttings from GN 018B maintained at low planting density showed higher rooting frequency than cuttings maintained at high planting density. Significant differences were not observed in September (p = 0.394), October (p = 0.589), June (p = 0.463) and July (p = 0.716) as the rooting rates of GN 018B were constantly improved under low planting density (Table 2.9). However, contrasting effects were observed with PP 2107 cuttings which showed low rooting rates at low planting density (Table 2.9) and low callusing (Table 2.10) in overall as compared to GN 018B. High rooting rates of GN 018B was usually correlated with high callus formation at high and low planting densities (Fig. 2.10). Cuttings of PP 2107 stockplants usually developed 'type three' root system as compared to cuttings of GN 018B stockplants which constantly developed 'type one' and 'type two' root systems (Fig. 2.13).

Table 2.9: Effect of season and planting density on mean rooting rates of GN clones

Season	Months	High Density		Low Density	
		GN 018B	PP 2107	GN 018B	PP 2107
Spring	Sept.10	27.4 ± 3.1^{a}	63.3 ± 3.1^{b}	46.2 ± 2.4^{b}	55.9 ± 2.4^{c}
	Oct. 10	23.3 ± 2.7^{a}	60.5 ± 2.7^{d}	41.0 ± 2.9^{b}	47.2 ± 2.9^{c}
Summer	Dec.10	10.4 ± 3.2^{a}	50.0 ± 3.2^{b}	3.8 ± 3.6^{a}	21.5 ± 3.6^{b}
	Jan.11	4.2 ± 2.1^{a}	21.9 ± 2.1^{b}	22.6 ± 4.6^{a}	38.9 ± 4.6^{b}
Autumn	Apr.11	15.3 ± 3.8^{b}	6.6 ± 3.8^{a}	39.2 ± 2.2^{b}	15.3 ± 2.2^{a}
	May.11	12.8 ± 1.9^{b}	5.2 ± 1.9^{a}	45.1 ± 3.1^{b}	9.4 ± 3.1^{a}
Winter	Jun. 11	15.6 ± 0.2^{a}	47.2 ± 0.2^{b}	19.4 ± 0.2^{a}	31.6 ± 0.2^{b}
	Jul. 11	17.3 ± 2.1^{a}	70.1 ± 2.1^{b}	28.1 ± 6.2^{a}	34.3 ± 6.2^{a}

a, b = mean separation within rows, Tukey's HSD test, ± S.E. ($p \leq 0.05$, n = 576 for high and low planting density respectively).

Further, season had a significant influence on overall rooting rates of GN clones. High rooting frequency of GN 018B was recorded in September (46.2%) at low planting density whereas low rooting rates were observed in December (3.8%) and January (4.2%) respectively. However, high rooting rates of PP 2107 were observed at high planting density in July (70.1%) and low rooting frequency was recorded in April (6.6%) and May (5.2%) (Table 2.9; Fig. 2.11). As previously mentioned, although GN stockplants were infected with pathogens, GN 018B saplings developed high rooting rates as compared to PP 2107 after disease infestations. Thus, fungal infestations in April and May 2011 impacted differently on rooting frequency of GN clones (Table 2.9). High rooting rates of GN 018B observed in September and May also showed high callusing in particular at low planting density. Low callusing on PP 2107 was also correlated with low rooting rates in the months of April and May 2011(Table 2.10). Further, 'type three' root system was usually observed on saplings of PP 2107 short stockplants, in particular when rooting rates were high in July, September and October. However, 'type three' root type was not observed during the periods of low rooting frequency of April and May 2011 (Fig. 2.13).

As discussed previously, short stokplants usually developed shorter mini-cuttings with shorter internodes i.e. more nodes as compared to tall stockplants which showed dominance on apical or middle cuttings. Although all the cuttings used in this study had

equal lengths, the number of nodes on each cutting was not standardised as size of stockplants, position of cuttings and planting density influenced mini-cutting morphology.

Fig. 2.10. Callus formation on *E. grandis* x *E. nitens* cuttings assessed through subjective visual inspection at the end of the root formation period.

Table 2.10. Effect of season and planting density on callus formation *of E. grandis* x *E. nitens* clones.

Season	Months	High Density		Low Density	
		GN 018B	PP 2107	GN 018B	PP 2107
Spring	Sept.10	2.33 ±0.66^{b}	1.00 ±0.66^{a}	4.17 ±0.44^{b}	1.08 ±0.44^{a}
	Oct.10	2.83 ±0.65^{b}	0.58 ±0.61^{a}	3.50 ±0.42^{b}	2.17 ±0.42^{a}
Summer	Dec.10	1.92 ±0.32^{b}	1.00 ±0.32^{a}	0.75 ±0.13^{b}	0.16 ±0.13^{a}
	Jan.11	0.25 ±0.30^{a}	1.00 ±0.30^{b}	1.08 ±0.34^{b}	0.25 ±0.34^{a}
Autumn	Apr.11	0.42 ±0.14^{b}	0.08 ±0.14^{a}	0.00 ± .00^{a}	0.00 ± .00^{a}
	May 11	3.17 ±0.25^{b}	0.08 ±0.25^{a}	1.08 ±0.13^{b}	0.00 ± 0.13^{a}
Winter	Jun. 11	2.20 ±0.28^{b}	1.25 ±0.28^{a}	3.45 ±0.12^{b}	0.54 ± 0.12^{a}
	Jul. 11	5.50 ±0.35^{b}	4.25 ±0.35^{a}	3.50 ±0.80^{a}	3.67 ± 0.80^{a}

a, b = mean separation within rows, Tukey's HSD test, ± S.E. ($p \leq 0.05$, n = 576 for high and low planting densities, respectively).

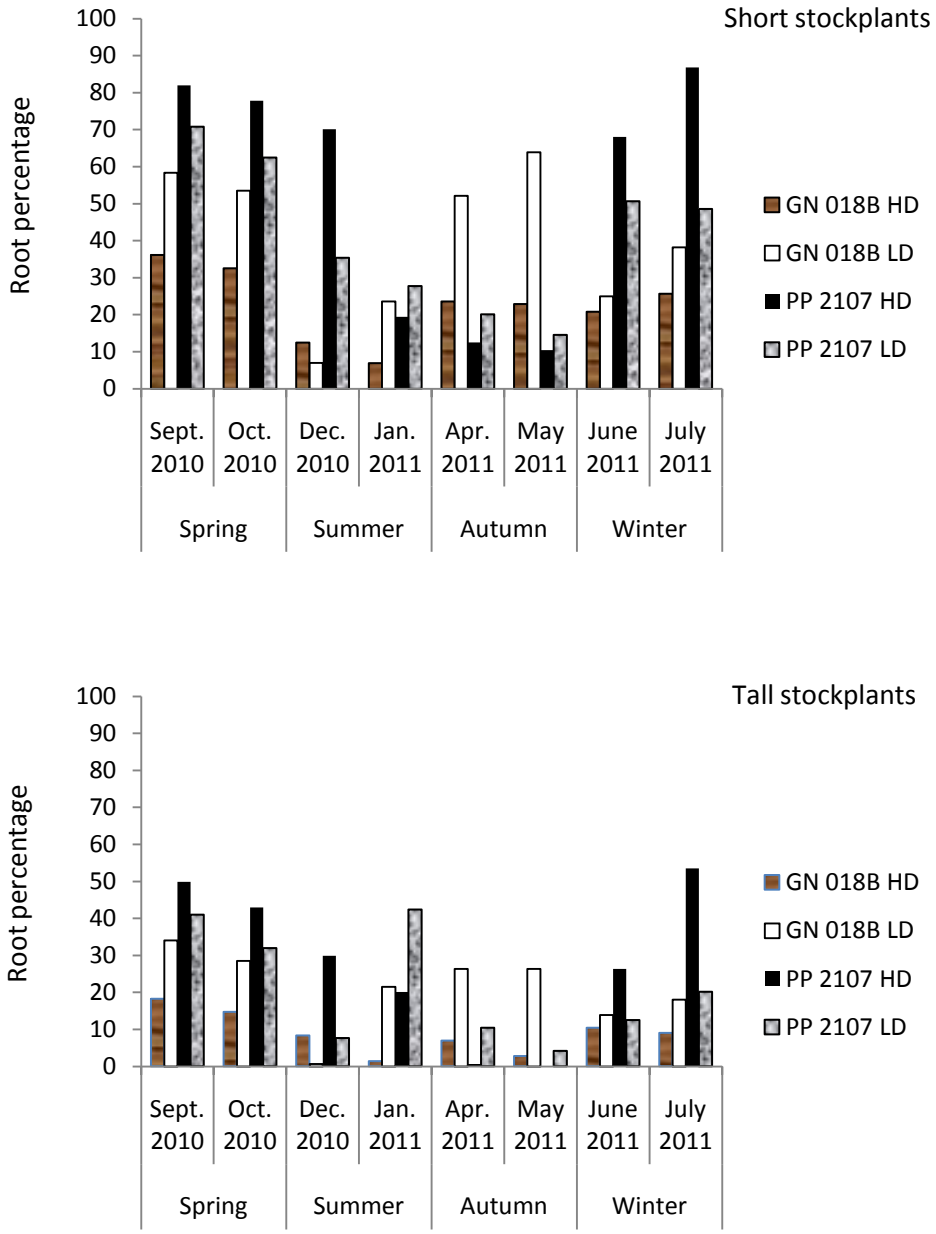


Fig. 2.11: Rooting rates of tall and short *E. grandis* x *E. nitens* (GN) clones evaluated separately per genotype and planting density over season.

The size of stockplants had a significant effect on overall rooting rates of GN clones (Fig. 2.11). Cuttings derived from short stockplants rooted better than cuttings from tall stockplants except in January where significant differences were not observed at high (p = 0.621) and low planting densities (p = 0.172). Short GN stockplants had higher rooting frequency than tall stockplants regardless of clone, spacing or season (Fig. 2.11). As

mentioned above, at low planting density GN 018B developed high rooting frequency and high callus formation regardless of size of stockplants. Cuttings of PP 2107 short stockplants maintained at high planting density had 86.8%, 81.9% and 77.7% rooting rates in July, September and October respectively, and 12.5% and 10.4% in April and May respectively (Fig. 2.11). However, cuttings of GN 018B short stockplants had rooting rates of 58.3%, 53.4% and 6.9% in September, October and December respectively, and 52% and 63.8% rooting in April and May respectively under low planting density (Fig. 2.11). In addition, 'type three' root system was usually observed on saplings of PP 2107 stockplants as compared to 'type one' and 'type two' commonly observed on GN 018B saplings (Fig. 2.5).

2.3.6. Impact of position of cuttings on rooting

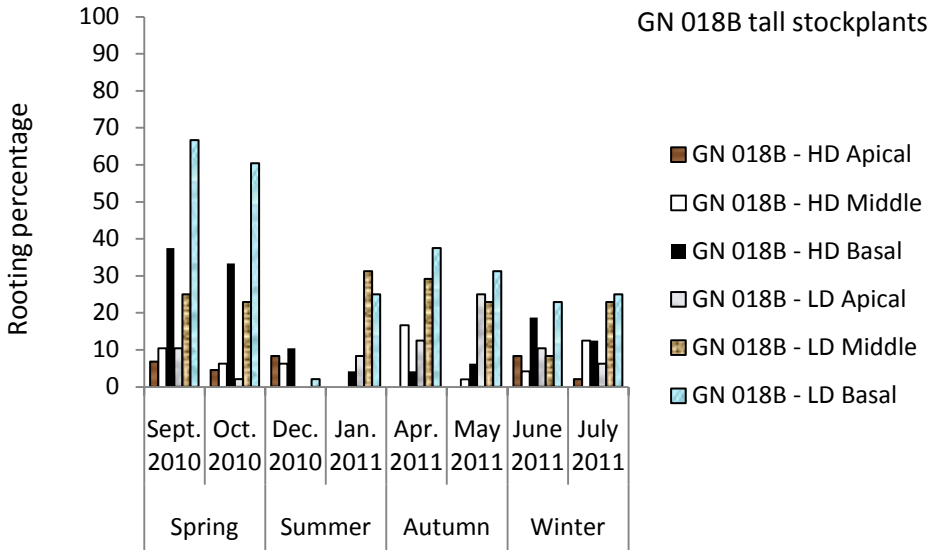
The position of cuttings on stockplants was significantly different at high and low planting densities except in December 2010 (HD: $p = 0.800$; LD: $p = 0.297$), April (HD: $p = 0.133$) and May 2011 (HD: $p = 0.245$) (Table 2.15; Fig. 2.12). In September, basal cuttings had high rooting rates of 80.7% compared to 46.4% and 26.0% rooting frequency for middle and apical cuttings respectively (Table 2.11). Therefore, apical, middle and basal cuttings have different rooting rates, with higher rooting frequency on basal cuttings despite seasonal variations (Fig. 2.12; Table 2.12). As discussed later, the morphological and developmental changes of stockplants as well as successive harvests usually had great impact on the number of basal cuttings than middle and apical cuttings (Fig. 2.2). In this regard, the rooting rates of basal cuttings also decreased over season. Although rooting of PP 2107 was high in July at high planting density, its basal cuttings had lower rooting frequency as compared to rooting rates of September, presumably because of successive harvests.

