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Verticordia micropropagation through direct ex vitro rooting

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Verticordia micropropagation through direct *ex vitro* rooting

Verticordia grandis

Belinda Delaney

BSc (Biological Sciences)

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Abstract

The objective of this study was to improve the existing shoot multiplication protocol for *Verticordia grandis* (McComb, Arthur & Newll, 1986; Newell, Growns & McComb, 2005) and to investigate and establish reliable root induction and acclimatisation protocols to enhance survival of micropropagated plantlets. It was envisaged that these protocols would be successful in micropropagation, growth and survival of different *V. grandis* clones and possibly applicable to other *Verticordia* species.

The elongation of *in vitro* *Verticordia* shoots on multiplication media was improved by reducing the concentration of BAP from 1µM to 0.25 µM, which resulted in a more uniform shoot length of 4.5 – 5 cm; necessary for root induction experiments. The root induction protocol was optimised by determining the appropriate auxin concentration (80µM indole butyric acid; IBA) with an exposure time of 6 days. Acclimatisation and survival was greatly improved by transferring the IBA pulsed shoots to *ex vitro* conditions consisting of a free draining and aerated substrate (a mixture of peat and perlite 1:3) in crack pots. These were initially placed into a greenhouse (with controlled temperature & light conditions) in order to maintain high humidity. Over time humidity was reduced and after 112 days the plantlets were transferred to larger pots, containing fresh soil (peat/perlite/sand = 1:1:1) and placed in a shade house with a regular watering regime. Long-term survival was monitored and after 252 days survival was over 70%.

The declining survival rates after this time has made it evident that field performance and long-term survival needs further investigation. The application of the improved shoot multiplication and root induction protocols on other *V. grandis* clones produced survival rates of 0 to 62.5% (depending upon clone) over 252 days.
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Acknowledgements

I dedicate this research to the memory of Edward ‘Ted’ Williams and Diane Forsythe

I hereby gratefully acknowledge the people who supported my research for this masters project, especially my supervisors Dr Ian Bennett and Dr Mary Boyce. I thank them for their support, advice, time, encouragement and influence, as these were greatly appreciated. Thanks are also due to Clay Millar and his laboratory staff who devoted time and energy into all practical aspects of my working in a laboratory, whilst I was researching for my project. I consider myself fortunate to have had the financial support for my research provided by the School of Natural Sciences at ECU. I also wish to thank my fellow students, volunteers and friends that I have met over the years at ECU, especially those who offered encouragement and were always ready to lend a helping hand.

This project would have not been possible without the dedication of Ted Williams who helped me discover Eneabba’s wildflower treasures, Alan Tinker who shared his vast knowledge of Beekeeper’s Reserve and his amazing botanical skills with me, and Diane Forsythe, who always encouraged my endeavours in any way possible.

I hope the research presented here may contribute to the conservation of the amazing scarlet feather flower – *Verticordia grandis.*
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ABBREVIATIONS

BAP – 6 benzylaminopurine
DDI – double de-ionised water
CO₂ – carbon dioxide
GF – *Verticordia grandis* from lignotuber regrowth after fire
GRD – *Verticordia grandis* clone
IAA – indole-3-acetic-acid
IBA – indole-3-butyric-acid
IVS – *in vitro* soil
MilliQ water – de-ionised water
MS – Murashige & Skoog basal salt mixture
NAA – naphthalene acetic acid
NaOH – sodium hydroxide
O₂ – oxygen
VOV – *Verticordia ovalifolia* clone
VFR – *Verticordia fragrans* clone
VDF - *Verticordia densiflora* clone
1. Introduction

1.1 Background

The Southwest Botanical Province of Western Australia is situated between latitudes 27° S and 35° S and longitudes 112° E and 128° E and extends from Shark Bay in the northwest to Esperance in the southeast. This region includes the narrow coastal land from east of Esperance stretching to the Western and South Australian border and is confined by oceans in the west and south and by arid landscapes in the north and east (Cole, 2006).

Figure 1.1: Map of the Southwest Botanical Region in Western Australia. The green area shows the ‘eco-region’ boundary; the dark yellow area shows the ‘transitional zone’ from Shark Bay in the northwest continuing to the SA border in the southeast (Cole, 2006).
It is recognized as one of the world’s 34 major biodiversity hotspots and is the only one in Australia; it consists of more than 480,000 km$^2$ (48.0 million hectares) with over 52% of vascular plants and 12.5% of genera being endemic to the region. This region is also classified as a ‘centre for plant diversity’ by the WWF and the International Union for Conservation of Nature and Natural Resources (IUCNN) because of its plant species richness (Hopper & Gioia, 2004). The southwest of Australia is one of the oldest landscapes in the world with very diverse vegetation communities that have adapted to the leached and nutrient-poor soils. The species richness and diversity of flora in this region is probably due to the combination of ecological and phylogenetic processes that have occurred over long periods of time. This could explain the diverse plant communities; ranging from kwongan (sclerophyllous shrubs) heath- and shrub lands on the coastal sand plains to temperate forests in the far southwest, and wood- and shrub lands further east. All these plant communities vary greatly over short distances and because of these varying features the region has become popular for its wild flower displays in spring, and eco-tourism has become an important industry (Cole, 2006). Many of these plants have become desirable for the domestic and export cut flower trade, ornamental horticulture and land restoration (Seaton, 2002).

The world’s plant biodiversity is in sharp decline with many species being listed as ‘critically endangered, endangered or vulnerable’ and requiring conservation (Sarasan, Cripps, Ramsay, Atherton, McMichen, Prendergast & Rowntree, 2006); this includes many taxa of the Southwest Botanical Province (Cole, 2006). Over the last century this region has been extensively degraded, resulting in landscape modification, fragmentation, biodiversity and habitat loss (Hobbs & Saunders, 1991). Human induced processes have altered over 90% of the original vegetation.
(Beard, 1980), which has been reduced to mostly scattered, fragmented vegetation remnants in various conditions. Land clearing for agricultural purposes, urban development, domestic stock grazing, logging and changes in fire regimes account for most of the native vegetation loss and habitat destruction (Cole, 2006). Salinisation of groundwater, due to altered landscape hydrology (Hobbs, 1992), susceptibility of remnant vegetation to weed invasion (Pigott, 2000) and (feral) grazing animals are other threats to the remaining bio-diversity of the Southwest Botanical Province (Denham & Auld, 2004). Another major threat to the flora of this region is a disease caused by *Phytophthora cinnamomi*, a soil-borne pathogen (water mould), which can cause irreversible damage to affected plant communities. The pathogen attacks the roots of susceptible host plants, thus significantly reducing the host plant’s ability for nutrient and water transport, and eventually kills the host (Shearer, Crane, Barrett & Cochrane, 2007).

The conservation significance of the highly diverse and endemic flora of the Southwest Botanical Region has a high priority with government conservation agencies, as many critically endangered taxa have been identified. Many of these endangered species require immediate remedial action to prevent extinction (Coates & Atkins, 2000), and *in situ* conservation by protecting natural habitats and efficient management of wild plant populations is very efficient with many species. But these sometimes need to be complimented with *ex situ* methods (Sarasan, *et al.*, 2006), as numerous species have recalcitrant seeds or produce poor quality seeds; so successful seed propagation and plant establishment is often very difficult or impossible (Bell, 1999). Many taxa from the Myrtaceae, including some *Verticordia*, are threatened by habitat loss.
1.2. Myrtaceae and Genus *Verticordia*

Myrtaceae is one of the ten largest plant families in the Southwest Botanical Province and of the 785 species 92% are endemic (Beard, Chapman & Gioia, 2000). The genus *Verticordia*, belongs to this family and is a member of the Australian sub-family *Leptospermoideae*, characterised by their dry capsular fruit (Cochrane, Brown, Cunneen & Kelly, 2001; Clark & Lee, 2002). It was named by Augustin Pyramus de Candolle in 1821, and is endemic to Australia (George, 1991). *Verticordia* belong to the *Chamelaucium* alliance with its closest relatives being *Chamelaucium, Darwinia, Homoranthus* and *Rylstonea* (George, 1991). These are commonly associated with many other myrtaceaous genera in Western Australia’s shrub and heath land communities (Cochrane, *et al.*, 2001).

1.2.1 *Verticordia* Habitat

All but three of the species, subspecies and varieties of verticordias are endemic to Western Australia, and the distribution largely depends on the annual rainfall, with many adapted to 350 mm to 850 mm rainfall zones (George, 1991; George, 2002; Seaton, 2002). They grow in a diversity of habitats and soils ranging from coastal regions to far inland arid regions. They occur as woody perennial understorey shrubs in open forests or as heath shrubs on sand plains and even as saline tolerant shrubs growing on the edges of salt lakes (George, 1991; Seaton, 2002). Although verticordias grow in this diversity of habitats and soils, some only occur in very particular locations and/or specific conditions, many have already been declared rare and endangered (George, 1991; George, 2002; Yates & Ladd, 2004).
1.2.2 *Verticordia* Morphology

Verticordias generally grow up to 2 m tall, with a few species growing taller, and have very diverse forms with considerable variation depending on species, although the bushy shrub is the most common form. Their shrub form may be dwarf, prostrate, erect or widely spreading with a compact, straggly or horizontally layered appearance (George, 1991). All verticordias, however, share one prominent characterising feature; the sepals are deeply divided in various ways, with most species having petals that are often dentated, divided, fimbriated or lobed and most flowers having a hairy hypanthium (Marchant, Wheeler, Rye, Bennett, Lander, MacFarlane, 1987), hence, the common name - feather flowers.

1.2.3 *Verticordia* Seed Germination and Reproduction

Seed production and germination processes are critical for the survival of most plant species (Bell, 1999), but poorly understood for *Verticordia* species. Bell (1999) emphasised that Australian plant species rely on several germination mechanisms that interact to interrupt seed dormancy and initiate germination when environmental conditions are suitable. Cochrane *et al.* (2001) reported that for many *Verticordia* species there is no adequate knowledge of the reproductive potential, although some germination trials with a few species have been successful (Cochrane, *et al.*, 2001). Generally, annual reproductive capacity in the form of seed production and viability in many *Verticordia* species is exceedingly low (Cochrane, *et al.*, 2001).