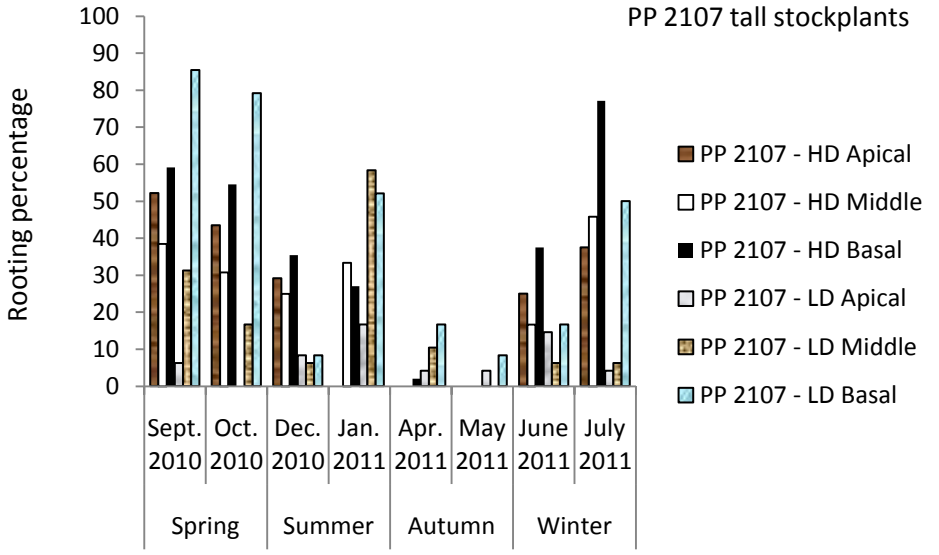
Table 2.11: Overall effect of position of cuttings on stockplants and planting density on GN clone rooting rates over different seasons.

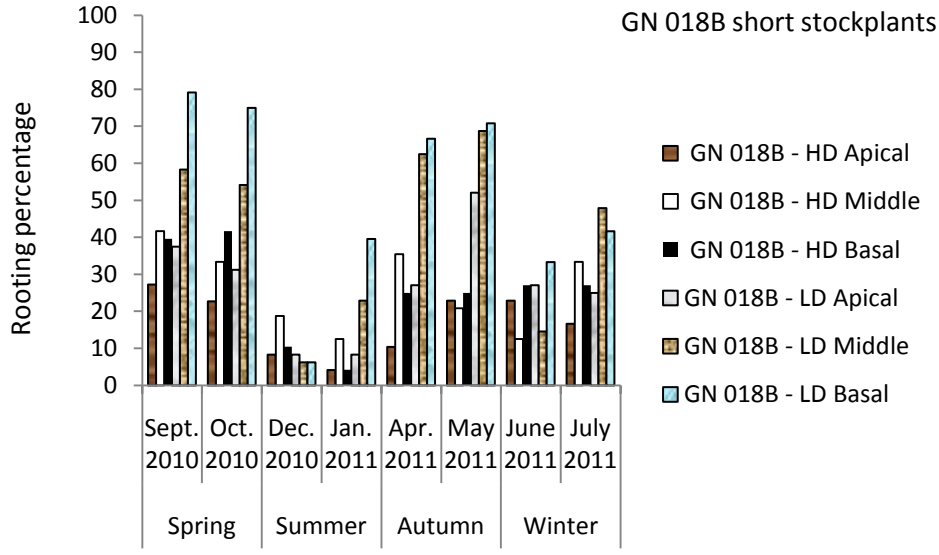
Season	Month/year	High Density			Low Density		
		Apical	Middle	Basal	Apical	Middle	Basal
Spring	Sept.10	42.3±3.8^{a}	45.8±3.8^{a}	47.9±3.8^{a}	26.0±2.9^{a}	46.4±2.9^{b}	80.7±2.9^{c}
	Oct.10	36.4±3.3^{a}	33.5±3.3^{a}	55.6±3.3^{b}	18.8±3.6^{a}	39.1±3.6^{b}	74.5±3.6^{c}
Summer	Dec.10	28.6±3.9^{a}	31.8±3.9^{a}	30.2±3.9^{a}	16.7±4.5^{b}	5.2±4.5^{a}	16.1±4.5^{b}
	Jan.11	4.2 ±2.5^{a}	17.1±2.5^{b}	17.7±2.5^{b}	15.6±5.6^{a}	35.9±5.6^{b}	40.1±5.6^{b}
Autumn	Apr.11	4.7 ±4.7^{a}	13.5±4.7^{b}	14.6±4.7^{b}	14.1±2.7^{a}	31.2±2.7^{b}	36.5±2.7^{b}
	May11	8.9 ±2.3^{a}	9.9±2.3^{a}	8.3 ±2.3^{a}	24.0±3.8^{a}	27.1±3.8^{a}	30.7±3.8^{a}
Winter	Jun. 11	29.1±0.2^{b}	22.9±0.2^{a}	42.1±0.2^{c}	23.9±0.2^{b}	17.7±0.2^{a}	34.8±0.2^{c}
	Jul. 11	32.3±2.6^{a}	46.3±2.6^{b}	52.6±2.6^{c}	16.1±7.6^{a}	31.2±7.6^{a}	46.3±7.6^{b}

a, b = mean separation within rows, Tukey's HSD test, ± S.E. ($p \leq 0.05$, n = 576 for high and low planting densities, respectively).

Although rooting frequency of PP 2107 saplings was higher when stockplants were maintained at high planting density, position of cuttings on stockplants did not have a significant effect on callus formation and root architecture (Fig. 2.10; 2.13). The influence of position was important on GN 018B rooting frequency in spring and summer, but not significant in autumn as basal and middle cuttings showed similar rooting frequencies (Fig. 2.12; Table 2.12). Thus, with successive harvests, middle and basal cuttings developed similar rooting rates, like in April and May 2011 position was not significant different (Fig. 2.12).


GN 018B tall stockplants
Rooting percentage
100
90
80
70
60
50
40
30
20
10
0
Sept. 2010
Oct. 2010
Dec. 2010
Jan. 2011
Apr. 2011
May 2011
June 2011
July 2011
Spring
Summer
Autumn
Winter
GN 018B - HD Apical
GN 018B - HD Middle
GN 018B - HD Basal
GN 018B - LD Apical
GN 018B - LD Middle
GN 018B - LD Basal


PP 2107 tall stockplants
Rooting percentage
100
90
80
70
60
50
40
30
20
10
0
Sept. 2010
Oct. 2010
Dec. 2010
Jan. 2011
Apr. 2011
May 2011
June 2011
July 2011
Spring
Summer
Autumn
Winter
PP 2107 - HD Apical
PP 2107 - HD Middle
PP 2107 - HD Basal
PP 2107 - LD Apical
PP 2107 - LD Middle
PP 2107 - LD Basal


GN 018B short stockplants
Rooting percentage
100
90
80
70
60
50
40
30
20
10
0
Sept. 2010
Oct. 2010
Dec. 2010
Jan. 2011
Apr. 2011
May 2011
June 2011
July 2011
Spring
Summer
Autumn
Winter
GN 018B - HD Apical
GN 018B - HD Middle
GN 018B - HD Basal
GN 018B - LD Apical
GN 018B - LD Middle
GN 018B - LD Basal

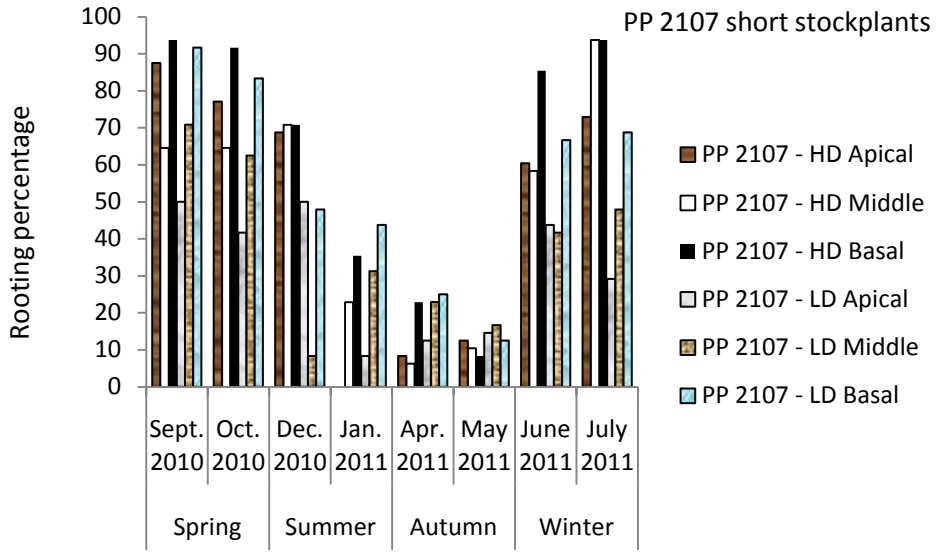


Fig. 2.12. Overall rooting rates of apical, middle and basal shoots of tall and short *E. grandis* x *E. nitens* (GN) clones maintained at high and low planting densities over season.

Table 2.12: Levels of significance on the impact of high and low planting densities, position of cuttings on stockplants and size on overall rooting rates of GN clones (HD: High planting density and LD: Low planting density).

Season	Month	*P - values*													
		Clone		Position		Size		Clone x position		Clone x size		Position x size		Clone x position x size	
		HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD	HD	LD
Spring	Sept. 10	<.001	0.394	0.039	<.001	<.001	<.001	0.008	0.339	0.096	0.204	0.012	<.001	0.304	0.145
	Oct. 10	<.001	0.589	<.001	<.001	<.001	<.001	0.005	0.584	0.04	0.216	0.036	<.001	0.088	0.412
Summer	Dec. 10	<.001	0.004	0.8	0.297	0.004	0.005	0.713	0.998	0.185	0.716	0.312	0.329	0.605	0.762
	Jan. 11	<.001	0.05	0.016	0.005	0.621	0.172	0.238	0.899	0.013	0.093	0.154	0.225	0.096	0.712
Autumn	Apr. 11	0.053	0.002	0.133	0.008	0.002	0.011	0.188	0.848	0.846	0.679	0.859	0.491	0.552	0.65
	May 11	0.003	<.001	0.245	0.039	<.001	<.001	0.119	0.097	0.511	0.179	0.14	0.012	0.304	0.054
Winter	Jun. 11	<.001	0.463	0.037	0.058	0.007	0.004	0.391	0.899	0.988	0.093	0.584	0.225	0.877	0.712
	Jul. 11	<.001	0.716	0.014	0.02	0.001	0.004	0.037	0.3	0.019	0.647	0.081	0.761	0.258	0.942

Analyses were performed using Two Way ANOVA after the data was transformed using arcsine transformation ($p \leq 0.05$, n = 576 cuttings at high planting density and low planting density separately).

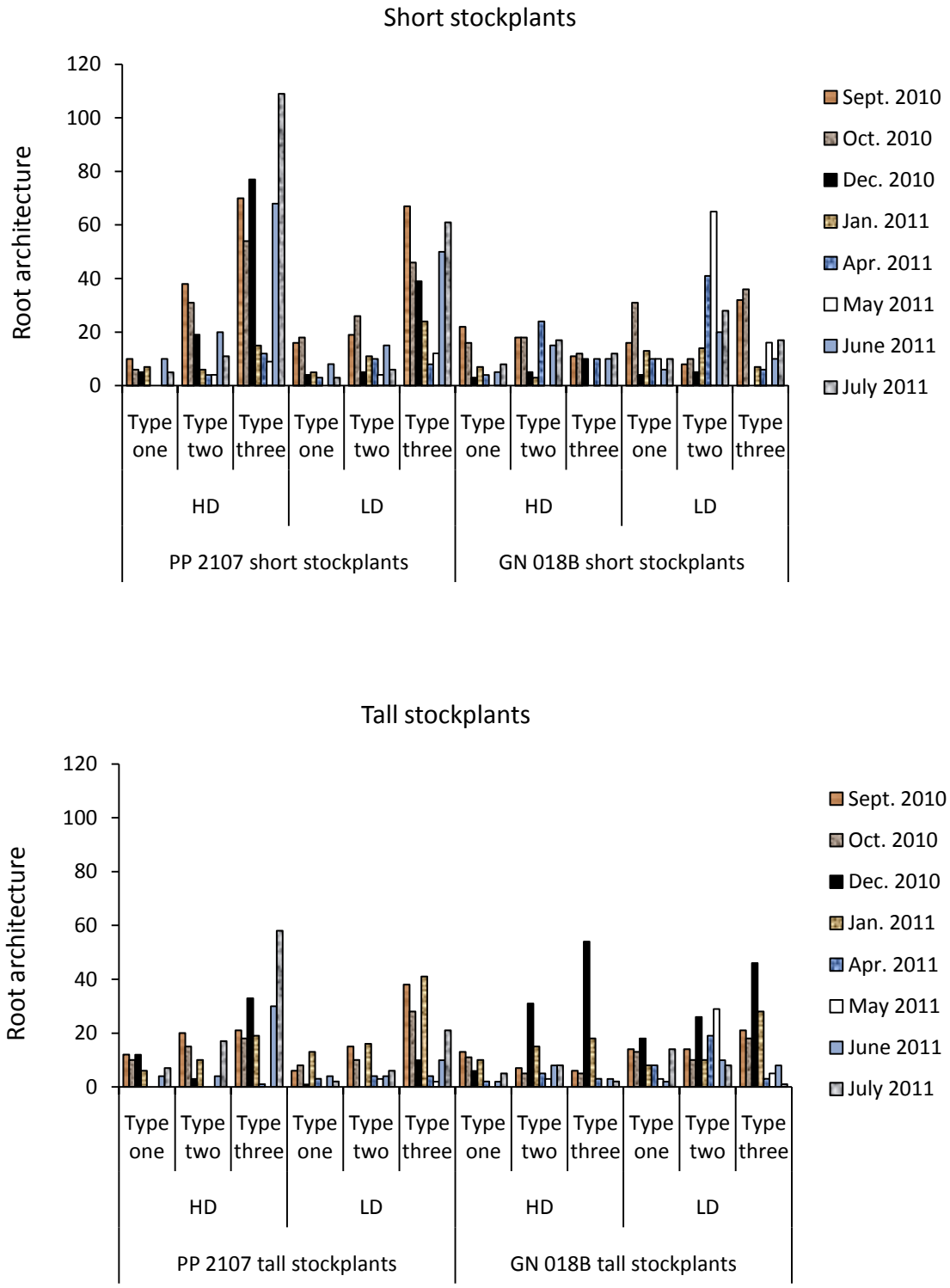


Fig. 2.13. Overall root types (architecture) developed by *E. grandis* x *E. nitens* between high and low planting densities at the end of the root formation period.

2.4. DISCUSSION

The aim of this study was to investigate the effect of management practices on the yield and subsequent rooting of cuttings of difficult-to-root vs. easy-to-root *E. grandis* x *E. nitens* (GN) clones under commercial nursery conditions. The specific objectives were (1) to determine whether the size of stockplants, planting density and season affected coppice yields and rooting frequency, (2) to determine whether rooting differences between GN 018B (difficult-to-root) and PP 2107 (easy-to-root) is dependent on position of cuttings on stockplants and seasonal variations, and (3) to ascertain whether stockplant-pathogen interactions affect the rooting rates of GN clones. Studies have shown varying propagule productivity in eucalypt clones per area per year, with production ranging from 114 cuttings to 25500 cuttings per m²/ year, depending on the species, spacing and clonal hedging systems (Campinhos and Ikemori, 1983; Carvalho *et al.,* 1991; Higashi *et al.,* 2000; Campinhos *et al.,* 2000; Aracruz Guaiba (unpublished)). However, production in the nurseries relies on how successful the produced cuttings can form roots. Similarly, there is a trade-off between the spacing density of stockplants and the yield and subsequent rooting efficiency of cuttings (de Assis *et al.,* 2004).

There are many cold-tolerant hybrids, and for which little or no research into the factors affecting their rooting potential has been undertaken. Rooting frequency of cuttings usually differs due to position of cuttings on stockplants (Paton *et al*., 1970; Wilson, 1993; Hartman *et al.,* 2002), size of stockplants (de Assis*et al.,* 2004) and season (Maile and Nieuwenhuis, 1996; Wilson, 1999). Further, stockplants morphology and development usually changes with successive harvests. Because little is known about how changes in the morphology of stockplants affect the rooting ability of subsequent cuttings, this study attempted to investigate the impact of spacing density, size of stockplants and the position of cuttings on stockplants, on the rooting capacity of GN cuttings. Many studies on *Eucalyptus* rooting rates have focused on the impact of the size of mini-cuttings on adventitious rooting rates during the root formation period. The use of mini-cuttings is part of a standard propagation practice at Sunshine Seedlings Services nursery, where GN stockplants are maintained at high planting density (100 stockplants/1.5 m^{-2}) with an average rooting frequency of 26.7% and 38.9% of GN 018B and PP 2107 respectively (Table 3.1) (Pollard *pers. com*.). However, little has been reported on the impact of the

seasons and management of stockplants on rooting rates of GN clones under commercial operations.

It is likely that management of stockplants would have an impact on the yield and subsequent rooting of GN clones. Although not significantly different, PP 2107 produced more cuttings than GN 018B, in particular at low planting density (Tables 2.5; 2.6), suggesting that the differences in coppice production may vary between the clones. Similarly, despite seasonal influences, the PP 2107clone had higher rooting rates when compared to GN 018B, expect during the periods of fungal infestations in autumn. PP 2107 stockplants maintained at high planting density had rooting frequencies of 70.1%, 63.3%, 60.5% and 50.0% in July, September, October and December respectively (Fig. 2.11; Table 2.9). GN 018B stockplants maintained at low planting density also showed rooting rates of 39.2%, 45.1%, 46.2% and 41.1% in April, May, September and October respectively (Fig. 2.11; Table 2.9). At Sunshine nursery, the reported rooting rates for PP 2107 were 58.4%, 46.8%, 50.2% and 32.9% in July, September, October and December respectively and 34.2%, 36.9%, 35.8%, 35.8% for July, September, October and December respectively for GN 018B (Fig. 2.1; Table 2.1). Thus, rooting rates of GN 018B and PP 2107 documented at Sunshine nursery were lower than rooting rates of GN 018B and PP 2107 reported in the present study depending on spacing density. However, low rooting frequency of PP 2107 cuttings were observed in summer and autumn following fungal infestations (Table 2.9), and nursery rooting records were higher than rooting frequency reported in this study (Table 2.1). In contrast, GN 018B maintained at low planting density increased its rooting frequency following fungal infestations in summer and autumn (Table 2.9), and was higher that rooting frequency reported in the nursery. Although PP 2107 rooting rates were lower in autumn, its rooting frequency improved in winter and was higher than GN 018B rooting rates.

Cuttings taken from three year old *E. nitens* stockplants have shown 56% and 30% rooting rates in September and March respectively (Maile and Nieuwenhuis, 1996), suggesting a seasonal influence on rooting. In contrast, Tibbits *et al.* (1997) showed that rooting frequency of *E. nitens* was high in winter and low in summer, although rooting frequency declined over time. Aimers-Halliday *et al*. (1999) determined that *E. grandis* x *E. nitens* (GN) hybrid behave more like the *E. grandis* parent than the *E. nitens* parent in its ability to coppice and produce saplings. Presumably, GN 018B in this study may have inherited more *E. nitens* than *E. grandis* genes, because it exhibited lower rooting rates than

PP 2107. Similarly, it could also be speculated that high coppicing and high rooting rates of PP 2107 may have been inherited from the *E. grandis* parent.

High callus formation was observed during the season of high rooting rates, and GN 018B developed more calli than PP 2107 at the end of the root formation period (Table 2.10). Murugan (2007) observed that high rooting rates of a GN clone deemed difficult-to-root correlated with high callus formation and were influenced by season. As GN 018B and the GN clone in Murugan's (2007) study were difficult-to-root clones and developed more calli, callus formation may be associated with difficult-to-root clones (Wilson 1988). This study has also speculated that stockplants at low planting density were exposed to more light and may have accumulated more fertiliser than stockplants at high planting density. More fertiliser accumulation is associated with high cytokinin, thus high sprouting (van der Werf and Nagel, 1996). It is not known how fertiliser differences at the two planting densities contributed to differences in rooting rates and callus formation on GN clones.

In addition to seasonal variation on rooting rates, high temperatures and high humidity of summer stimulated high growth of stockplants although more harvests were recorded in spring. Less number of harvests recorded in summer was mainly due to the impact of fungal infestations which degraded the morphology and development of stockplants. Following these infestations, additional harvests were not recorded as the harvested coppices were below the nursery propagation standards. Thus, it is more likely that the presence of *Quambalaria eucalypti* (*Sporothrix eucalypti*) and grasshoppers (Acrididae) infestations degraded the development and physiology of stockplants. In support of this interpretation, studies on GN hybrids in a nursery in KwaZulu-Natal have shown that these hybrids are susceptible to diseases which cause dieback of young shoots and leaf blight (Coutinho *et al*., 2002). Furthermore, susceptibility to fungal infestations was different between the two clones as rooting rates of PP 2107 decreased significantly in April and May (autumn), suggesting that GN 018B may have high resistance to fungal disease (Fig. 2.11; Table 2.9). In KwaZulu-Natal nurseries, Coutinho *et al*. (2002) documented that pathogens on ramets of *E. grandis* x *E. nitens* (GN) hybrid clone may reduce its rooting ability. In that study, Coutinho *et al*. (2002) also showed that the levels of susceptibility and resistance among *E. grandis* clones were significantly different, suggesting that such factors should be used for the selection of tolerant material.

A roughly exponential decrease in rooting rates of GN clones from spring to summer at high and low planting density was observed in this study (Fig. 2.10; Table 2.10). At low planting density, great improvement of rooting frequency was observed on cuttings from GN 018B but contrasting effects were observed on cuttings from PP 2107. This observation suggested that the differences in light intensity on stockplants, due to spacing density, could have caused variations in rooting ability of GN clones. Experimental evidence under *in vitro*tissue culture conditions have shown different light effects on *E. globulus* (difficult-to-root species) and *E. saligna* (easy-to-root species), suggesting that light induces changes in the metabolism of cuttings, and thus producing different rooting responses (Fogaça and Fett-Neto, 2005). Two points can be inferred from the findings of those experiments when compared to the results presented in this study. Firstly, *E. globulus*, just as GN 018B, depends on the presence of exogenous auxin to root, independent of light intensity. In contrast, in *E. saligna*, just like PP 2107, auxin promoted high rooting in the presence of light (Fogaça and Fett-Neto, 2005), although PP 2107 was reported to root without exogenous auxin application (CSIR *internal comm.*[6]). Even though the results of that experiment are not in good agreement with this study, GN 018B and PP 2107 are not pure species as compared to *E. globulus* and *E. saligna.* Direct comparisons are difficult because many studies on rooting usually evaluate responses of cuttings to light, plant growth regulators or nutrition regime during the root formation period. However, little is known on the rooting responses of *Eucalyptus* hybrid cuttings due to light effects on stockplants. This study has shown that the planting density of stockplants was critical in influencing rooting ability of difficult-to and easy-to-root GN clones. Therefore, light intensity on stockplants influenced GN rooting rates in the presence of exogenous auxin, suggesting that the two GN clones be maintained at different spacing clonal-garden.

The observed growth vigour was associated with the spring season where a higher number of cuttings were produced than in summer. Other conceivable explanations are that stockplants were planted in autumn (2010) and the first experimental coppice was harvested at the end of winter dormancy (spring). The number of available material for cuttings decreases during winter because of cold temperatures (Pollard, *pers. comm.*[7]).

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During dormancy, chilling temperatures are associated with changes in nucleic acids, proteins, polyamines, amino acids, organic acids and carbohydrates content which may be related with bud break (Wang and Faust, 1987). Carbohydrates are the main source of the metabolism changes that occurred during the dormant period and for spring sprouting (Flore and Layne, 1996; Bonhomme *et al.,* 2005).

Although short stockplants produced fewer cuttings, those cuttings had better rooting rates as compared to cuttings harvested from tall stockplants (Fig. 2.7; Table 2.9). Furthermore, significant differences were observed at low planting density, suggesting that planting density (light intensity) and size of stockplants impacted on the differences observed in cuttings production of the two GN clones. Reports in the literature have indicated that the spectral quality of light has a major effect on plant growth, development and morphology (Morgan and Smith, 1981; Morgan *et al.,* 1985; Moe, 1988; Warrington *et al.,* 1989; Newton *et al.,* 1996). During growth of a number of tree species, including *E. grandis*, light intensity was found to influence aspects of cutting morphology, such as stem length and specific leaf area of stock plants (Leakey and Storeton-West, 1992; Hoad and Leakey, 1992). In the present study, stockplants planted at low density (both tall and short) received more light than those in the high planting density, and produced more cuttings which usually had short internodes. Furthermore, the presence of an apical dominant sprout was usually observed on cuttings maintained at high planting density whereas such dominance was not observed at low planting density , and in particular on short stockplants. Similar responses were reported in *Pinus radiata* (Washington *et al.,* 1989) and *Eucalyptus grandis* (Hoad and Leakey, 1992) showing a relative increase in height of the dominant shoot relative to the other shoots due to lower red: far-red ratios. Further, studies on woody plants have shown that branch growth is determined by the relative competitive abilities for assimilates. High light intensity could increase growth of lateral shoots either for more photosynthesis and more available assimilates, changes in hormone production or both, if apical control of the parent shoot is removed (Wilson, 2000). Thus, because of interactions between light intensity and stockplants due to spacing density, it is possible that endogenous factors play a role in sprouting.