Many *Verticordia* species are capable of superfluous flower production and the low quantity of seeds produced is disproportionate to the number of flowers. This low seed to flower ratio is common in the genus (Houston, Lamont, Radford & Errington, 1993; Cochrane, *et al.*, 2001) and
Cochrane et al. (2001) concluded that the low seed set could be a result of the self-incompatibility common in Myrtaceae. However, as Tyagi, McComb & Considine, (1991) and others (Bellairs & Bell, 1990; Speer, 1993; McEvoy & True, 1995; Bell, 1999) suggest an array of environmental and physiological factors could contribute to the low seed set and viability in the genus (Rye & James, 1992; George, 2002; Yates & Ladd, 2004). It is not unusual for Australian native species to have limited seed production as many of these plants reproduce vegetatively (Johnson & Burchett, 1996; Kimpton, James & Drinnan, 2002). Cochrane, et al (2001) also proposed that another biological constraint that could play a part in the reproductive success is that protein and lipid creation required for seed production requires more energy than flower production. This may be a survival strategy for taxa during adverse environmental conditions, e.g. changes in pollinator activity, predation, competition and other unpredictable events (Cochrane, et al., 2001).

Tyagi, Considine & McComb (1992) researched the remarkable longevity of some verticordia pollen at different temperatures for species both with and without a pollen presenter, but did not discover the physiological basis for this. It was suggested that this long viability could facilitate artificial hybridisation of species with breeding barriers such as different flowering times, but it was not determined if in vitro germination levels could match levels of in vivo germination of the Verticordia species examined (Tyagi, et al., 1992).

While verticordias have limited reproductive success from seed germination, Bell (1999) states, that the secondary dormancy mechanisms, which can prevent germination of many Australian native species are not fully understood. Several Western Australian recalcitrant plant seeds have
been investigated on with seed dormancy-relieving treatments, including smoke treatments, to promote and increase germination (Dixon, Roche & Pate, 1995; Roche, Dixon & Pate, 1997; Roche, Koch & Dixon, 1997), and it was suggested that research on seed germination and recalcitrance needs to be further investigated. Bell (1999) concluded that it was vital to understand the eco-physiology of seed germination processes and that further research would be needed in order to improve conservation of Australian plant species for improved land management, rehabilitation and for progress in horticultural practices.

1.2.4 Verticordia Conservation & Cultivation Value

The long lasting feather flowers are highly sought after for the commercial flower markets and many species have been bush-picked to the extent that this had adversely impacted on wild populations (e.g. Verticordia eriocephala). Commercial bush-picking, therefore, has become unsustainable for most native populations (Tyagi et al., 1991; Atkins, 1998; Cochrane, et al., 2001) and the harvesting from natural bush populations is now restricted (George, 2002).

Verticordias are not widely cultivated for the cut flower trade (Seaton, 2002), but some success has provided an alternative to bush-picked plants. However, this has been limited to only a few species, such as V. plumosa (Seaton, 2002).

1.2.5 Verticordia Propagation

In the past, asexual propagation techniques have been successfully used on some Verticordia species (e.g. V. plumosa, V. chrysanthella), with the focus mainly on cuttings and some success
with tissue culture (George, 2002). In addition, Egerton-Warburton, Ghisalberti, & Burton, (1998) found that intergeneric hybridisation facilitated by synchronous flowering can be useful (e.g. *C. floriferum* x *V. plumosa*), and grafting on to *Chamelaucium* root stock (Yan, 2001) has also produced some useful results. Grafting of several *Verticordia* species, for commercial applications in field conditions, however, has not been successful (Ben-Jaacov, Ackerman & Evenor, 2000). Grafting is also considered uneconomical due to its slower production rate and additional costs (Lullfitz, 2001).

1.2.6 Micropropagation of *Verticordia grandis*

The application of *ex situ* conservation practices for plant species can compliment *in situ* methods, or replace them all together with species that are too difficult to propagate by conventional methods (Sarasan, *et al*., 2006). *Verticordia grandis*, with its large scarlet red flowers, is an attractive species for the cut flower industry, but has proven to be difficult to propagate by conventional methods (Speer, 1993), due to recalcitrance and low viability of its seeds (Bell, 1999). Pollination of this species is poorly understood (Bell, 1999), but it can regenerate from a lignotuber; it is one of the few verticordias that possess this capacity (George & George, 2003). A reliable alternative to conventional propagation methods is tissue culture, which can successfully produce viable clones of difficult to propagate species (Ilan & Khayat, 1997). Micropropagation of *V. grandis* could produce numerous, disease free plants for horticultural purposes, the cut flower trade and for the restoration of degraded habitats.
1.3 Micropropagation

1.3.1 Principles of in vitro Propagation

The development of the elementary methods of plant tissue culture took place in the 1950s and 1960s and has since advanced to commercial plant biotechnology, which has become an invaluable tool in the plant propagation industry; e.g. ornamental horticulture and agriculture (DeKlerk, 2002). One distinct advantage of producing plants by tissue culture is the raising of disease free true-to-type plantlets and minimising diseases and pests, which include internal pathogens, phytoplasma, viruses, viroids, pathogenic fungi, moulds, yeasts, bacteria and insects. (Bandyopadhyay, Cane, Rasmussen & Hamill, 1999; De Klerk, 2002). In general it is based on the principle of establishing and maintaining healthy micro-shoots in culture for multiplication so that these produce rooted micro-cuttings and plantlets (DeKlerk, 2002; Liu & Bao, 2003).

Conventional propagation methods are often inefficient for multiplying many mature and/or difficult-to-propagate plant species, but tissue culture can successfully produce viable clones (Ilan & Khayat, 1997). However, it has also been well documented that root induction can be problematic in woody plants (DeKlerk, 2002; Liu & Bao, 2003; Seth, Kendurkar, & Nadgauda, 2007). A major benefit of micropropagation applied to slow growing woody perennials is the propagation and multiplication of uniform and numerous disease free plantlets in a short time, as there are no effectual vegetative propagation techniques for reproducing mature plants that can equal this. Micropropagation also allows for faster and greater production of numerous plantlets compared to the slower, conventional seed or vegetative propagation methods (Franclet, 1991; Haapala, Pakkanen, Pulkinnen, 2004).
The basic principle of plant micropropagation of meristematic shoot tissues is to grow, manipulate and multiply identical plant cells, tissues and organs, which have been isolated from the mother plant. This approach involves techniques that are conducted in a controlled and aseptic environment; the cells, tissues and organs of a selected plant are isolated, surface sterilised (Teng, Sin & Teng, 2002) and cultured in a growth-promoting environment (Franclet, 1991; van Acker & Scholten, 1995; George, Hall & de Klerk, 2007). There are generally four distinct phases applied in micropropagation; 1. Establishment, 2. Multiplication, 3. Root Induction and 4. Acclimatisation. In the ‘Establishment Phase’ a plant part is surface sterilised and then introduced to a sterile culture media. This phase determines the suitability of the explants; e.g. being free of exo- and endogenous contamination. In the ‘Multiplication Phase’ the explants are aseptically transferred to media containing growth hormones that promote shoot proliferation (usually) and elongation. This phase can generate numerous, stabilised explants in a very short time, which can be kept in culture (Multiplication Phase) as long as needed by periodic sub-culturing on to new ‘multiplication media, or the explants can be aseptically transferred to root induction media (Root Induction Phase). In the ‘Root Induction Phase’ elongated explants are aseptically transferred to a growth media that usually contains auxin/s to initiate and promote root growth in preparation for autotrophic growth of explants. In the ‘Acclimatisation Phase’ rooted explants are transferred to a suitable growth substrate and placed in the controlled environment of a glasshouse for gradual acclimatisation to ex vitro conditions. The plants are initially kept in high humidity, which is gradually decreased as plantlets acclimatise; they become more capable of controlling water loss.
1.3.2 Composition of *in vitro* Culture Media

An optimised culture media is essential for shoot growth, multiplication and root induction in tissue culture, as the media has to provide all nutrients and elements for *in vitro* growth of plants. Most culture media contain the following:

- **Minerals (as macro- and micro salts):** Most plants respond favourably to the widely applied Murashige & Skoog (1962) MS salts. Lloyd & McCowan’s (1980) Woody Plant Medium is also widely used. The application quantity depends on the *in vitro* cultivation objectives; e.g. high formulations (full strength) are better suited for shoot multiplication, whilst lower concentrations (1/2 or 1/4 strength) are generally used to promote root induction depending on plant species (Kim, Oh, Jee & Chung, 2003).

- **Carbon (energy) source:** Sucrose in the form of commercial sugar is the most preferred carbon source in tissue culture because it is cheap and readily available. Carbon (e.g. sucrose, glucose, maltose or galactose) for *in vitro* plant metabolism has to be added to many culture media as the plantlets are not fully autotrophic and photosynthesis is not adequate (Rahman, Islam, Hossain & Islam, 2010).

- **Plant growth regulators (auxins, cytokinins, gibberellins and others):** Several growth regulators consist of natural and/or synthetic compounds that manipulate development and growth of *in vitro* plants. The application and quantities of growth regulators depends on the *in vitro* cultivation objectives; e.g. callus formation, shoot multiplication and elongation or root induction. Auxins regulate several physiological process; e.g. formation of shoots and adventitious and lateral roots (Ibrahim & DeBergh, 2001).

- **Other organics:** Vitamins are not necessarily added to tissue culture media, but most formulations contain vitamins; e.g. in MS salts the included vitamins are thiamine,
pyridoxine, nicotinic acid, with myo-inositol (sugar alcohol) and thiamine considered to be essential to plant growth (Abrahamian & Kantharajah, 2011).