High growth observed at low planting density could be attributed to more cytokinins in the stockplant tissue. Cytokinins were shown to interact with different phytohormones such as auxins (Crowell *et al*., 1990), ethylene (Simmons *et al.,* 1992), and abscisic acid (Izhaki *et al.,* 1996). Interactions were shown to exist between cytokinins and light (Lerbs *et al.,*

1984), sugars (Sheen *et al.,* 1999) and pathogen infection (Memelink *et al.,* 1987). Bollmark and Eliasson (1990) reported that a cytokinin-like compound induced under high light intensity was related to high rooting of difficult-to-root *Picea abies* cuttings. Tall and short stockplants in the low planting density most likely received more fertiliser than plants maintained at high planting density. Van der Werf and Nagel (1996) suggested that low nitrogen supply as under high planting density, leads to low cytokinin production and decreases rate of export from the roots to the shoot, resulting in changes in carbon allocation . With regards to shoot and root mass, root dry mass was higher at high planting density than at low planting density. Usually, plants invest more biomass in shoots than in roots when grown at high nitrogen supply whereas the opposite reaction is observed at low nitrogen supply (van der Werf, 1996). Although there were no signs of nitrogen deficiency on stockplants under high planting density, the present study has shown the role of spacing density on cuttings production and growth of GN stockplants.

The pattern of rooting efficiency of cuttings is regulated by environmental and endogenous factors (da Rocha Corrêa and Fett-Neto, 2004) and auxins seem to be the main class of regulatory molecules acting on the process (Blakesley, 1994). Studies on *E. grandis* and *E. nitens* have reported that each species has its own requirements of exogenous auxins to promote rooting (Maile and Nieuwenhuis, 1996). In *E. nitens,* rooting rates varied from 30% to 56.3% when cuttings were treated with 0.8% to 0.2% IBA concentrations. However, similar rooting rates were observed in *E. grandis* when cuttings were treated with 0.2% to 1% IBA concentrations (Maile and Nieuwenhuis, 1996). Although the present study did not investigate the impact of auxin concentrations on GN clones rooting rates, there were indications that GN 018B and PP 2107 may require different concentrations of auxin treatments.

The observed high rooting rates on basal cuttings of GN clones was explained based on seasonal variations, number of harvests, planting density and the size differences of stockplants (Fig. 2.12). Studies on *E. camaldulensis* have reported that middle and basal cuttings root better than tip cuttings (Bachelard and Stowe, 1963). Similarly, basal cuttings of *Osyris lanceolata* set in winter and spring were shown to root better than apical cuttings and cuttings set in other seasons of the year (Teklehaimanot *et al.,* 2004). Although our planting density treatments caused differences in rooting responses between the two clones, PP 2107 maintained at high planting density usually had higher rooting rates for apical, middle and basal cuttings. However, at low planting density, the two clones showed

higher rooting frequency from basal cuttings (Fig. 2.12). From personal observations, shorter stockplants yielded cuttings with shorter internodes (more nodes) than taller stockplants and apical dominance was not usually observed in particular at low planting density. Although this study did not investigate the impact of the number of nodes on the rooting potential of cuttings, a possible hypothesis is that the number of nodes may influence rooting of GN cuttings. In cuttings of *Populus* x *deltoids* 'Walker' and *Populusjackii* 'Northwest'Schroeder and Walker (1991) found that the number of buds varied in relation to stem position and clone. Basal cuttings had a greater number of buds than mid or distal cuttings and that indicated that bud development was related to rooting (Schroeder and Walker, 1991). Although *Populus* clones are not directly comparable to eucalypt, that study indicated the role of node and position of cuttings on stockplants in relation to rooting efficiency. Furthermore, Wilson (1988) reported that the presence of buds on *Eucalyptus grandis* cuttings may produce more auxins, which in turn, facilitate the rooting process. Poor rooting of cuttings from the apical shoot may be related to bud development (powerful sinks for assimilates (Wilson 1988)) and hormonal levels (Smith and Wareing, 1972). Thus, there are a large number of factors, together with their interactions, contributing to the differences in rooting of cuttings (Wilson, 1994).

Differences in coppice yields and rooting rates of GN 018B and PP 2107 were observed at the two planting densities, size of stockplants and position of cuttings on stockplants. If light intensity on stockplants determined auxins content this caused the mobilisation of soluble sugars that activity should be reflected on the distribution and number of roots in the mung bean bioassay. A different number of roots would be expected to form in relation to planting density, the size of stockplants and position of cuttings on stockplants. That possibility is considered in the next chapter.

CHAPTER 3: ANALYSIS OF ENDOGENOUS SOLUBLE SUGARS AND ROOTING STIMULANTS

3.1. INTRODUCTION

Carbohydrates are synthesized in source leaves and translocated to sink tissues in most species in the form of sucrose to sustain metabolism and growth, or to be stored as starch (Roitsch and Gonzalez, 2004). Sugars are the primary products of photosynthesis and the source of materials from which plants make proteins, polysaccharides, oils and woody materials. Sugars modulate many vital processes that are also controlled by plant growth regulators during plant growth and development (León and Sheen, 2003). The disaccharide sucrose and the cleavage products glucose and fructose are the central molecules for carbohydrate translocation, metabolism and sensing in higher plants (Roitsch and Gonzalez, 2004). Sucrose plays a role as a messenger of information on the assimilate status of both sinks and sources (Ferrar, 1992).

The size of stockplants, planting density and position has different growth and developmental effects on coppice yields and rooting frequency of *E. grandis* x *E. nitens* clones (Chapter 2). Changes in the growth activity of stockplants have also been correlated with seasonal variation in rooting success due to sensitivity to or variation of endogenous auxin levels (Howard, 1991). Furthermore, studies have suggested that the fertilizer concentration applied to stockplants at high/low planting density generates interactions of soluble sugars with plant growth regulators and light (van der Werf and Nagel, 1996; Roitsch and Ehneß, 2000), which may explain the sprouting and rooting responses.In addition, cytokinins play a major role in the regulation of various processes associated with active growth and thus an enhanced demand for carbohydrates (Roitsch and Ehneß, 2000). Auxin and cytokinin antagonistically affect the expression of genes in the initiation of lateral roots in the pericycle (Vanneste *et al.*, 2005) and their ratios promote high or low rooting of cuttings (Hartman *et al.*, 2002).

Certain indication of mechanisms by which the size of stockplants, planting density and position of cuttings impact on the rooting rates of GN clones have been emanated in

Chapter 2. It has been proposed that GN 018B and PP 2107 stockplants require different planting densities which affect how much light individual stockplants received in order to achieve high rooting rates. The light intensity supplied to stockplants may affect soluble sugar concentration and auxin metabolism or sensitivity thereby promoting or inhibiting coppice yield and rooting rate. While evaluating coppice yields and rooting frequency of GN 018B and PP 2107 cuttings, it was frequently observed that PP 2107 clone produced high number of cuttings as well as high rooting rates at high and low planting densities respectively. PP 2107 clone was also severely affected by fungal diseases in summer because of its high growth, and this impacted negatively on its development and rooting rates as compared to GN 018B clone. This prompted an investigation into evaluating the management effects of stockplants on soluble sugars concentration and root initiation and growth, towards providing insight into size of stockplants and planting density needed to optimise rooting frequency in the nursery.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Eucalyptus grandis x *E. nitens* (GN) cuttings harvested from stockplants maintained at high and low planting densities in mini-clonal hedges located at Sunshine Seedling nursery (29º 31.709'S; 30º 28.583'E), KwaZulu-Natal, South Africa were used for all experiments. All experiments used separately freeze-dried tissue samples harvested from apical, middle and basal shoots of tall and short GN 018B and PP 2107 stockplants maintained at high and low planting densities. At each harvest (for rooting experiments), some cuttings were freeze-dried for the analysis of soluble sugars and for the mung bean bioassay. These experiments were repeated in spring (September 2010) and in summer (December 2010).

3.2.2. Experimental techniques

3.2.2.1. Extraction and analysis of soluble sugars

Freeze-dried material (0.10 g) of *E. grandis* x *E. nitens* leaves and stems were mixed with 10 mL 80% (v/v) ethanol and homogenized for 1 minute using an Ultra-Turrax®. Thereafter, the mixture was incubated in an 80ºC water bath for 60 minutes. The mixture was then kept at 4ºC overnight (16 hours) to extract the soluble sugars. After centrifugation at 10 000 rpm for 10 min at 4ºC, the supernatant was filtered through glass wool and dried in a vacuum concentrator. Dried samples were resuspended in 2 mL ultra-pure water, and then filtered through a 0.45 µm nylon filter. Sugar concentrations were determined according to Liu *et al.* (1999) using an isocratic HPLC system (LC-20AT; Shimadzu Corp., Kyoto, Japan) equipped with a refractive index detector (RID-10A; Shimadzu Corp.) and a 300 mm × 7.8 mm Rezex RCM-Monosaccharide column (8 µm pore size; Phenomenex®, Torrance, CA, USA). The concentrations of individual sugars were determined by calibrating the diluted 25 mg of authentic sugar standards (0-100% dilution) of the pick area (P A) in the HPLC.

3.2.2.2. Auxin standard curve

A mass of 33 mg IBA was diluted into 16.24 mL of 98% ethanol to make a 10^{-2} M IBA stock solution. Thereafter, 2 mL of the stock solution (10^{-2} M IBA) was diluted into 18 mL of distilled water to form the 10^{-3} M IBA standard. A series of dilutions (10^{-4} M IBA, 10^{-5} M IBA, 10^{-6} M IBA and 10^{-7} M IBA) were prepared in the same manner. Mung bean seeds (*Vigna radiata* L. Wilczek) were sown into trays filled with moist vermiculite and germinated under continuous light (32.1 µmol m^{-2} s^{-1}) at 27°C. After 9 days the hypocotyls including two primary leaves, were cut to a length of 7-9 cm, and the cotyledons removed (Taylor and van Staden, 1996). After four hours (dipping period), cuttings were rinsed and placed in vials containing distilled water. These were placed randomly in trays and returned to the growth cabinet for a period of 9 days. The root 'intensity' for each hypocotyl cutting was then recorded (Fig. 3.1).

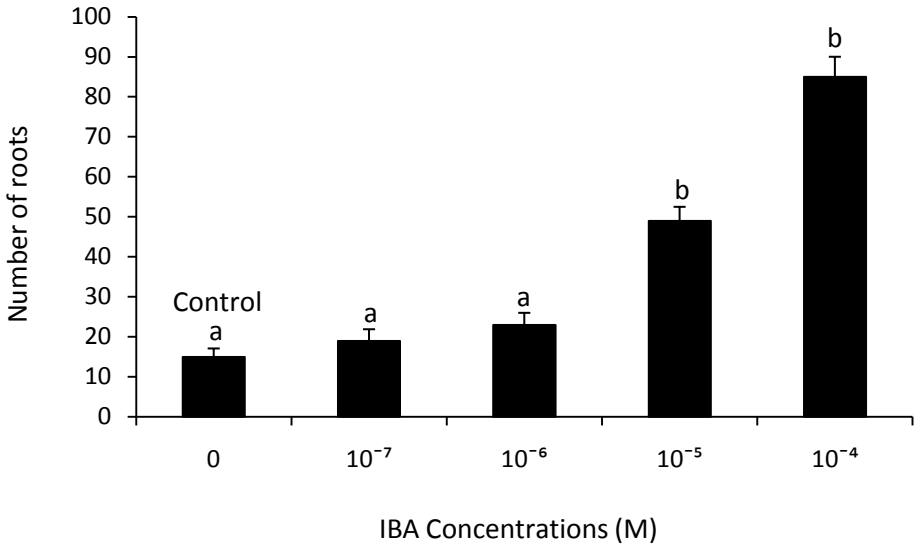

Fig. 3.1: Effect of IBA concentration on root initiation and growth of *Vigna radiata* hypocotyl cuttings. Bars bearing different letters are significantly different at $P \leq 0.05$.

3.2.2.3. Mung bean bioassay

A 0.5 g sample of ground freeze-dried tissue was placed into a plastic test tube and homogenized with 40 mL 80% ethanol using an Ultra-Turrax for 30 seconds. The sample was then transferred into a 250 mL volumetric flask, covered and left overnight (16 hours) at 4°C. The extract was transferred back again into test tubes, centrifuged at 10 000 rpm for 10 minutes and the precipitates were discarded. The supernatant was filtered and gently decanted into rotary evaporator flasks. The extract was dried at 35-40 °C and taken up in 4 mL of 0.1 M acetic acid and applied to a preconditioned column combination consisting of a polyvinylpyrrolidone (PVP) as well as a DEAE-Sephadex (Pharmacia) column and a C18 SEP-PAK cartridge (Waters). The PVP column retained the phenols and other impurities.