- pH; the pH is adjusted to 5.8 using 1M NaOH for shoot multiplication to approximate what would be suitable for plant metabolism and to ensure agar solidification.

- Gelling agents (purified agar or gellan gum products): In micropropagation traditionally solid media is favoured over liquid media with agar often being the preferred gelling agent. The concentration of the gelling agent is associated with the water potential from the medium to the cultured plants, depending on the objectives of cultivation; e.g. shoot multiplication or root induction (DeBergh, 1983). Gellan gum (Gelrite) produces a clear gel, which allows for accurate observation of root induction, and it has been observed that many Australian woody plants have a greater survival rate when cultured in a Gelrite medium (Williams & Taji, 1987).

- De-ionised water; water is the universal solvent for all solutes required for micropropagation culture media (DeBergh, 1983).

- The media needs to then be dispensed into appropriate culture vessels and sterilised in an autoclave generally at 121°C and 1.2kg/cm² for 20 minutes.

### 1.3.3 Physical environment for micropropagation

The physical environment for micropropagation is as important as the appropriate culture media formulation. Light and temperature cycles, necessary for optimum growth of cultured plantlets, are programmable and controllable according to the individual plant species’ requirements (Johnson, 1996).
• Light: Plant development and growth is influenced by wavelength, intensity and the duration of light and this can be controlled in micropropagation according to plant species and the in vitro culture objectives. Light is also an important factor for phototropism, morphogenesis and photosynthesis (Read & Preece, 2003). It has been observed that light can enhance shoot growth and root formation in some species, whilst other species preferred darkness for root induction (Kumar, Palni & Nandi, 2003).

• Temperature: Plant development in micropropagation also depends on the optimum temperature range for the physiological process of respiration, but these optimum temperatures vary according to species and genotypes. Culture temperatures between 20° and 27° C are most commonly applied (Read & Preece, 2003).

• Gas exchange: In vitro plantlets are grown in culture vessels that present a closed system to prevent microbial contamination. This can limit the inflow of CO₂ and the outflow of O₂ and ethylene, although some gas exchange may occur depending on the type of culture vessels used (Park, Jeon, Kim, Park, Aswath & Joung, 2004). The relative humidity inside the culture vessel is usually very high, approximately 98-100% (Gribaudo, Restagno & Novello, 2003), which can be problematic for initial acclimatisation of plantlets from in vitro to ex vitro conditions.
1.3.4 Selection of Source Plants for Micropropagtion

Cuttings from different individual explants are likely to behave differently under culture conditions (Geneve, 1989; Anthony, McLean & Lawrie, 2000). Nevertheless, it is important to carefully select plant source material that is of vigorous, healthy appearance with the desired phenotypic attributes (Kartsonas & Papafotiou, 2007). The initial explant source selection is vital with regard to epigenetic and genetic characteristics of the source plants (Wiltshire, Potts & Reid, 1998), so the careful selection of source plants is crucial (Idczak, & Brielmaier-Liebetanz, 2003). This can ensure the cloning of the desired ‘true-to-type’ phenotypic plant material, although this can only be confirmed by close molecular examination (Arora, Sharma, Srivastava, Ranade & Sharma, 2011).

Epigenetic and genetic stability of micropropagated plants are an important pre-requisite and this has been investigated with various molecular techniques. Hop plants displayed epigenetic differences between the field grown mother plants and the micropropagated clones (Peredo, Arroyo-Garcia & Revilla, 2009). Kaeppler, Kaeppler & Rhee (2000) observed that there was phenotypic variation and temporary alterations in the physiology of the shoots in micropropagated plants compared to seed grown plants. But it was also concluded that over time, with repeated subculture, the phenotypic variation would initially accumulate but then reduce; the micropropagated material would become more similar to the source plant with culture age (Rodriguez Lopez, Wetten & Wilkinson, 2010). The long-term genetic stability was also confirmed in a study for banana clones (Lakshmanan, Venkataramareddy & Neelwarne, 2007).
1.3.5 Juvenility & Rejuvenation in Woody Plants in Tissue Culture

Several studies of the different phases in plant development have verified that there are four phases; embryonic phase (establishment of shoot and root meristems), juvenile phase (no reproduction capabilities), adult vegetative phase (establishment of reproductive capacity) and the adult reproductive phase (Hackett & Murray, 1992; Greenwood, 1995; Wiltshire, et al., 1998). These phases are stable and comparatively inconspicuous as the characteristics of one phase are usually replaced by traits that characterise the next phase (Conway & Poethig, 1993; Kerstetter & Poethig, 1998).

As with other methods of propagation, the micropropagation success of woody plant species can depend largely on the source plant’s juvenility status (Hackett, 1985), as the more juvenile explant is easier to reproduce than a more mature plant (Lullfitz, 2001). A cutting from a woody plant in the juvenile phase has greater ability to root and this is regarded as evidence that the plant has not yet reached maturity (Hackett, 1985; Jones, 1999). The juvenile stage is defined by gradual changes, e.g. biochemical, morphological and physiological characteristics, that occur in the young plant after germination (Haapala, et al., 2004).

One of the greatest limitations in slow growing woody perennials is their recalcitrance due to complex seasonal and life cycles, which can make it difficult to establish stabilised shoot cultures (McCown, 2000). This could be overcome with the generation of shoot culture by micropropagation techniques that utilise the plant’s meristematic tissues (Irish & Karlen, 1998) that can renew and sustain growth, and produce new plants from the existing vegetative structures (Schmidt, 1997; Franclet, 1991; Hackett & Murray, 1992). Each step of
micropropagation can be manipulated and has become the preferred method for production of woody plants that typically have long juvenility phases and slow breeding processes (Stokes, 1980; de Jeu & Cadic, 2000), as observed in many *Verticordia* species. Tissue culture can be a method to restore the juvenile phase in large amounts of explant material, as studies suggest that sub-culturing over time can re-establish juvenility in clones cultured from mature woody plants (McCown, 2000; Andreu & Marín 2005).

Hackett (1985) stipulates that juvenility of a woody species ends with the onset of flowering. Andreu & Marín (2005) discussed reinvigoration of mature woody plants during *in vitro* culture and established that the best multiplication results for shoot increase were gained from micropropagated plants that originated from cutting derived plants. These rejuvenated plants differ in physiology and often have a restored rooting capability that may only be temporary (Hackett & Murray, 1992; Haapala, *et al.*, 2004).

### 1.3.6 Morphological Characteristics of Micropropagated Plantlets

Most research suggests that genotypic and phenotypic variation seems to be a common occurrence observed in micropropagated plants. These variations can pre-exist in plant material from natural populations that has been used for tissue culture initiation or they can result from tissue culture conditions. They can be beneficial, as somaclonal variation can lead to the development of new varieties and/or the development and utilisation of desired traits (Skirvin, 1978; Larkin & Scowcroft, 1981; Perez, Mbohuli, Sagarra, Aragon, Gonzalez, Isidron & Lorenzo, 2011). But repeated sub-culturing of micropropagation can also result in observed
malformation of leaves, stems and roots; e.g. changes in leaf morphology resulting in thinner leaves, undifferentiated mesophyll, flattened chloroplasts and other changes at sub-cellular level which could affect some vital physiological processes like photosynthesis (Skirvin, 1978; Sebastiani, Minnocci, Vitagliano, Gribaudo & Novello, 2001; Romano & Loucao, 2003). Most micropropagated plantlets lack a well-developed and functional waxy cuticle, which acts as a barrier to prevent water loss, although the cuticle is present. This is problematic for ex vitro acclimatisation as in vitro plantlets cannot regulate their water balance and experience water stress (Skirvin, 1978; Fila, Ghashghaie, Hoarau & Cornic, 1998). Morphological changes observed in stems can include shorter internodes, thickened stems and stunted growth (Skirvin, 1978; Martin, 2003). Callus formation, deformed and brittle roots have been observed with in vitro root induction of shoots which can prove to be problematic when transferring rooted shoots from in vitro to ex vitro conditions, causing new roots to break (Kim, Klopfenstein & Cregg, 1998; Metivier, Yeung, Patel & Thorpe, 2007).

After successful acclimatisation most of these described morphological deficiencies and malformations improve with plant age and the plant’s functions (leaf structure, water relations, photosynthetic parameters) become comparable to the ‘stock’ plant in the field (Pospislova, Ticha, Kadlecak, Haisel & Plzakova, 1999; Agnihotri, Mishra & Nandi, 2009). DNA analysis can be used to confirm that in vitro cultured plants are genetically comparable and true-to-type of the wild host plant (Arora, et al., 2011).
1.3.7 Root Induction

In micropropagation of woody plants, adventitious root induction and formation are fundamental for successful production of viable plantlets. Many woody plants are recalcitrant and do not propagate easily due to the lack of morphological plasticity in their cells and tissues and require the application of exogenous auxins to induce adventitious root induction.

Auxins - The most commonly used auxins for root induction includes indole-3-butyric-acid (IBA), indole-3-acetic-acid (IAA) and α-naphthalene-acetic-acid (NAA), with IBA being the most effective and most commonly used. The different effects depend on the plant species, the concentration and the purpose of application (Ibrahim & Debergh, 2001; De Klerk, Van Krieken & De Jong, 1999). Different auxins, concentrations or combinations, may have to be applied depending on rooting treatments (in vitro or ex vitro) and on plant species (DeKlerk, terBrugge & Marinova, 1997). The application methods vary greatly, depending on the culture methods; for in vitro root induction the auxin is added to the culture medium, for ex vitro root induction the stems can be dipped in a highly concentrated auxin formula (Ibrahim & Debergh, 2001).