To prepare these columns, insoluble PVP was washed twice with deionized water to remove the fine particles and DEAE-Sephadex (0.5g) was washed twice with 0.1 M ammonium acetate (pH 8). PVP (2.5 g) and DEAE-Sephadex (0.5 g) were placed in separate 5 mL syringes equipped with a disc of filter paper on the bottom. The DEAE-Sephadex column was attached below the PVP column and equilibrated with 20 mL 0.1 M acetic acid. Thereafter, a SEP-PAK cartridge was activated by washing with 8 mL of 100% methanol, equilibrated with 4 mL 0.1 M acetic acid and attached below the DEAE-Sephadex column to retain cytokinins. The extract loaded on to this system was eluted with

4 mL 0.1 M acetic acid followed by 4 mL 20% methanol to remove polar impurities and compounds were eluted with 4 mL40% methanol. Aliquots of the extracted compounds were pipetted into short vials, evaporated under vacuum and kept frozen.

The freeze-dried sample extract was diluted with 10 mL100% ethanol followed by 4 mL distilled water in the dark. The solution was mixed using a Varlex®. Thereafter, aliquots of the solution 1 mL and 0.1 mL were transferred into separate vials. In the 1 mL vial, an additional 1.8 mL of 100% methanol was added, followed by 17.2 mL of water to make up a total volume of 20 mL. Also, in the vial containing 0.1 mL stock solution, an additional 1.98 mL of 100% methanol was added followed by 17.94 mL of water to totalise a final volume of 20 mL (Bertling and Bangerth, 1995).

Mung bean seeds (*Vigna radiata* L. Wilczek) were germinated (see section 3.2.2.2) and three hypocotyl cuttings were repeated three times for apical, middle and basal tissue samples and evaluated for high and low planting densities between the two sizes of the two clones. These cuttings were then placed (3-4 cm) for 4 hours into the vials containing 1 mL (100%), 0.1 mL (10%) of the stock solution and distilled water was used as the control. The stock solution was the diluted extract of DM (dry mass) of the eucalypt cuttings. These vials were kept at 26°C in the growth cabinet. After four hours (dipping period) in the diluted extract, mung bean cuttings were rinsed in distilled water and placed in vials containing distilled water. These were placed randomly in trays and returned to the growth cabined for a period of 9 days. The rooting ‘intensity’ for each hypocotyl cutting was then recorded and compared to the standard IBA curve.

3.2.3. Data collection and statistical analysis

The analysis of soluble sugars and the mung bean bioassay experiments were repeated three times in spring and in summer. The rooting ‘intensity’ of mung bean hypocotyl cuttings was recorded at the end of the experiment. At the end of the dipping period (four hours), each of the nine test tubes contained one hypocotyl cutting at 100% and 10% concentration per treatment. Each treatment was made of one position, one size, one clone and one density. Overall, one treatment had 18 cuttings (replicates) and 216 cuttings were evaluated per planting density. A total of 864 mung bean hypocotyl cuttings were assessed

in the bioassay experiments, in spring and in summer for the two planting densities (see section 3.2.2.3). Soluble sugar concentration (sucrose, glucose and fructose) was determined using GN tissue samples from apical, middle and basal shoots of tall and short stockplants maintained at high and low planting densities. Twelve treatments were evaluated in spring and in summer separately.

Statistical analyses were carried out with GenStat software package (version 13.2; VSN International, Hemel Hempstead, UK). All data were subjected to Two-Way ANOVA, and soluble sugar concentration and rooting data which were not normally distributed were log-transformed. The analyses were performed on transformed data, with means presented as non-transformed data plus standard error (S.E) values. Differences among treatments were separated by Tukey's Honestly Significant Difference (HSD) test ($p \leq 0.05$).

3.3. RESULTS

To gather more knowledge on the management effects of *E. grandis* x *E. nitens* (GN) stockplants affecting coppice yield and rooting rate, harvested cuttings were freeze-dried in spring and in summer. These cuttings were used for the extraction of soluble sugars, towards providing an insight into understanding endogenous factors impacting sprouting and coppice yield, and root initiation and growth through the mung bean bioassay. Since PP 2107 stockplants were severely affected by fungal diseases in January 2011, further harvests from the two clones were not recorded because these cuttings did not meet the nursery standards.

3.3.1. Stockplant management effects on soluble sugar concentration

Laboratory analysis of *E. grandis* x *E. nitens* (GN) tissue samples derived from tall and short stockplants revealed that short stockplants accumulated more sugars, in particular at the low planting density. Significant differences were observed at lower planting than higher planting density (Table 3.1). Further, the sucrose concentration in tissue samples was higher than glucose and fructose at low planting density, and samples from apical cuttings showed higher soluble sugars than middle and basal cuttings (Fig. 3.2). Similarly, higher soluble sugars were accumulated in spring (September 2010) than in summer (December 2010), regardless of planting density and genotype. The PP 2107 clone tissue samples showed contained higher concentration of soluble sugars and higher number of roots than the GN 018B clone, particularly in spring at high density (Fig. 3.3). However, for the GN 018B clone, high rooting rates were observed in summer whereas high sugar concentrations were observed in spring regardless of the planting density (Fig. 3.3). Although tissue samples from apical cuttings showed higher sucrose concentration as compared to middle and basal cuttings, their rooting rates in the mung bean bioassay were similar (Tables 3.2; 3.3; 3.4; 3.5).

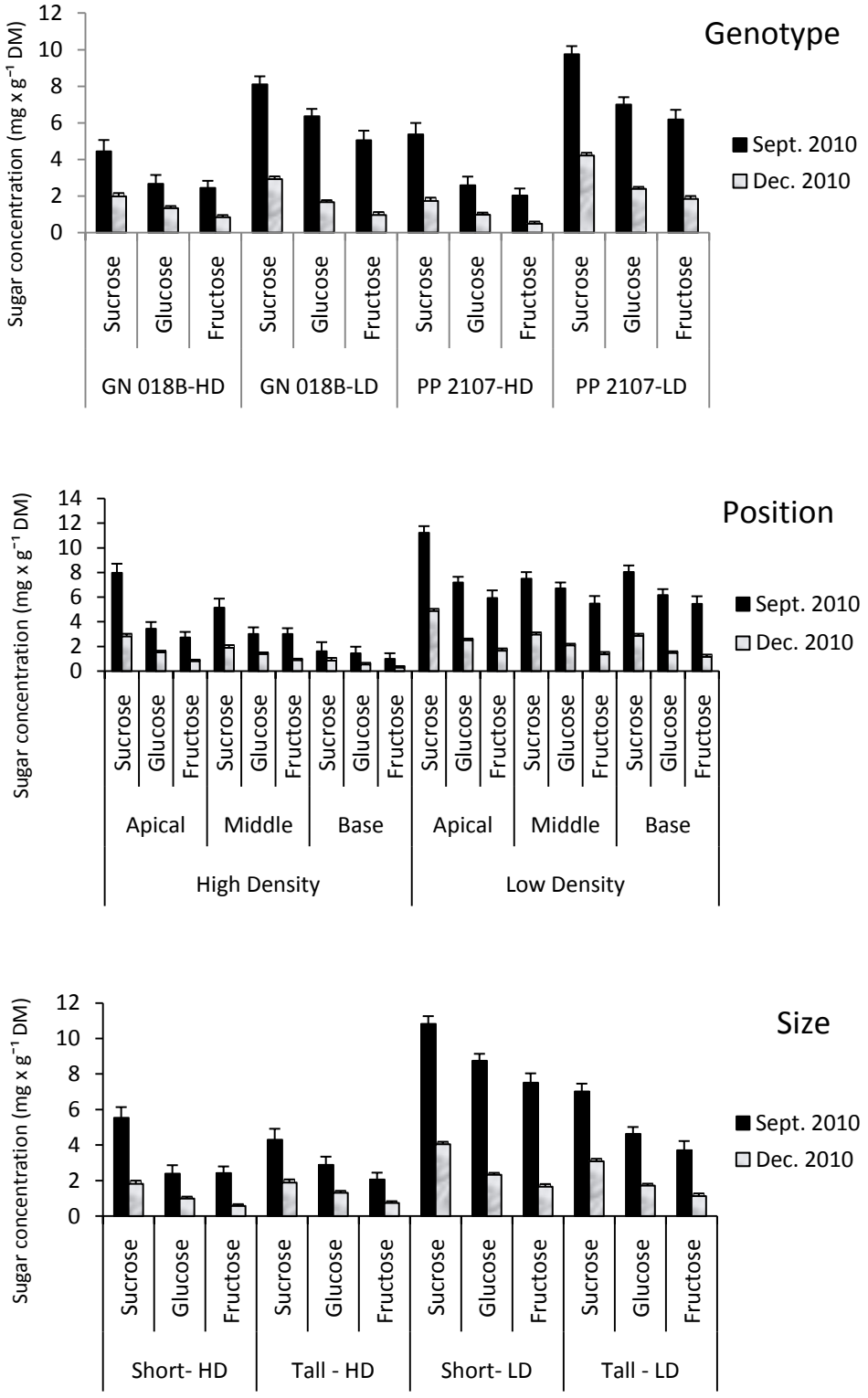

Fig. 3.2: Soluble sugar concentration of apical, middle and basal cuttings of tall (20 cm) and short (10 cm) stockplants of the GN 018B and PP 2107 clones maintained at high (HD) and low (LD) planting densities in September 2010 and December 2010. Vertical bars are ± S.E.

Table 3.1: Levels of significance in sucrose, glucose and fructose concentrations (mg x g^{-1} DM) by spacing density over season

Variation		*P* - Value						
		Clone	Position	Size	Clone	Clone × Size	Position × Size	Clone × Position × Size
HD								
Sep-10	Sucrose	0.293	<.001	0.172	0.016	0.217	0.265	0.377
Dec-10		0.337	<.001	0.792	0.846	0.018	0.950	0.017
Sep-10	Glucose	0.892	0.052	0.482	0.958	0.580	0.307	0.775
Dec-10		0.044	<.001	0.066	0.259	<.001	0.495	0.013
Sep-10	Fructose	0.449	0.012	0.532	0.037	0.833	0.715	0.984
Dec-10		0.042	0.013	0.322	0.930	0.005	0.058	0.155
LD								
Sep-10	Sucrose	0.014	<.001	<.001	0.637	0.094	0.008	0.120
Dec-10		<.001	<.001	<.001	0.362	0.110	<.001	0.007
Sep-10	Glucose	0.262	0.364	<.001	0.915	0.425	0.032	0.360
Dec-10		<.001	<.001	<.001	0.876	0.148	<.001	0.867
Sep-10	Fructose	0.135	0.833	<.001	0.042	0.470	0.024	0.399
Dec-10		<.001	0.203	0.024	0.743	0.352	0.129	0.405

Analyses were performed using Two Way ANOVA after the data was transformed using arcsine transformation ($p \leq 0.05$, n = 36 replicates evaluated in spring and summer separately). HD = High Density, LD = Low Density.

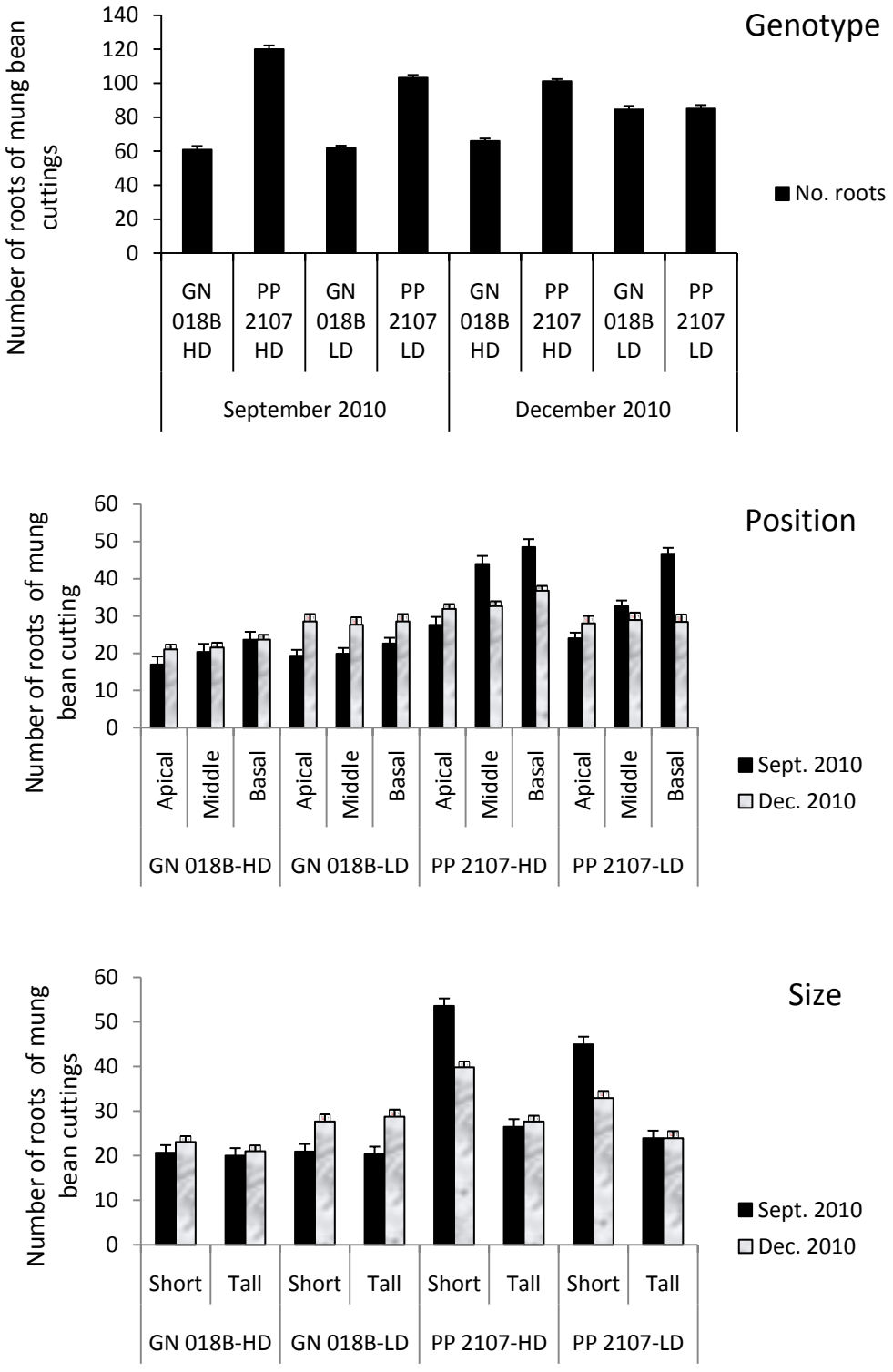

Fig. 3.3: Number of roots recorded on mung bean hypocotyl cuttings between apical, middle and basal cuttings derived from tall (20cm) and short (10 cm) GN 018B and PP 2107 clones maintained at high (HD) and low (LD) planting densities in September 2010 and December 2010.

Table 3.2. Effect of high planting density and position of cuttings on the number of roots of mung bean hypocotyl cuttings and soluble sugar concentration in GN cuttings (mg x g^{-1}DM) in September 2010 and in December 2010.

	GN 018B-HD				PP 2107-HD			
	Apical	Middle	Base	Total	Apical	Middle	Base	Total
Sept.								
No. roots	17.0±2.1^{a}	20.3±2.1^{b}	23.6±2.1^{b}	60.9±2.1^{b}	27.6±2.1^{bc}	44.0±2.1^{d}	48.5±2.1^{e}	120.1±2.1^{a}
Sucrose	5.6 ± 1.0^{b}	6.1 ± 1.0^{b}	1.5 ± 1.0^{a}	13.2±1.0^{b}	10.2±1.0^{a}	4.2 ± 1.0^{a}	1.6±1.0^{bc}	16 ± 1.0^{a}
Glucose	3.4 ± 0.8^{b}	3.2 ± 0.8^{b}	1.4 ± 0.8^{b}	8.0 ± 0.8^{a}	3.4 ± 0.8^{b}	2.8 ± 0.8^{b}	1.4 ± 0.8^{b}	7.6 ± 0.8^{a}
Fructose	2.0 ± 0.6^{a}	4.1 ± 0.6^{a}	1.2 ± 0.6^{a}	7.3 ± 0.6^{a}	3.4 ± 0.6^{a}	1.8 ± 0.6^{a}	0.7 ± 0.6^{a}	5.9 ± 0.6^{b}
Dec.								
No. roots	21.0±1.3^{a}	21.5±1.3^{a}	23.6±1.3^{a}	66.1±1.3^{b}	31.8±1.3^{b}	32.6±1.3^{b}	36.7±1.3^{a}	101.1±1.3^{a}
Sucrose	2.9 ± 0.3^{a}	2.1 ± 0.3^{a}	0.8 ± 0.3^{b}	5.8 ± 0.3^{a}	2.6 ± 0.3^{a}	1.7 ± 0.3^{a}	0.8 ± 0.3^{a}	5.1 ± 0.3^{b}
Glucose	1.6 ± 0.2^{a}	1.2 ± 0.2^{a}	0.6 ± 0.2^{b}	3.4 ± 0.2^{a}	1.4 ± 0.2^{a}	1.1 ± 0.2^{a}	0.5 ± 0.2^{a}	3.0 ± 0.2^{a}
Fructose	1.0 ± 0.2^{a}	1.1 ± 0.2^{a}	0.4 ± 0.2^{b}	2.5 ± 0.2^{a}	0.6 ± 0.2^{a}	0.6 ± 0.2^{a}	0.2 ± 0.2^{a}	1.4 ± 0.2^{b}

a, b = mean separation within rows (no. of roots) and sugars (columns) of different cutting position as well as total number of roots or sugars between clones, Tukey's HSD test, ± S.E. ($p \leq 0.05$, n = 216 cuttings per planting density/ month and n = 12 for sucrose, glucose and fructose separately).

Table 3.3: Effect of low planting density and position of cuttings on the number of roots of mung bean hypocotyl cuttings and soluble sugar concentration in GN cuttings (mg x g^{-1}DM) in September 2010 and in December 2010.

	GN 018B-LD				PP 2107-LD			
	Apical	Middle	Base	Total	Apical	Middle	Base	Total
Sept.								
No. roots	19.3±1.5^{a}	19.8±1.5^{a}	22.6±1.5^{a}	61.7±1.5^{b}	24.0±1.5^{a}	32.6±1.5^{b}	46.7±1.5^{c}	103.3±1.5^{a}
Sucrose	10.2±0.7^{a}	7.1 ± 0.7^{b}	7.0 ± 0.7^{b}	24.3±0.7^{b}	12.2±0.7^{a}	7.9 ± 0.7^{b}	9.0±0.7^{b}	29.1 ± 0.7^{a}
Glucose	6.6±0.6^{a}	6.4 ± 0.6^{a}	5.9 ± 0.6^{a}	18.9±0.6^{b}	7.6±0.6^{a}	6.9±0.6^{a}	6.4±0.6^{a}	20.9 ± 0.6^{a}
Fructose	5.1±0.9^{a}	6.2 ± 0.9^{a}	3.8 ± 0.9^{b}	15.1±0.9^{b}	6.7±0.9^{a}	4.7±0.9^{b}	7.1±0.9^{a}	18.5 ± 0.9^{b}
Dec.								
No. roots	28.5±2.0^{a}	27.6±2.0^{a}	28.5±2.0^{a}	84.6±2.0^{a}	28.0±2.0^{a}	28.8±2.0^{a}	28.3±2.0^{a}	85.1± 2.0^{a}
Sucrose	4.2 ± 0.2^{a}	2.5 ± 0.2^{b}	2.0 ± 0.2^{c}	8.7 ± 0.2^{b}	5.5 ± 0.2^{a}	3.4±0.2^{b}	3.6 ± 0.2^{b}	12.5± 0.2^{a}
Glucose	2.2 ± 0.1^{a}	1.7 ± 0.1^{b}	1.1 ± 0.1^{c}	5.0 ± 0.1^{b}	2.8 ± 0.1^{a}	2.4±0.1^{b}	1.8 ± 0.1^{c}	7.0 ± 0.1^{a}
Fructose	1.1 ± 0.2^{a}	1.0 ± 0.2^{a}	0.7 ± 0.2^{a}	2.8 ± 0.2^{b}	2.2 ± 0.2^{a}	1.7±0.2^{b}	1.6 ± 0.2^{b}	5.5 ± 0.2^{a}

a, b = mean separation within rows (no. of roots) and sugars (columns) of different cutting position as well as total number of roots or sugars between clones, Tukey's HSD test, ± S.E. ($p \leq 0.05$, n = 216 cuttings per planting density/ month and n = 12 for sucrose, glucose and fructose separately).

Table 3.4: Effect of high planting density and size of stockplants on the number of roots of mung bean hypocotyl cuttings and soluble sugar concentration (mg x g^{-1} DM) in September 2010 and in December 2010.

	GN 018B-HD			PP 2107-HD		
	Short	Tall	Total	Short	Tall	Total
Sept.						
No. roots	20.6±1.7[a]	20.0±1.7[a]	40.6±1.7[b]	53.5±1.7[a]	26.5±1.7[b]	80.0±1.7[a]
Sucrose	5.6 ± 0.8[a]	3.3 ± 0.8[b]	8.9 ± 0.8[b]	5.4 ± 0.8[a]	5.3±0.8[a]	10.7±0.8[a]
Glucose	2.3 ± 0.6[a]	3.1 ± 0.6[a]	5.4 ± 0.6[a]	2.5 ± 0.6[a]	2.6±0.6[a]	5.1 ± 0.6[a]
Fructose	2.7 ± 0.5[a]	2.2 ± 0.5[a]	4.9 ± 0.5[a]	2.2 ± 0.5[a]	1.9±0.5[a]	4.1 ± 0.5[a]
Dec.						
No. roots	23.2±1.1[a]	21.0±1.1[a]	44.2±1.1[b]	39.8±1.1[a]	27.6±1.1[b]	67.4±1.1[a]
Sucrose	1.6±0.2[b]	2.4 ± 0.2[a]	4.0 ± 0.2[a]	2.0±0.2[a]	1.4 ± 0.2[b]	3.4 ± 0.2[b]
Glucose	0.8 ±0.1[b]	1.8 ± 0.1[a]	2.6 ± 0.1[b]	1.2±0.1[a]	0.7 ± 0.1[b]	3.4 ± 0.1[a]
Fructose	0.5±0.1[b]	1.2 ± 0.1[a]	1.7 ± 0.1[b]	0.7±0.1[a]	0.3 ± 0.1[b]	3.4 ± 0.1[a]

a, b = mean separation within rows (no. of roots) and sugars (columns) of different cutting size as well as total number of roots or sugars between clones, Tukey's HSD test, ± S.E. ($p \leq 0.05$, n = 216 cuttings per planting density/ month and n = 12 for sucrose, glucose and fructose separately).

Table 3.5: Effect of low planting density and size of stockplants on the number of roots of mung bean hypocotyl cuttings and soluble sugar concentration (mg x g^{-1} DM) in September 2010 and in December 2010.

	GN 018B-LD			PP 2107-LD		
	Short	Tall	Total	Short	Tall	Total
Sept.						
No. roots	20.9±1.2[a]	20.3±1.2[a]	41.2±1.2[b]	45.0±1.2[a]	23.9±1.2[b]	68.9±1.2[a]
Sucrose	10.5±0.6[a]	5.6±0.6[b]	16.1±0.6[b]	11.1±0.6[a]	8.4± 0.6[b]	19.5±0.6[a]
Glucose	8.6 ± 0.5[a]	4.1± 0.5[b]	12.7±0.5[b]	8.8±0.5[a]	5.2± 0.5[b]	14.0±0.5[a]
Fructose	7.2±0.7[a]	2.8± 0.7[b]	10.0±0.7[b]	7.8±0.7[a]	4.5±0.7[b]	12.3±0.7[a]
Dec.						
No. roots	27.6±1.6[a]	28.7±1.6[a]	56.3±1.6[a]	32.9±1.6[a]	23.9±1.6[b]	56.8±1.6[a]
Sucrose	3.6±0.2[a]	2.3±0.2[b]	5.9±0.2[b]	4.5±0.2[a]	3.9±0.2[b]	8.4±0.2[a]
Glucose	2.1±0.1[a]	1.2±0.1[b]	3.3±0.1[b]	2.6±0.1[ca]	2.2±0.1[b]	4.8±0.1[a]
Fructose	1.1±0.2[a]	0.8±0.2[a]	1.9±0.2[b]	2.2±0.2[a]	1.5±0.2[b]	3.7±0.2[a]

a, b = mean separation within rows (no. of roots) and sugars (columns) of different cutting size as well as total number of roots or sugars between clones, Tukey's HSD test, ± S.E. ($p \leq 0.05$, n = 216 cuttings per planting density/ month and n = 12 for sucrose, glucose and fructose separately).

3.3.2.Effect of stockplant management on root initiation and growth

There were variations in root initiation and growth of mung bean hypocotyls cuttings treated with *E. grandis* x *E. nitens* tissue samples (Fig. 3.4). Root initiation and growth was high, as mung bean cuttings treated with GN tissue extracts from shorter stockplants rooted better than if supplied with extracts from taller stockplants (Fig. 3.3). The clone PP 2107 stockplants harvested in September had higher rooting potential than those taken in December (Fig. 3.3). However, the rooting potential of the GN 018B clone harvested in December from stockplants maintained at low planting density had higher rooting rates than those of September samples. Overall, the clone PP 2107 had higher rooting potential than clone GN 018B, regardless of planting density, size of stockplants and season. Samples from basal GN tissue also had higher rooting potential regardless of season and planting density than apical and middle GN tissues (Fig. 3.3).