Substrate - For in vitro root induction the microcuttings are maintained in a culture media with low mineral concentrations (½ MS or less) and low auxin concentrations to stimulate adventitious root formation (Kim, et al., 2003). For ex vitro root induction, microcuttings can be pulsed on high auxin concentrations in a culture media with low mineral concentrations (½ MS or less) for usually 7 days (Newell, Growns & McComb, 2005) or the cuttings are dipped in high concentrations of auxins before being transferred to an organic and aerobic growing substrate (peat/perlite/sand) for root formation and simultaneous acclimatisation (Martin, 2003; Martin, 2003/04; Hazarika, 2003; Meiners, Schwab & Szankowski 2007).
1.3.8 Acclimatisation of in vitro Microcuttings to ex vitro Conditions

The transfer of microcuttings from in vitro to ex vitro greenhouse conditions has been problematic for a number of woody species including those related to Verticordia, e.g. Eucalyptus, Chamelaucium and Scholtzia (Beardsell, 1996; Johnston, 1996; Ben-Jaacov, et al., 2000; Lullfitz, 2001). Examination of the plantlet condition has resulted in the recognition that plants coming from tissue culture do not necessarily have normal physiological and morphological anatomy (Santamaria & Kertsins, 1994; Dami & Hughes, 1997). These functional differences include lack of junction between adventitious roots and stem vascular tissue, stomatal malfunction and the microcutting’s lack of a well-developed and functional cuticle that acts as a barrier to prevent water loss; this can cause plantlets to die of water stress (Skirvin, 1978; Fila, et al., 1998; Romano & Loucao, 2003). The survival rate of microcuttings to be acclimatised ex vitro can be increased significantly by maintaining high relative humidity (~ 90% - 98%) by using systems that apply water vapour, fog or mist periodically. In particular, the need for high humidity but low water droplet size has been emphasised by several authors (De Bergh, 1991; Drew, Smith, Moisander & James, 1991; Read & Preece, 2003; Johnson & Armstrong, 2003). This has been facilitated by the availability of fog generators that can deliver water droplets of less than 5µ, rather than misting systems.

The use of humidity tents can increase the retention of relative humidity and create a microclimate. This watering regime can be reduced as the microcuttings acclimatise (Offord & Campbell, 1992; Aragon, Escalona, Capote, Pena, Cejas, Rodrigues, Canal, Sandoval, Roels, DeBergh, & Gonzalez-Olmedo, 2005).
IBA pulsed \textit{in vitro} shoots of woody plants have successfully been tested for their ability to root under high humidity (Yan & Sedgley, 2006). New areas of micropropagation were being investigated and in 2003 Newell and co-workers reported work on shoot microcuttings of a \textit{Verticordia} species in a porous agar medium that contained air filled porosity from 10\% to 29\%. The researchers concluded that root induction and length was significantly increased compared to standard agar solidified media (Newell, 2003). Newell \textit{et al}. (2005) have proposed that an aerobic medium base may be important in facilitating transfer of \textit{in vitro} plants to \textit{ex vitro} conditions. It was concluded that for several woody perennials the rooting performance in \textit{In Vitro Soil} (IVS) was successful and this method could reduce the stress and plant loss during the acclimatisation stage of micro-cuttings from sterile laboratory conditions to field conditions (Newell, \textit{et al}.., 2005).

Alternatives to the \textit{in vitro} shoot multiplication (Phase 2) followed by \textit{in vitro} root induction (Phase 3) have also been examined. The simultaneous \textit{ex vitro} root induction and acclimatisation have been trialled as a cost and time reducing strategy (McClelland, Smith & Carthers, 1990; Kim, \textit{et al}.., 1998; Martin, 2003; Thomas & Schiefelbein, 2005; Feyissa, Welander & Negash, 2007; Xu, Wang & Zhang, 2008). Under these circumstances root induction and acclimatisation (Hazarika, 2003) occur simultaneously in a controlled environment such as a greenhouse, under conditions similar to those used for acclimatisation of \textit{in vitro} rooted plants. A major advantage of \textit{ex vitro} root induction is that root damage from de-flasking and transfer to soil, that can preclude growth and vigour, is avoided (Yan & Sedgley, 2006).
1.3.9 Acclimatisation

The transfer of plants from culture to soil has, in the past, caused considerable problems and has been the subject of discussion (Rohr, Iliev, Scaltsosyiannes & Tsoulpha, 2003) and even conferences on the topic (e.g. International Society of Horticultural Science has organised three international conferences on this topic in 2003, 2005 and again in 2007). In general, the requirements are dependent upon two major aspects: plantlet condition and the environmental conditions required for transfer. More recently, where these aspects have not been important or overcome (Newell, et al., 2005), other aspects have been concentrated upon; such as the media from which the plants have been derived and/or the number of roots produced per plantlet (McClelland, et al., 1990; Bonal & Monteuuis, 1997; Kim, et al., 1998; Bennett, McDavid, McComb, 2004).

The environmental conditions have received considerable attention and in recent years these have become more standardised for many species. The ultimate requirement of propagated plants is that they survive under the field condition for which they have been produced. To this end, while there has been some research done on the survival of plants produced from different modes of propagation, relatively little has been produced regarding the long-term growth and production. Comparisons between micropropagated plants and seedlings (Bell, van der Moezel, Bennett, McComb, Wilkins, Marshall & Morgan, 1993) and micrpropagated plants and cuttings (Bergman, 1998; Mutataya, Wilson, Ong, Deans & Sprent, 2002) have shown that any initial differences between plants are generally lost after long-term growth. This is particularly
important for forest species where it has been suggested that root development of micropropagated plants may lead to trees being susceptible to wind-throw (Bell, et al., 1993).

1.4 Aims

Research on verticordias has been fairly limited (McComb, Arthur & Newll, 1986; George, 1991; Tyagi, Considine & McComb, 1991; Tyagi, Considine & McComb, 1992; Housten, Lamont, Radford & Errington, 1993; Speer, 1993; McEvoy & True, 1995; Stummer, Smith & Langridge, 1995; Egerton-Warburton, Ghisalberti & Burton, 1998; George, 2002; George & George, 2003; Yates & Ladd, 2004), when considering the diversity of this genus and the common threat of habitat loss. The main objective of this research was to investigate and establish reliable and appropriate micropropagation, root induction and acclimatisation protocols for *V. grandis* in order to increase the quality and survival *ex vitro*, as results varied in previous studies.

1.4.1 Limitations of conventional propagation methods

There are several limitations in producing verticordias for horticultural, land restoration and conservation purposes; e.g. slow growth, limited seed production, seed recalcitrance and poor germination rates (Bell, 1999; Cochrane, et al., 2001). Conventional means of asexual propagation (cuttings and grafting) have been useful for a limited number of species, but most are not amenable to these processes. This is related to the availability of suitable cutting material from wild populations and genetic and environmental factors (Johnson, 1996). Often it
is difficult to obtain an adequate amount of the appropriate material to establish well-replicated and controlled experiments on the development protocols; e.g. it is very difficult, if not impossible, to obtain a large number of shoots, at the same stage of development (seasonal or ontogenetic age) and from a single genotype, for adequate replication.

Most available verticordia clones, such as *V. chrysanthella*, *V. mitchelliana* and *V. plumosa* have limited genetic variation, and have probably been selected on the basis of ease of propagation. In contrast, *Verticordia grandis* is a difficult-to-root species and micropropagation with consequent *in vitro* subcultures can induce rejuvenation or increased re-invigoration, which can increase rooting capacity.

Micropropagation has become a popular technique to overcome many of these limitations; stock plant material from difficult to propagate plants can be used to micropropagate large numbers of progeny plants.

1.4.2 Research aims

This research investigated the capacity to produce numerous explants by improving the existing micropropagation protocol (McComb, Newell & Arthur, 1986) for *Verticordia grandis*, as well as investigating the most successful root induction protocol for acclimatisation and survival of plantlets. This was achieved by determining the best method to achieve optimal shoots for micropropagation from mature woody cuttings. Plantlets from the clones were observed for juvenile growth phase traits in the growth of roots and shoots and the different modes of propagation were compared for the development of parameters that can increase growth and survival in field conditions.
For this study 2 clones of *Verticordia grandis*, which have been in culture for several years in the tissue culture laboratory at Edith Cowan University, were used, and 21 different *Verticordia* cuttings were introduced into culture.

Cuttings were taken from 4 different *Verticordia grandis* plants sprouting after a fire, 10 from new growth on different mature *Verticordia grandis* plants from various locations around Eneabba in Western Australia and cuttings from new growth of 7 different verticordia plants (5 from *V. ovalifolia*, 1 from *V. fragans* and 1 from *V. densiflora*).

The aim was to initially experiment with the existing clones of *Verticordia grandis* to establish reliable shoot multiplication, root induction and acclimatisation protocols and then to test the effectiveness of these on the newly introduced clones of verticordias for comparison.

### 1.4.3 Benefits of this research

In particular, the findings of this research could be beneficial to the reconstruction and rehabilitation of plant communities on disused mine sites, drilling exploration sites and other land cleared sites in the northern sand plain kwongan vegetation around Eneabba, Western Australia, the natural habitat of *Verticordia grandis*. The results of this research could also benefit growers of West Australian wild flowers, as especially *Verticordia grandis* is an attractive species for the export cut flower market (Seaton, 2002).
2. MATERIALS & METHODS

2.1 Plant material

All micro-cuttings (shoots) used initially for the optimisation of the existing protocol for shoot multiplication of *V. grandis* were obtained from two *V. grandis* clones (GRD 1; GRD 3) that had been in culture (at Edith Cowan University’s School of Natural Sciences Tissue Culture Laboratory) for over 5 years before experiments began.

In July 2007 explants of *V. grandis* (GRD) were collected from the Eneabba area; locations were recorded with GPS readings, host plant and site descriptions were noted and all material was cut from new shoot growth. Explant material from different new shoots of *V. grandis* (GF) that had resprouted after a fire were collected, as well as explant cuttings from *V. ovalifolia* (VOV), *V. densiflora* (VDF) and *V. fragrans* (VFR) (Table 2.1).

For each explant, a minimum of 5 - 10 cuttings of ~12cm length were collected, depending on the size of the host plant; e.g. the new shoots of the VGF clones were small, and the lignotuber was exposed ensuring that cuttings were taken from the same root. The cuttings were placed in a zip lock bag, kept on ice or at 4°C and processed in the laboratory the next day.

2.2 Process of initiation of new clones into culture

The cuttings were surface sterilised in a 2% benzalkonium chloride in 10% ethanol solution for 20 minutes, before being rinsed 3x in sterilized milliQ water, after which they were aseptically cut into ~0.5-1cm pieces at internodes. These were then transferred onto the 5 ml of shoot multiplication media in 50 ml flasks. Initially, a total of 548 flasks were prepared for each microcutting. Each uncontaminated, surviving microcutting was transferred daily on to fresh 5ml
of shoot multiplication media in 50 ml flasks in aseptic conditions for 28 days. After 4 weeks the surviving shoots were transferred to 250 ml culture vessels.

Table 2.1: The explant material for clones that were collected at different locations and habitats are as follows:

<table>
<thead>
<tr>
<th>Clone</th>
<th>Location</th>
<th>Habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRD4</td>
<td>S29° 58’ 19.2&quot;, E115° 18’ 51.7&quot;</td>
<td>Sand over laterite, heath</td>
</tr>
<tr>
<td>GRD5</td>
<td>S29° 58’ 19.2&quot;, E115° 18’ 48.4&quot;</td>
<td>Sand over laterite, heath</td>
</tr>
<tr>
<td>GRD6</td>
<td>S29° 58’ 13.8&quot;, E115° 18’ 51.7&quot;</td>
<td>Sand over laterite, heath</td>
</tr>
<tr>
<td>GRD7</td>
<td>S29° 54’ 22.7&quot;, E115° 15’ 02.9&quot;</td>
<td>Sand over laterite, heath</td>
</tr>
<tr>
<td>GRD8</td>
<td>S29° 43’ 12.9&quot;, E115° 13’ 33.6&quot;</td>
<td>Sand, heath, banksia</td>
</tr>
<tr>
<td>GRD9</td>
<td>S29° 43’ 23.4&quot;, E115° 13’ 21.8&quot;</td>
<td>Sand, heath, banksia</td>
</tr>
<tr>
<td>GRD10</td>
<td>S29° 43’ 5.5&quot;, E115° 13’ 18.1&quot;</td>
<td>Sand, heath, banksia</td>
</tr>
<tr>
<td>GRD11</td>
<td>S29° 42’ 12.1&quot;, E115° 13’ 29.3&quot;</td>
<td>Sand, heath, banksia</td>
</tr>
<tr>
<td>GRD12</td>
<td>S29° 42’ 10.5&quot;, E115° 13’ 29.6&quot;</td>
<td>Sand, heath, banksia</td>
</tr>
<tr>
<td>GRD13</td>
<td>S29° 42’ 09.8&quot;, E115° 13’ 27.1&quot;</td>
<td>Sand, heath, banksia</td>
</tr>
<tr>
<td>GF1</td>
<td>S29° 41’ 12.9&quot;, E115° 12’ 33.6&quot;</td>
<td>Burnt heath, woodland*</td>
</tr>
<tr>
<td>GF2</td>
<td>S29° 41’ 13.6&quot;, E115° 12’ 32.9&quot;</td>
<td>Burnt heath, woodland*</td>
</tr>
<tr>
<td>GF3</td>
<td>S29° 41’ 21.4&quot;, E115° 12’ 31.6&quot;</td>
<td>Burnt heath, woodland*</td>
</tr>
<tr>
<td>GF4</td>
<td>S29° 41’ 24.8&quot;, E115° 12’ 30.7&quot;</td>
<td>Burnt heath, woodland*</td>
</tr>
<tr>
<td>VOV1</td>
<td>S29° 57’ 13.1&quot;, E115° 16’ 15.8&quot;</td>
<td>Sand, heath, verge</td>
</tr>
<tr>
<td>VOV2</td>
<td>S29° 57’ 13.9&quot;, E115° 16’ 16.7&quot;</td>
<td>Sand, heath, verge</td>
</tr>
<tr>
<td>VOV3</td>
<td>S29° 57’ 14.3&quot;, E115° 16’ 16.8&quot;</td>
<td>Sand, heath, verge</td>
</tr>
<tr>
<td>VOV4</td>
<td>S29° 57’ 14.8&quot;, E115° 16’ 17.1&quot;</td>
<td>Sand, heath, verge</td>
</tr>
<tr>
<td>VOV5</td>
<td>S29° 57’ 16.4&quot;, E115° 16’ 19.9&quot;</td>
<td>Sand, heath, verge</td>
</tr>
<tr>
<td>VFR1</td>
<td>S29° 58’ 10.8&quot;, E115° 17’ 12.2&quot;</td>
<td>Remnant heath</td>
</tr>
<tr>
<td>VDF1</td>
<td>S29° 59’ 24.3&quot;, E115° 18’ 23.1&quot;</td>
<td>Private Property</td>
</tr>
</tbody>
</table>

Clones GRD1, 2 & 3 were in culture at ECU; GRD2 became contaminated and could not be used in experiments. *New shoot growth from roots after original plant was destroyed by fire. All others were from new seasonal shoots.

2.3 Media preparation

All micro-cuttings (shoots) used for the optimisation of the existing protocol for shoot multiplication of *V. grandis* were obtained from two *V. grandis* clones (GRD1; GRD3). The initial protocol was as follows: full strength Murashige & Skoog (MS) (Murashige & Skoog, 1962) medium supplemented with 1µM 6-benzylaminopurine (BAP), 1.5 µM Kinetin, 1.25 µM naphthalene acetic acid (NAA), 20 g L⁻¹ sucrose, pH adjusted to 5.8, solidified with 2.5 g L⁻¹
agar and 2.5 g L\(^{-1}\) gelrite. The media was dispensed (50 ml) into 250 ml culture vessels and autoclaved at 121°C for 20 minutes.

### 2.4 Culture conditions

All prepared liquid media, nutrient solutions and growth substrates were sterilised by autoclaving at 121°C for 20 minutes prior to application for all *in vitro* experiments conducted in the laboratory. All *in vitro* cultures were maintained in standardised conditions (under a 16h day with irradiance of 90µmol m\(^{-2}\) s\(^{-1}\) / 8h night photoperiod, with temperature at 25°C ± 1°C) for 28 days, before being aseptically sub-cultured for shoot multiplication or utilised for further experiments.

All *ex vitro* cultures (explants/plantlets) were maintained in standardised conditions (10 seconds of water misting at 7000 lux accumulated light, with temperatures at 25°C ± 5°C) in enclosed tents for high humidity (~90% - 98%) in the greenhouse for 28 days. Then the *ex vitro* cultures were moved out of the tents, assessed for root growth, and continued to be maintained in standardised conditions without the enclosed tent (10 seconds of water misting at 7000 lux accumulated light, with temperatures constant at 25°C ± 1°C and reduced humidity). The plants were assessed every 28 days and at 84 days the surviving plants were moved to the shade house for further hardening. The shade house did not have temperature or light control, but had automated, periodical watering (morning and evening sprinklers for 10 minutes). At 112 days the surviving plants were re-potted in a soil mixture (pasteurised) consisting of peat, perlite and white sand (1:1:1) and survival was assessed every 28 days.
2.5 Changes to shoot multiplication media composition

It was observed that using the original media formulation resulted in shoot multiplication of GRD1 clones being unvarying in shoot length and prolific, and in GRD 3 shoot multiplication was prolific, but with no consistent elongation; most of the shoots were less than 1cm long. Sub-cultured shoots of uniform length (~ 2 - 5cm) from both clones were needed for further experimentation and comparisons.

2.5.1 Testing various BAP concentrations to improve elongation in clones

The original shoot sterilised multiplication media was used as a control (1 µM, BAP) against media supplemented with 0.1 µM, 0.15 µM, 0.2 µM, 0.25 µM, 0.3 µM, 0.35 µM, 0.4 µM, 0.45 µM and 0.5 µM BAP. Twenty five randomly selected shoots of clones GRD1 and GRD3 were kept on the various media for 28 days and then harvested, after which shoot growth was measured; this experiment was repeated 2 times, with all the results combined. The mean growth for each concentration was calculated and the variance was analysed. The shoot multiplication media was changed, accordingly due to the results that were achieved, for all following media preparations.
2.6 Original root induction protocol

The success of micropropagated plantlets depends on viable adventitious root formation on shoots (with the help of exogenous growth regulators such as auxins), as this determines the survival of the new plants (Martin, 2003), but root induction for micropropagated *V. grandis* has proven to be difficult. The shoot length could also determine the efficiency of root formation, as longer shoots are in a better physiological condition for root induction (Xu, *et al.*, 2008).

For root induction experiments shoots of GRD1 (each 4.5 - 5 cm long) were randomly chosen from numerous culture vessels, leaves removed from the lower part of the stem (~1 cm) as wounding of the stem tissue can affect the balance of growth regulators, which can enhance root induction (Marks & Simpson, 2000). Shoots were grown in sterile rooting media (50 ml) modified from Bennett, *et al.*, (2004); it contained ¼ strength MS macronutrients and full strength micronutrients supplemented with 10 µM indol-3-butyric acid (IBA concentration depending on experiment), 2% sucrose, pH adjusted to 5.5, solidified with 2.5 g L⁻¹ agar and 2.5 g L⁻¹ gelrite in 250 ml clear culture vessels. Each vessel contained 50 ml of sterilised medium. The control rooting medium had no IBA. Each culture vessel contained 5 shoots and these were maintained in the growth room at standard conditions for 28 days, after which the results were analysed.