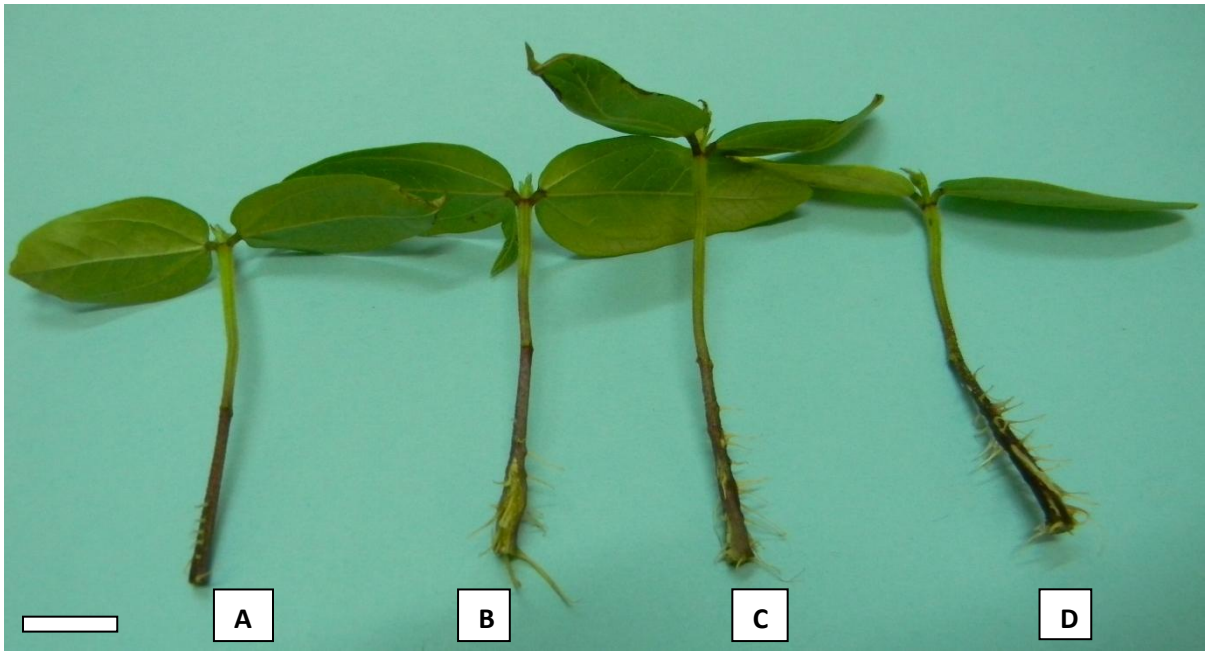

Fig. 3.4: Variations in root initiation and growth of mung bean hypocotyls cuttings treated with *E. grandis* x *E. nitens* cutting extracts (B; C; D) compared with control (water) (A). Bar = 2 cm.

However, as cutting tissue samples of GN 018B maintained at low planting density promoted high rooting rates in December, significant differences were not therefore observed (Fig. 3.3; Table 3.6). As discussed later in this study, mung bean cutting rooting potential observed in the bioassay experiments were high as the rooting rates recorded in September under the nursery conditions. Thus, the rooting rates of GN cuttings and mung bean hypocotyls cuttings recorded in September for both experiments were usually higher than the rooting rates of December. However, similar effects were not observed in December because the rooting potential of mung bean cuttings treated with tissue extracts from GN 018B clone were unexpectedly higher compared to nursery rooting in December.

Table 3.6: Levels of significance on the number of roots of mung bean hypocotyl cuttings among spacing, genotype, position, size and season (HD: High Density, LD: Low Density).

Variation	*P* - value						
	Clone	Position	Size	Clone × position	Clone × size	Position × size	Clone × position × size
HD							
Sep.10	<.001	<.001	<.001	0.003	<.001	<.001	<.001
Dec.10	<.001	0.023	<.001	0.678	<.001	0.407	0.094
LD							
Sep.10	<.001	<.001	<.001	<.001	<.001	<.001	<.001
Dec.10	0.902	0.994	0.024	0.905	0.005	0.400	0.227

Analyses were performed using Two Way ANOVA after the data was transformed using arcsine transformation ($p \leq 0.05$, n = 216 cuttings per planting density/ month evaluated separately).

3.4. DISCUSSION

The aim of this study was to evaluate the management effects of stockplants on soluble sugars concentration and root initiation and growth, towards providing insight into size of stockplants and planting density needed to optimise rooting frequency in the nursery. Studies on *Eucalyptus* rooting frequency under the nursery protocols have suggested that rooting ability of cuttings varies with position of cutting, season, size and planting density of the stockplants (Paton *et al*., 1970; Wilson, 1993; Maile and Nieuwenhuis, 1996; Wilson, 1999; Hartman *et al.*, 2002; de Assis *et al.*, 2004). Investigations were thereafter conducted in the laboratory to understand the endogenous factors influencing rooting differences of GN 018B and PP 2107 clones, particularly in September and December at high and low planting densities. In this study, endogenous IAA extracted from GN 018B clone planted at low planting density improved the rooting rates of mung bean hypocotyl cuttings. Contrasting effects were observed in the bioassay experiment when mung bean cuttings were treated with PP 2107 clone tissues maintained at low planting density in September (Fig. 3.3). Hence, at high planting density as observed in Chapter 2, high rooting frequency of mung bean cuttings treated with PP 2107 tissue was recorded.

This suggests that light intensity (planting density) on stockplant cutting tissues had a significant rooting effect on GN clones (Tables 3.2; 3.3; 3.4; 3.5). Further, the rooting rates of mung bean cuttings were different because of the origin of GN tissue samples; suggesting that the two GN clones may necessitate different planting densities (light growing conditions). These results showed similar rooting responses as the rooting frequency in Chapter 2, which were also higher in September regardless of planting density, size of stockplant and position of cuttings. However, the mung bean hypocotyl cuttings treated with GN 018B tissue extracts in December showed high rooting in particular at low planting density whereas low rooting was recorded in September. Thus, low rooting rates of GN 018B cuttings observed in December (Chapter 2) did not necessarily correspond to the rooting rates observed in the bioassay experiment of December tissue extracts. Although the rooting rates from the two studies are not directly comparable, season may affect endogenous chemicals which promote/inhibit rooting of GN clones in the presence of light.

In the mung bean bioassay, the association of injury at the hypocotyl cutting base and rooting frequency is observable in the presence of light and exclusion of exogenous auxin application. Presumably, tissue extracts in the mung bean bioassay exhibit rooting promotory activity depending on the concentration of endogenous root stimulants in the plant tissues (Wilson, 1988). Cuttings of PP 2107 clone maintained at high planting density usually root readily without exogenous auxins. However, management factors on rooting rates of PP 2107 clone and its impacts on the interactions between endogenous rooting stimulants and Seradix 2 are not fully understood (CSIR *internal com*.). Similarly, the impact of planting density on the rooting rates of PP 2107 has not yet been fully investigated. In the absence of exogenous IBA, light exposure during the root formation period had no effect on rooting of *E. saligna* (easy-to-root species) cuttings, but contrasting rooting responses were observed with *E. globulus* (difficult-to-root species) cuttings (Fett-Neto *et al*., 2001). Although pure *Eucalyptus* species were used in those studies, light-induced rooting response may explain rooting rates of GN clones relative to auxin application on cuttings under different light growing conditions of stockplants. In many plants, evidence also suggests that light and dark conditions influence the rate of entry of exogenous IAA, the endogenous level of auxins, and processes of conjugation and oxidative breakdown of IAA (Tam *et al.*, 1998).

Furthermore, GN 018B extracts from the low planting density promoted high rooting rates in the mung bean bioassay and nursery rooting experiments, suggesting that environmental changes may alter IAA metabolism. MTR1 plants, a mutagenized population of 3F7-11 duckweed *Lemna gibba*, grown in continuous light had twice the free IAA levels than MTR1 plants grown without any light condition (Tam*et al.*, 1998). However, PP 2107 cuttings maintained at low planting density and treated with Seradix 2 exhibited low rooting rates, suggesting possible interactions of endogenous and exogenous auxins inhibiting rooting. Cuttings of *E. saligna* (difficult-to-root species) were shown to either be sensitive to exogenous auxin or nearly had optimal auxin supply for rooting (Fett-Neto *et al*., 2001), suggesting that not all cuttings require auxins for rooting. Other possible explanations could be that cuttings of stockplants under high irradiance experience metabolic breakdown of endogenous auxin. The inhibitory effect of high irradiance on rooting rates of mung bean hypocotyls cuttings grown without auxin supply was due to possible photochemical and metabolism breakdown of endogenous auxin (Jarvis and Shaheed, 1987). Auxin plays a

central role in the determination of rooting capacity, and light conditions are known to affect auxin metabolism and tissue sensitivity (Reid *et al*., 1991).

The previous chapter also suggested that in the PP 2107 clone, high rooting frequency was possibly inherited from *E. grandis*, as *E. grandis* usually roots better than *E. nitens*. Aimers-Halliday *et al*. (1999) determined that the *E. grandis* x *E. nitens* hybrid behaved more like the *E. grandis* parent than the *E. nitens* parent in its ability to coppice and produce saplings. Although GN 018B and PP 2107 clones are both GN clones, PP 2107 clone had high branch growths because of high soluble sugar concentrations, suggesting its possible inheritance of more *E. grandis* genes, considering that *E. nitens* does not sprout readily (Hartney, 1980; Zobel, 1993). Thus, if this is the case, whether high light quality or intensity on PP 2107 stockplants triggered inhibitor growth regulators which decreased the rooting rates in the mung bean bioassay is not fully known. *Eucalyptus grandis* adult and juvenile leaves have been shown to contain the G growth regulator, which inhibits or promotes rooting at high or low concentrations (Dhawan *et al.*, 1979; Milborrow, 1984). Low concentrations of G promote rooting in mung bean hypocotyls cuttings and root elongation in *Avena* coleoptile sections (Dhawan *et al.*, 1979). Possibly, similar type of inhibitor could contribute to the recalcitrance rooting responses of PP 2107 cuttings maintained at low planting density, depending on the concentration of G in PP 2107 tissues. Other alternative explanations are that PP 2107 stockplants (tall and short) maintained at low planting density accumulated more soluble sugars, in particular sucrose and tended to grow more vigorously than GN 018B stockplants (Fig. 3.2; 3.3). Although low soluble sugar concentrations were accumulated in summer, both GN clones exhibited high rooting rates, suggesting that rooting potential of these two GN clones has nothing to do with soluble sugars. This study speculates that PP 2107 leaves may develop G inhibitor faster than GN 018B leaves under the same spacing, and therefore reduce its rooting frequency. If this is the case, season would have a different impact on sprouting and rooting rates of the two GN clones. In addition, the previous chapter showed that high summer temperatures and high humidity promoted high sprouts although plants were affected by fungal diseases, and GN rooting rates was usually low. The rooting rates of mung bean cuttings from the extracts of GN 018B tissues was higher in December compared to September, whereas the rooting rates of mung beans from PP 2107 extracts were higher in September and lower in December (Fig. 3.3). Therefore, high growths of PP 2107 clone

observed at low planting density may promote the G inhibitor in leaves thereby decreasing rooting ability of this clone.

The correlation between high branch growths and low planting density may also indicate high cytokinin concentration in GN stockplant tissues. If high light intensity favours high cytokinin accumulation in GN hybrids grown at high light intensity (low planting density), this could explain the suppression of rooting of these cuttings. Suppression of rooting in the nursery and bioassay experiments was usually observed on cuttings and tissue samples of PP 2107 stockplants grown at low planting density. However, GN 018B cuttings had high rooting rates and its tissue extractives promoted high rooting at low planting density in nursery and in mung bean bioassay. In the previous chapter, GN stockplants (tall/short) maintained at low planting density were assumed to accumulate more fertilizer, water and assimilates than stockplants at high planting density. That observation suggested that interactions of more than one factor at the two spacing densities may determine GN stockplant sprouting behaviour and rooting ability. Kosh (1996) advocated that the interactions between plant growth regulators (especially auxin/sugar antagonisms) and other nutrients such as nitrogen or phosphorus suggest that sugars may affect development at the cell, organ, and whole-plant levels. If at low planting density high cytokinins synthesis was associated with high shoot branching, sugar signals could initiate photosynthetic processes inducing high cutting yields (Chapter 2). Nett-Neto *et al.* (2001) assumed that auxins supplementation should overcome the light-mediated and cytokinin-induced inhibition of rooting. Therefore, variables associated with these contrasting rooting responses are not fully attributed to one factor but rather to interactions of different factors.