This protocol was used for all experiments – auxin pulsing duration and concentrations were changed in some of the following experiments. The shoots were always kept on the rooting maintenance media with no auxins for 28 days before being evaluated.
2.6.1 Optimisation of duration of IBA treatment for root induction

IBA has long been one of the preferred and active auxins to promote root formation in \textit{in vitro} cultured plant shoots (Epstein & Muller, 1993). Previous root initiation trials with the existing root induction protocol for \textit{V. grandis} by McComb, Newell & Arthur (1986) showed inconsistent and poor root growth of the \textit{V. grandis} clone for each trial. In order to determine the optimum IBA pulsing period for root induction, shoot replicates were grown on rooting media supplemented with 10µM of IBA (as per root induction protocol by Bennett, \textit{et al.}, 2004).

For each day of treatment 25 shoots of GRD1 were randomly chosen and the media of the control was not supplemented with IBA. The experiment was designed for 7 days; on each day 5 shoots, from 5 randomly selected vessels, were transferred to rooting media that contained no IBA. These were then kept in this media for 28 days, the experiment was repeated twice; the numbers of roots were counted and the means calculated for each day of pulsing. For this experiment only the root numbers/shoot were analysed in order to determine the most effect IBA pulsing time. This was repeated with GRD3 clones.

2.6.2 Optimisation of IBA concentration for root induction

The root formation performance is affected by the concentration of auxin, the type of auxin and the plant species. IBA is often the preferred auxin for root induction, as plant tissues can rapidly oxidise other auxins such as IAA (DeKlerk, \textit{et al.}, 1997). IBA can either promote the formation
of adventitious and lateral roots, or it can inhibit this process – this variation depends on the IBA concentration and the plant species (Muller, 2000).

For this experiment different IBA concentrations were tested to determine the optimal concentration to achieve the greatest number of shoots with root induction and growth. The randomly selected shoots (GRD1), 25 for each treatment, were pulsed for 6 days on media supplemented with different IBA concentrations (0, 2.5, 5, 10, 20, 40, 80 and 160µM). After this, all shoots were randomly transferred to culture vessels rooting media without IBA, visible root formation was recorded for each shoot on a daily basis and then totals scored after 28 days for statistical analysis and evaluation.

2.6.3 Trials with auxin combinations for root induction

In contemporary plant science research it has become evident that endogenous plant auxins interact with exogenous auxins resulting in diverse fast and slow responses (Woodward & Bartel, 2005). Different auxin combinations have been successful for root induction in numerous plant species due to their various properties (Martin, 2003; Martin, 2003/4; Pati, Rath, Sharma, Sood & Ahuja, 2006; Nandagopal & Kumari, 2007), so several combinations of auxins were experimented with in order to achieve more overall consistency in root response and to determine the optimal exogenous auxin treatment for root induction.
For this experiment it was assumed that 80 µM of an auxin combination would give a comparable result, as was achieved with 80 µM IBA, in order to determine greatest effectiveness for root induction and formation. The randomly selected shoots (GRD1), 25 for each treatment, were pulsed for 6 days on media supplemented with different auxin treatments: Treatments: no auxin, IAA (80µM), NAA (80µM), IAA (40µM) + NAA (40µM), IAA (40µM) + IBA (40µM), NAA (54µM) + IAA (26µM), NAA (40µM) + IBA (40µM), IAA (54µM) + NAA (26µM), IAA (26µM) + NAA (26µM) + IBA (26µM) or IBA (80µM - control). After this, all shoots were randomly transferred to culture vessels rooting media without auxins, visible root formation was recorded for each shoot on a daily basis and then totals scored after 28 days for statistical analysis and evaluation.

2.7 Trials with different in vitro substrates for root induction

In 2003, Newell and co-workers reported results of experiments on shoot microcuttings of Verticordia species using a porous agar medium that contained air filled porosity of 10% to 29% to enhance root formation. The researchers concluded that root induction and length was significantly increased compared to standard agar solidified media (Newell, 2003). Another alternative in vitro rooting substrate being researched by Newell et al., (2005) involves in vitro – soil medium (IVS), which consists of an aerobic rooting medium and the results were compared with agar-solidified media. It was concluded that for numerous woody perennials (including species from Myrtaceae) the rooting performance in IVS was successful and this method could reduce the stress and plant loss during the acclimatisation stage of micro cuttings from sterile in vitro conditions to in vivo field conditions (Newell, 2006). Newell, et al., (2005) and Yan &
Sedgley (2006) experimented with different substrates including IVS (\textit{in vitro} soil media) instead of agar based media on difficult to root recalcitrant woody plants to increase root formation and vigour.

For this experiment different substrates were trialled; some were commercially available whilst others were prepared prior to the experiment. The shoots (25 for each treatment, randomly chosen) were pulsed on agar/gelrite media supplemented with 80 µM IBA and after 6 days these were randomly transferred to culture vessels that contained a uniform amount of sterilised substrate (autoclaved at 121°C for 20 minutes for each different substrate). This was supplemented with 50 ml sterilised liquid nutrient solution containing ¼ strength MS macronutrients and full strength micronutrients supplemented with 2% sucrose, pH adjusted to 5.5. The substrates included: Agar/gelrite, White sand (Ø ≤ 1 mm), white sand (Ø ≥ 1 mm), sterilised soil (2 peat: 1perlite), Jiffy pots®, black sand (Ø ≤ 1 mm) and rockwool®. This was repeated with fewer treatments that included: Agar/gelrite, blacks and sterile soil (2 peat: 1 perlite).

2.7.1 Comparison of sterilized soil with different nutrients

In order to test if GRD1 clones will initiate roots (after a 6 day IBA pulsing) in sterilized soil without additional nutrients, the following experiment was conducted: 25 shoots for each treatment (randomly chosen) were pulsed on agar/gelrite media supplemented with IBA and after 6 days these were randomly transferred to culture vessels that contained a uniform amount of sterilised sterilised soil (2 peat: 1 perlite). According to treatment some were supplemented with
50 ml sterilised liquid nutrient solution containing ¼ strength MS macronutrients and full strength micronutrients supplemented with 2% sucrose, pH adjusted to 5.5 (control), some with just 50 ml sterilised milliQ water supplemented with 2% sucrose, pH adjusted to 5.5 and the others just with 50 ml sterilised milliQ water. These culture vessels were kept in the culture room for 28 days, after which the roots were scored. The entire root system of each shoot was carefully separated and scanned for root surface area and length for comparison and statistical analysis.

2.7.2 Comparison of in vitro sterilized soil to ex vitro soil

In order to test if GRD1 clones will form roots (after a 6 day IBA pulsing) in soil ex vitro without additional nutrients, the following experiment was conducted: 25 shoots of GRD1 for each treatment (randomly chosen) were pulsed on agar/gelrite media supplemented with IBA and after 6 days these were randomly transferred to crackpots (direct soil) containing the same soil composition as the control (sterilised soil in culture vessels that contained a uniform amount of soil media supplemented with 50ml sterilised milliQ water, no nutrients). The culture vessels were kept in the culture room and the other shoots were transferred to the greenhouse in seed trays with vented lids at a temperature regime of 20-30 °C in high humidity (~90% - 98%). After 28 days the roots were scored; the entire root system of each shoot was carefully separated, scanned for root surface area and length and root numbers were counted for comparison and statistical analysis. All fresh shoots were separated from all roots and weighed then all shoots and roots were dried at 70º C for 24 hours and weighed again to determine the shoot and root biomass for statistical analysis.
2.8 Survival in greenhouse during acclimatisation phase

For comparison 180 shoots of GRD1 clones (after *in vitro* IBA pulsing) were transferred to sterilized soil (with just 50ml millQ water) *in vitro* and then after 28 days were re-potted in to crackpots containing the same soil mixture, which were placed randomly in humidity tents that allowed for retention of high humidity (90-98%) with periodical misting. In order to facilitate rooting simultaneously with acclimatisation 180 shoots of GRD1 clones (after *in vitro* IBA pulsing) were, at the same time, placed randomly (arranged in a randomised complete block design) in crackpots containing a uniform soil mixture in humidity tents that allowed for retention of high humidity (90-98%) with periodical misting. The survival rates for both treatments were scored in intervals of 28 days for the initial acclimatisation in humidity tents, and then the shoots were moved out of the humidity tents to reduce humidity (~60%). All shoots remained in the greenhouse until roots were visible at the bottom of the crackpots or until they died. All were scored after 56 days. The surviving plantlets were scored every 28 days, and then remaining shoots were moved to a shady area in the glasshouse (at 84 days) where the humidity was less, but a periodical watering regime was in place. After 112 days the surviving shoots were scored again and then moved to the shade house for further *in vivo* acclimatisation. The shade house had a restricted water regime of once in the morning (10 mins) and once in the afternoon (10 mins). These surviving plantlets were then re-potted into larger pots containing the peat and perlite mixed with sand (1:1:1) for additional drainage. Long-term survival after re-potting continued to be assessed every 28 days.

The pulsed shoots were transferred to the greenhouse on March 8\(^{th}\), 2008, and this process was repeated 6 times. Again after 112 days all surviving plants were moved to the shade house with
ambient temperatures and regular watering. Long-term survival was monitored for over 252 days in total.

2.9 *In vitro* shoot multiplication of new GRD clones

Following the success of the shoot multiplication protocol for GRD1 & 3 the effectiveness was tested for other clones. Shoot multiplication for new clones introduced into culture (Table 2.1) was not achieved for all clones; VOV, VDF and VFR did not produce shoots that could be sub-cultured over time. Shoot multiplication proved to be difficult for GRD 4, 5, 7, 8 and 13. The most successful shoots for multiplication were GRD 6, 9, 10, 11, 12 and GF1, 2, 3 and 4 (no results shown here), as these clones continuously produced multiple and uniform shoots. The improved protocol for shoot multiplication was used on all new clones to produce comparable shoots with a uniform length of ~4.5-5cm for root induction.