The extracts from *Eucalyptus grandis* x *E. nitens* stockplants maintained at high or low planting density had contrasting rooting effects in the mung bean bioassay (Fig. 3.2, 3.3; Tables 3.2, 3.3; 3.4; 3.5). In this study, low planting density promoted high accumulation of soluble sugars. Similarly, short stockplants accumulated more soluble sugars than tall stockplants at low planting density, although tall stockplants maintained at high planting density showed high soluble sugar accumulation. This suggests that soluble sugar concentration and light conditions on stockplants may have different effects on root formation of mung bean cuttings, and thus of GN clone cuttings. Sucrose, glucose and fructose stimulate

the formation of adventitious roots on the hypocotyls of dark-grown wild type *Arabidopsis* seedlings (Gibson, 2005). However, high concentrations of glucose and sucrose inhibit true leaf formation and root growth of young *Arabidopsis* seedlings, suggesting that the effects of sugars (and sugar type) on adventitious root formation are concentration dependent (Gibson, 2005). Although GN cuttings may not be compared to *Arabidopsis* seedlings, these studies showed the role of sugar concentration and light intensity on plant development and rooting responses. High light intensity may promote high sucrose levels which may also be reflected in high growth rates (Leyser, *pers. comm.*[8]). As a product of photosynthesis, sucrose levels also reflect environmental and physiological conditions. Its transfer from source to sink tissues can clearly influence patterns of growth and development, and its partitioning and sensing is vital for all stages of the plant life cycle (Roitsch and Gonzalez, 2004). Sucrose and its cleavage products glucose and fructose are also important metabolic signals that affect the expression of different classes of genes (Koch, 1996; Rolland *et al.*, 2002) and are involved in the regulation of plant development (Wobus and Weber, 1999). Thus, low growths of tall and short stockplants at high planting density could be explained by low sugar concentration in shoot tissues. Van der Werf and Nagel (1996) reviewed the role of cytokinin-sucrose interactions on biomass partitioning and accumulation, and suggested that low nitrogen supply is related to low cytokinin production, which decreases cell division in young leaves in the presence of auxins.

Although apical GN extractives had higher sugar concentration from tissues under high/low planting density, (tall/short stockplants), their rooting rates were usually lower than middle or basal ones for cuttings in the mung bean bioassay (Fig. 3.2, 3.3; Tables 3.2, 3.3). This suggests that adventitious root formation of GN clones may be related to plant growth regulators and light interactions rather than sugars concentration alone. Fogaça and Fett-Neto (2005) found that rooting of microcuttings of *E. saligna* depended on the presence of light than exogenous supplementation of sucrose. Therefore, in those studies they suggested that light effects on adventitious root development were related to phytohormone uptake and metabolism, rather than carbon source availability (Fogaça and Fett-Neto, 2005).

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While the present study did not evaluate the impact of sucrose during the root formation period, such studies could contribute to our understanding of auxin conjugation and metabolism due to light intensity. Although basal GN tissue extracts had lower soluble sugar concentration than apical and middle cuttings, these extracts constantly promoted high rooting rates of mung bean hypocotyl cuttings.

Season and size of stockplants had similar effects on GN rooting frequency under nursery experiments and in the mung bean bioassay. High rooting rates of mung bean cuttings were observed in September and in December for PP 2107 and GN 018B clone tissue extracts respectively. Furthermore, soluble sugar concentration of the two GN clone tissues was high in September and low in December and mung bean cuttings rooting rates were also different (Fig. 3.2, 3.3; Table 3.2, 3.3). Although plant tissue extractives used in the laboratory did not show any nutritional deficiency sign, it could be speculated that successive harvests may have affected the physiological status of GN stockplants. From my personal observations, the falling of older mung bean leaves during the bioassay experiments was related to leaf age and necrosis on leaf tissues. Distilled water used in the bioassay did not have boron, thus necrosis and leaf fall of mung bean cuttings could presumably due to boron deficiency and light interactions. The distinct effects of size of stockplants, cutting position and season on soluble sugars and root initiation of mung bean cuttings may provide useful management information thereby optimizing rooting rates of GN clones. The possibility of manipulating planting density in the early developmental stage of *E. grandis* x *E. nitens* stockplants may provide a valuable system for evaluating rooting rates, and soluble sugars have nothing to do with rooting of GN 018B and PP 2107 clones in the nursery.

CHAPTER 4: OVERVIEW AND MANAGEMENT IMPLICATIONS

4.1 Overview

The aim of this study was to investigate the effect of size and planting density of stockplants in mini-hedges, on the yield and subsequent rooting of cuttings from various positions of GN clones of known rooting potential (i.e. GN 018B: difficult-to-root and PP 2107: easy-to-root clones). The particular interests investigated were the seasonal variations on rooting rates of GN clones, the analysis of soluble sugars and rooting stimulants on growth and rooting of mung bean hypocotyl cuttings. At the Sunshine Seedlings Services nursery, GN clones (e.g. GN 018B and PP 2107) are usually maintained at seven cm in length and spaced 5 x 5 cm apart. Long-term nursery observations have shown that rooting rates of GN clones vary seasonally, and very little is known about the impact of the position of cuttings on stockplants on overall rooting rates. Furthermore, the impact of stockplant morphological and developmental changes over season due to successive harvests on GN rooting rates is still largely unknown. In addition, many studies on adventitious rooting have focused on the performance of cuttings (*in vitro*/*ex-vitro*) during the root formation period. Therefore, this study comparatively analysed GN 018B and PP 2107 tall (20 cm) and short (10 cm) stockplants grown at high (100 x 1.5 m^{-2}) and at low (25 x 1.5 m^{-2}) densities under commercial nursery conditions in a polyethylene tunnel. Cuttings were harvested every two to three weeks in September, October (spring); December 2010, January 2011 (summer); April, May 2011 (autumn), and June and July (winter). The harvested material was 5 – 7 cm in length and the light intensity received by individual shoots at the two planting density levels was recorded. Harvested cuttings from the three positions (apical, middle and basal shoots) were used for: (i) rooting experiments under nursery conditions and (ii) bio-stimulant analysis using the mung bean bioassay and (iii) soluble sugar analysis. This study has shown that PP 2107 clone had higher growths and higher rooting rates than GN 018B clone although no significant differences were observed in terms of number of cuttings produced.

Eucalyptus grandis x *E. nitens* stockplants grown at low planting density grew better and produced more cuttings than stockplants maintained at high planting density. For the two GN clones, short stockplants produced fewer cuttings but had a higher rooting frequency than cuttings from tall stockplants, and basal cuttings had higher rooting frequency. Cuttings produced from taller stockplants maintained at high planting density, usually had longer internodes and were similar to macro-cuttings from macro-hedges. Winter and spring promoted high rooting rates of PP 2107 clone maintained at high planting density whereas autumn promoted low rooting mainly due to fungal infestations which occurred in summer. In contrast, spring and autumn promoted high rooting rates of GN 018B clone maintained at low planting density as this clone showed higher resistance to fungal diseases than PP 2107 clone. However, rooting rates were usually lower in summer for PP 2107 clone but higher for GN 018B, and in particular at low planting density. Similar results were observed from the mung bean bioassay which showed high rooting frequency for IAA extracted from PP 2107 tissues maintained at high planting density. Sucrose concentration was the highest sugar present in stockplants grown at low planting density. Position, size and genotype had a significant impact on the type and concentration of sugar (i.e. sucrose, glucose and fructose), particularly of PP 2107 tissues. However, rooting rates of hypocotyl cuttings had nothing to do with endogenous soluble sugar concentration.

4.2 Performance of GN clones and management implications

Both *E. grandis* x *E. nitens* (GN) clones propagated under commercial nursery conditions produced similar number of cuttings in spring and in summer although PP 2107 clone showed higher growths due to higher soluble sugars. However, the two GN clones responded differently to planting density effects because GN 018B clone showed high rooting rates at low density whereas high planting density was suitable for PP 2107 clone. For GN 018B and PP 2107 clones, basal cuttings showed high rooting as compared to middle and apical cuttings, regardless of planting density. Furthermore, high rooting frequency of both GN clones was recorded in spring from apical, middle and basal cuttings. Although the rooting frequency of PP 2107 maintained at high planting density was high in winter, the rooting rates of GN 018B

were lower at low planting density of the same season largely due to successive harvests. This suggests that the age of GN stockplants may be considered when evaluating the rooting potential of *Eucalyptus* clone. In this study, stockplants were grown under polyethylene tunnels during winter. Higher coppice production and higher rooting rates of GN clones were higher in spring than in summer, and basal cuttings showed higher rooting rates. However, with successive harvests, the number of basal cuttings continuously decreased as compared to apical and middle cuttings. For instance, after a year of harvest, apical, middle and basal cuttings were observed occupying similar position on stockplants maintained under nursery conditions. Therefore, as GN 018B clone rooting rates were lower in July, it is possible that the age of stockplants and successive harvests had a greater impact on basal cuttings, thereby decreasing the overall rooting rates. However, apical, middle and basal cuttings of PP 2107 clone had high rooting rates in July, suggesting that successive harvests and age of stockplants had less impact on PP 2107 rooting rates. Other possible explanations are that variations of light intensity received by tall stockplant cuttings in particular maintained at high planting density impacted on cutting development and physiology. Basal cuttings from tall stockplants usually received less light as compared to apical cuttings, which received more light. Because of differences in light intensity between apical and basal cuttings, apical or middle cuttings usually showed signs of apical dominance. Although basal GN cutting tissues had lower soluble sugar concentration than apical and middle cuttings, the extracts from these tissues constantly promoted high rooting rates of mung bean hypocotyl cuttings. This suggested that rooting of GN 018B and PP 2107 had nothing to do with endogenous soluble sugars. Similarly, light intensity on stockplants due to differences in planting density influenced GN rooting rates in the presence of exogenous auxin, suggesting that the two GN clones should be maintained at different planting densities. High growth rates of tall stockplants maintained at high planting density not only promoted low rooting rates but also formed a reservoir of fungi, which was spread by generalist grasshoppers (Acrididae) to mini-stockplants clonal-gardens. Interestingly, the disease was observed in summer due to high temperatures and high humidity and its spread was only recorded on experimental stockplants, although nursery GN stockplants were also grown in the same polyethylene tunnel. The PP 2107 stockplants were severely affected by the fungal disease *Quambalaria eucalypti (Sporothrix eucalypti)* because of high sugar concentration in their tissues. Therefore, fungal infestations observed in summer

were mainly due to high branch growths of tall GN stockplant and its impact on cutting yields and rooting rates were observed in February, March and April 2011. Therefore, the concept of size of stockplants and planting density provides useful management information in the control of pests and pathogens on stockplants. Work cited in this study also suggested that GN clones show resistance or susceptibility to fungal attacks and soluble sugars may contribute to high sprouting and high fungal susceptibility. Short stockplants on the other hand, although fewer sprouts were yielded, their cuttings were usually shorter, similar in size, and developed more nodes per cutting, all of which contributed towards higher rooting rates of subsequent cuttings. The rooting rates of GN 018B and PP 2107 clones recorded in this study were usually higher that the rooting rates recorded under the nursery operations, particularly in spring and in summer.

Furthermore, seasonal effects were observed on coppice production and rooting rates of GN 018B and PP 2107 clones from commercial nursery records and in this study. Based on nursery and laboratory experiments, this study has also recommended that GN 018B and PP 2107 clones be planted at low planting and high planting densities respectively for optimum rooting rates. Thus, maintaining stockplants short could be the best practice as the harvested cuttings are usually short and develop more nodes which are presumed to contribute to higher rooting of short GN stockplants. However, the experimental set-up used in this study which combined tall/short and high/low planting density on similar beds may also create intra-clonal competition and unequal resource partitioning among stockplants. Observations made in this study also suggested that stockplants which are maintained at low planting density received more fertilizer in addition to high light intensity. It is not clear whether fertilizer concentration on stockplants influenced the rooting rates of GN cuttings as the latter was not investigated in this study. This area requires further investigation in future studies on management effects on rooting efficiency of GN clones. The nursery set-up for this study did not allow the application of equal amount of fertilizer as the two planting densities and sizes of the stockplants were maintained on the same bed and in the same tunnel. Therefore, it would be ideal that the two stockplant planting densities and sizes are maintained in different polyethylene tunnels and randomly scattered on bed so as to maintain different management regimes when necessary. That may be necessary in ensuring that experimental stockplants experienced less intra-clonal competition, the spread of fungal diseases is minimized, fertilizer

application is efficiently controlled and light intensity is less variable on cuttings. In addition, this would also reduce labour costs and induce less physical stress on stockplants.

Although this study did not determine the cause of mortality of stockplants maintained at low planting density, it was assumed that high light conditions on stockplants may also promote mortality if stockplants are stressed. It also remains to be investigated why short stockplants maintained at low planting density experienced higher mortality than taller stockplants, why PP 2107 clone developed a better rooting system and lower callusing. As the two clones are a GN hybrid, the observed rooting differences would also be caused by genetic differences, planting density and size of stockplants and position of cuttings on stockplants. Since GN 018B clone is still under commercial nursery production, maintaining its stockplants short under a low planting density may be a better option to optimize its rooting rates. Furthermore, although GN 018B clone showed signs of resistance to pathogens, further investigations are recommended to test its rooting rates versus common pathogens in the nursery. In this respect, GN 018B clone is of great interest in eucalypt hybrid improvement programme as it shows less susceptibility to fungal diseases as compared to PP 2107 clone. Since PP 2107 cuttings root readily without Seradix 2 application (CSIR *intern. com.*), a comparative study on the impact of position, planting density and size of stockplants on rooting rates using Seradix and non-Seradix is required. In conclusion, position of cuttings and size of stockplants maintained at high/low planting density experiments can provide useful management information in optimizing rooting rates in nurseries.

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