2.9.1 Root induction of successful new clones

Root induction trials with the most successful new clones commenced approximately after 1 year in culture. The optimised root induction protocol was applied to shoots that were pulsed on IBA/agar media and then transferred to crackpots with a uniform soil mixture, placed in the humidity tents in the greenhouse and monitored. In order to facilitate rooting simultaneously with acclimatisation, 40 shoots of various clones (after *in vitro* IBA pulsing) were placed randomly (arranged in a randomised complete block design) in crackpots containing the uniform soil mixture in humidity tents that allowed for retention of high humidity (90-98%) with periodic
misting. The results were scored every 28 days for analysis. These trials were repeated 3 times during 2008/09. The means for survival were calculated (rounded to a complete number) and results were combined for final analysis for all plantlets that performed well in culture. The following clones were used because of their similar physiology; e.g. fairly uniform length, large leaves and vigorous appearance – GRD 6, 9, 10, 11, 12, GF1, 2, 3, and 4 (although 1 and 2 had longer internodes than 3 and 4). All surviving plantlets were re-potted at 140 days (28 days later than the experiments with GRD1) in peat and perlite mixed with sand (1:1:1) for extra drainage. Re-potting was done later (compared to trails with GRD1) because not all plantlets had roots visible simultaneously by 56 days.

2.10 Statistical analysis

All experiments were randomised (or complete randomised block design was used) and analysis of variance (ANOVA), as a linear model, was applied to determine differences of means between clones and treatments. Levene’s test of homogeneity of variance was applied to all data sets and where variance were found to be unequal a log + 1 transformation was performed. Different post hoc tests were used to determine differences between treatments; Tukey’s B multiple-range test (P ≤ 0.05), or Dunnet’s t-test, where all groups were compared against the control group. All root lengths and surface areas were measured using an automatic root analyser – Delta-T-Scan and Delta-T-Scan software. For the analysis of long-term survival of rooted explants in the shadehouse a $\chi^2$ test was used and resulting values were compared to a $\chi^2$ critical table to determine significant probability values (P ≤ 0.05). SPSS version 17 was used for all statistical analysis.
3. RESULTS

3.1 Survival of new clones initiated into culture

A total of 380 shoots did not survive the first 2 weeks because of shoot death or contamination. Many shoots had varying axillary bud formation and shoot elongation, some to the extent that sub-culturing was not possible; these shoots were just transferred to fresh media every 28 days. Following subsequent sub-culturing of the clones every 28 days many more shoots died; after 6 months there were no *V. ovalifolia* (VOV), *V. fragrans* (VFR) or *V. densiflora* (VDF) shoots remaining (Table 3.1).

**Table 3.1:** Percentage Survival of new microcuttings over a 28 day period with cuttings being transferred to new media in 50ml flasks daily.

<table>
<thead>
<tr>
<th>Clone</th>
<th>Start (n)</th>
<th>Survival (%)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRD4</td>
<td>18</td>
<td>39 33 22 22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRD5</td>
<td>25</td>
<td>64 40 28 24</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>GRD6</td>
<td>35</td>
<td>69 69 69 69</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRD7</td>
<td>42</td>
<td>50 45 38 31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRD8</td>
<td>34</td>
<td>68 65 56 56</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRD9</td>
<td>17</td>
<td>71 59 53 47</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRD10</td>
<td>28</td>
<td>89 86 79 79</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRD11</td>
<td>18</td>
<td>56 28 22 17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GRD12</td>
<td>23</td>
<td>65 30 13 13</td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>27</td>
<td>70 41 37 37</td>
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</tr>
<tr>
<td>GRD17</td>
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<td>44 33 25 23</td>
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<td></td>
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</tr>
<tr>
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<td>27</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>VOV3</td>
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<td>57 28 28 7</td>
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</tr>
<tr>
<td>VOV4</td>
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<td>94 29 24 0</td>
<td></td>
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</tr>
<tr>
<td>VOV5</td>
<td>21</td>
<td>57 43 43 5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VFR1</td>
<td>29</td>
<td>52 52 45 45</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VDF1</td>
<td>24</td>
<td>54 46 42 37</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.2 Changes to shoot multiplication protocol

It was observed that using the original media formulation resulted in shoot multiplication of GRD1 clones having variable shoot length and proliferation, and in GRD 3 shoot multiplication was prolific, with but with no consistent elongation; most of the numerous shoots were less than 1 cm in length. Sub-cultured shoots of uniform length (~ 2 - 5 cm) from both clones were needed for further experimentation and comparisons. In order to improve shoot elongation necessary for shoot multiplication and proliferation of unvarying shoot lengths it was decided to modify the BAP concentration supplemented to the growth media.

The highest number of shoots with a more uniform shoot height was achieved at 0.25µM BAP, as the shoots of both clones (GRD1 – 2.3 cm & GRD3 – 2.1cm) were comparable for shoot elongation (Fig 3.2). All other BAP concentrations produced shoots of varying lengths (the shortest growth 0.7cm for GRD3 at 1µM and GRD1 was 1.1cm at 0.45 µM). The new shoots appeared much more vigorous and healthier in growth (a more visibly uniform leaf size and uniform shoot lengths) compared to most explant shoots before the experiment. The most unvarying height for both GRD1 & GRD3 shoots were achieved at 0.25 µM of BAP added to the shoot multiplication media. These results substantiated research that suggests that axillary bud formation and shoot height could be promoted by using low concentrations of BAP. Consequently the BAP concentration was changed to 0.25 µM for all following shoot multiplication media.
Figure 3.2: Comparisons of different BAP concentrations to improve shoot heights in vitro. The concentrations of 0.25 and 0.3µM BAP were significantly different (P ≤ 0.042) for GRD 1. For GRD 3 the concentration of 0.25µM BAP was significantly different (P ≤ 0.029). Means were analysed using Anova; n=25; different letters above bars indicate significant differences (ANOVA, Tukey’s test P ≤ 0.05); Error bars = ± SE.
3.3 Changes to root induction media composition

The most commonly used auxins for root induction includes indole-3-butyric-acid (IBA), indole-3-acetic-acid (IAA) and α-naphthalene-acetic-acid (NAA), with IBA often being the most common one used for root induction. In previous root initiation trials, using the existing root induction protocol (root induction media supplemented with 10µM of IBA) for *V. grandis* by McComb, Newell & Arthur (1986), the results showed inconsistent and poor root growth of the *V. grandis* clone (GRD1) for each trial. It was decided to determine optimal IBA pulsing duration and concentration.

3.3.1 Optimisation of duration of IBA pulsing

In order to determine the optimum IBA pulsing period for root induction, shoot replicates were grown on rooting media supplemented with 10µM of IBA. Root formation differed for the shoots that were pulsed with IBA for 5 and 6 days; root initials became visible just 7 days after the transfer from IBA pulsing media to non-IBA root growth media, and most shoots in all of these culture vessels had adventitious root growth visible after 17 days (Figure 3.3a). Shoots that were pulsed with IBA for 4 days showed root growth on most shoots after 23 days, whereas the shoots that were pulsed with IBA for 1, 2, 3 and 7 days showed poor root induction, with many of these shoots not forming roots at all. The highest percentage of root formation after 28 days was only 45% after 6 days pulsing on 10 µM IBA, which was very low when compared to the original root induction protocol results (McComb, *et al.*, 1986).
**Figure 3.3:** Comparison of duration of IBA pulsing for root formation and growth *in vitro*. a) The means of roots for days 1, 2, 3 & 7 were not statistically different ($P \leq 0.125$), but different to the means of roots ($P \leq 0.031$) for days 4, 5 & 6 which had no statistical difference. b) The greatest percentage of roots after 28 days resulted with shoots pulsed for 6 days. Means were analysed using Anova; $n=25$; different letters above bars indicate significant differences (ANOVA, Tukey’s test $P \leq 0.05$); Error bars = ± SE.
Some shoots of GRD3 clones readily displayed root formation on shoot multiplication media so this experiment was repeated twice for GRD3 clones. For GRD3 only the control treatment had one shoot with visible roots compared to the other treatments, which had no results (after 28 days all shoots had no visible root formation); consequently no analysis was possible.

All further experiments for root induction only used GRD1 clones and 6 days auxin pulsing for root induction.

### 3.3.2 Optimisation of IBA concentration

Root formation was greatest for the concentration of 80 µM IBA with a mean of 7 ± 1.2 roots/shoot, followed by 6.3 ± 1.0 roots/shoot for 40 µM IBA and 5.7 ± 1.1 roots/shoot at 20 µM IBA (Fig 3.4). Root initials for these treatments became visible by day 5 and 6, after transfer to media without IBA. Root formation on shoots pulsed on media with the other IBA concentrations became visible much later (day 18-25), if at all, with many shoots not producing any roots. The lower concentrations (0=control, 2.5µM, 5 µM & 10 µM) of IBA had little effect on root response and formation. All further experiments used 80 µM of auxin for root induction pulsing.
Figure 3.4: Comparison of in vitro root induction and growth using different IBA concentrations. a) Mean roots per shoot. Treatments with 0, 2.5, 5, 10 and 160 µM IBA were not statistically different (P ≤ 0.237), but different to the means of roots (P ≤ 0.029) for treatments with 20, 40 and 80 µM IBA were not statistically different. b) Mean percentage rooting. Means were analysed using Anova; n=25; different letters above bars indicate significant differences (ANOVA, Tukey’s test P ≤ 0.05); Error bars = ± SE.
3.3.3 Auxin combinations for *in vitro* root induction

Root formation in the control (80 µM IBA) was again visible 6 days after transfer to an auxin free media and after 17 days 24/25 shoots had roots from this treatment (Fig 3.5). Root formation for all other treatments were significantly lower and most roots appeared visible after 19 days. The treatment with no auxin had 1/25 shoots with 2 small (>0.3cm) roots. Treatments containing NAA (3, 4, 6, 8 and 9) produced large calluses at the shoot base and means of 0.8 \(\pm\) 0.3 and 1.7\(\pm\)0.4 roots/shoot. IAA did was not very effective for root induction; Treatments 2 and 4 only produced a mean of less than 1 \(\pm\) 0.2 root/shoot. Treatment 5 and 7 contained an auxin combination with IBA and produced means of 3.4 \(\pm\) 0.5 and 3.8 \(\pm\) 0.7 roots/shoot, which were more roots than treatments without IBA, but these results were not statistically different. For all further experiments 80 µM IBA was used for root induction.

Commercial auxin applications (Clonex powder & gel for soft and semi-hard wood) and prepared auxin solutions for dipping the shoots prior to transfer to soil for root induction and simultaneous acclimatisation was also trialled. However, within a few days all shoots were dead, so this was not further investigated and as no results were obtained, they were not recorded.
**Figure 3.5:** Comparison of *in vitro* root induction and growth using different auxin combinations and concentrations. There were no differences (P ≤ 0.351) between treatments 1 – 9, compared to the control treatment 10 that was significantly different (P ≤ 0.017). Means were analysed using Anova; n=25; different letters above bars indicate significant differences (ANOVA, Tukey’s test P ≤ 0.05); Error bars = ± SE.
3.4 Different substrates

Many alternatives to gel media have been investigated; e.g. foam, vermiculite, vermiculite and gelrite, rock wool, filter paper bridges, glass beads, coir, *luffa* –sponge, jute fibre and *in vitro* – soil / IVS, as these substrates can facilitate improved gas exchange conditions.

Trials with different substrates (Figure 3.6) resulted in good rooting response with black sand (8.1 ±2.7 roots/shoot, many branched with lateral roots), rock wool (6.6 ±2.9 roots/shoot) and agar (4.4 ±2.0 roots/shoot, few branched with lateral roots). Also shoots grown in black sand and rock wool appeared different to those grown in agar medium by having more, longer and some laterally branched roots. The first roots became visible in agar after 7 days, in rock wool several roots protruded at the sides after 13 days and in black sand some roots were visible at the side and bottom of the culture vessels by day 10. In the other treatments, the first roots became visible later; white sand (0.9 ±2.7 roots/shoot) by day 17, coarse white sand (1.7 ±2.9 roots/shoot) by day 16, sterile soil (2.7 ±2.8 roots/shoot) by day 14 and roots protruded from soil jiffies (3.3 ±2.8 roots/shoot) by day 14. Overall, the early visibility of roots in the different substrates suggests a faster rooting response (compared to previous trials with agar). However, the individual roots growing from shoots in agar were the only ones that could be clearly observed on a daily basis.
The roots from shoots grown in soil jiffies and rock wool were problematic to score, as each contained 5 shoots and the roots were difficult to completely separate and remove intact after 4 weeks of growth. However, each shoot had formed at least 1 root (often branched with lateral roots), and some shoots had small white outgrowths from the swelling at the shoot base; it is possible that these could have developed into roots if given more time.

**Figure 3.6:** Comparison of *in vitro* root induction and growth in different substrates. The means of roots between treatments white sand (≤1mmØ), coarse white sand (≥2mmØ), sterile soil (peat:perlite = 1:3) and commercial soil jiffies were not statistically different (P ≤ 0.276), but different compared to the means (P ≤ 0.022) of the roots between treatments black sand (≤1mmØ), commercial rock wool cubes (5cm³) and the control (agar/gelrite medium) which were not significantly different. This experiment was repeated twice to compare treatments Control, Sand 3 and Rockwool only, as they had higher means of root numbers per shoot, confirming no significant difference (P ≤ 0.153). Means were analysed using Anova; n=25; different letters above bars indicate significant differences (ANOVA, Tukey’s test P ≤ 0.05); Error bars = ± SE.

The roots from shoots grown in soil jiffies and rock wool were problematic to score, as each contained 5 shoots and the roots were difficult to completely separate and remove intact after 4 weeks of growth. However, each shoot had formed at least 1 root (often branched with lateral roots), and some shoots had small white outgrowths from the swelling at the shoot base; it is possible that these could have developed into roots if given more time.
3.4.1 Comparison of agar, black sand and sterile soil

The root formation of GRD1 clones in black sand was statistically comparable with agar (Figure 3.6), so the affect of root induction in these substrates was compared to sterile soil rooted shoots. Root numbers alone, where short and long roots are counted as equal, are not always an ideal parameter when comparing plant root systems and therefore root surface area and root length were also measured.

There were no statistical differences for means of root numbers (P=0.012) between treatments (Figure 3.7a). The root surface area means in Figure 3.7b (13.9cm² ± 1.1) for shoots maintained in sterile soil were statistically different compared to root surface area means of roots grown in agar (8.4cm² ± 1.6) and in black sand (11.9cm² ± 1.8). The sterile soil and black sand roots (less in black sand) were visibly different as well; these had more branched lateral root formation compared to roots from agar that consisted of single roots of varying lengths. It was also observed, whilst separating the roots from the substrate, that not all roots were submersed in the nutrient liquid. The mean lengths (Figure 3.7c) were for roots grown in sterile soil (6.2cm ± 2.0) were statistically different compared to roots grown in black sand (5.7cm ± 1.8) and agar (4.3 ± 1.7). Roots grown in agar were mostly single roots with no branching, compared to the branched roots of various thicknesses from the other treatments of in vitro black sand and in vitro sterile soil (Fig 3.8).
Figure 3.7: Comparison of *in vitro* root induction and growth in different substrates
a) Mean roots per shoot. nsd (P=0.012). b) Mean root surface areas grown in agar/gelrite = control were statistically not different to black sand (P=0.113) compared to roots grown in sterile soil (P=0.014). c) Means of root length grown in agar/gelrite = control were statistically not different to black sand (P=0.245) compared to roots grown in sterile soil (P=0.037). Means were analysed using Anova; n=25; different letters above bars indicate significant differences (ANOVA, Tukey’s test P ≤ 0.05); Error bars = ± SE.
Figure 3.8: Comparison of harvested and washed roots grown in different in vitro substrates. a) Shoot with root formation grown in agar. b) Shoot with root formation grown in black sand. c) Shoot with root formation grown in sterile soil.

For further in vitro experiments only sterile soil was used.

3.5 Comparison of sterile soil with different nutrients

The previous experiment achieved good root induction on GRD1 clones using the aerated, porous substrate of sterile soil instead of an agar medium. This led to experimenting root induction with sterile soil supplemented with just sucrose, with complete nutrients and with just DDI water as a control.

The mean root numbers for sterile soil +DDI (4.1 ± 1.2), sterile soil + sucrose (4.6 ± 1.8) and for the control, sterile soil + nutrients (5.6 ±1.7) were not statistically different (Fig 3.9a). The means for root surface area (Fig 3.9b) for shoots grown in sterile soil + DDI (3.9 cm² ± 1.6) and for shoots grown in sterile soil + sucrose (5 cm² ± 2.0) were statistically different from shoots grown in the control of sterile soil + nutrients (11.8 cm² ± 1.9). The root surface area for the control treatment (sterile soil + nutrients) was also less than in the previous experiment (Fig 3.7b, 13.9 cm² ± 2.0). The mean root lengths for sterile soil +DDI (4.4 cm ±0.4), sterile soil + sucrose (4.6 cm ±0.1) and for
the control, sterile soil + nutrients (6 cm ± 0.6) were not statistically different (Fig 3.9c). This experiment showed that root induction and growth was possible without added nutrients in the substrate, so just soil was used for further experiments.

Figure 3.9: Comparison of *in vitro* sterile soil with different nutrients (no nutrients). a) Mean root numbers b) Mean root surface c) Mean root Means were analysed using Anova; n=25; different letters above bars indicate significant differences (ANOVA, Tukey’s test P ≤ 0.05); Error bars = ± SE.
3.6 *Ex vitro* root formation and acclimatization

Combined *in vitro* shoot multiplication with *ex vitro* root formation and acclimatisation has been trialled to enhance survival of plantlets, as well as a cost and time reducing strategy. For this experiment *in vitro* (sterile soil) and *ex vitro* (direct soil in greenhouse) root formations were compared.

It was not possible to observe when root initials began to appear and compare for either treatments. Root numbers (Fig 3.10a) were not statistically different (sterile soil 2.1 ± 0.2 roots/shoot, direct soil 1.9 ± 0.1 root / shoot). The mean root surface area for sterile soil was 7.9 cm² ±0.9 compared to 6.3 cm² ± SE 1.0 for direct soil. Even though the results were statistically different (Figure 3.10b), each shoot had roots after 28 days. After 28 days of growth the mean root length in sterile soil was 4.8 cm ±0.4 and the mean length in direct soil was 4.7 cm ± 0.4, which were statistically not different (Figure 3.10c). Root morphology appeared similar, as root branching were visible on roots from both treatments. Means of dry shoot biomass (Figure 3.10d) were not statistically different between treatments (sterile soil 0.88g ± 0.3, direct soil 0.64g ± 0.3) and the means for dry root biomass (Fig 3.10e) were also not statistically different (sterile soil 0.53g ± 0.2, direct soil 0.48g ± 0.2).
Figure 3.10: Comparison of *in vitro* and *ex vitro* root induction and growth after being in soil for 28 days. a) The mean number of roots per shoot b) Mean root surface areas c) Mean root d) The mean shoot dry weights e) Mean root dry weights. Means were analysed using Anova; n=25; different letters above bars indicate significant differences (ANOVA, Tukey’s test \( P \leq 0.05 \)); Error bars = ± SE.
3.7 Survival

The greenhouse environment consists of a septic environment with higher light levels and lower relative humidity which are the opposite to *in vitro* conditions. Due to these different ambient conditions transfer from *in vitro* to *ex vitro* is stressful for the plantlets, but the main success of micropropagation largely depends on the plantlets capability to resume growth during and after acclimatisation. This experiment was started simultaneously for all shoots at 28 days following the completion of IBA pulsing and long-term survivals were recorded (Figure 3.11).

Shoots kept in *in vitro* conditions for root growth, then transferred to soil in crackpots after 28 days, did not acclimatize easily to the greenhouse conditions; many wilted early which often resulted in death. After 140 days there was 100% mortality recorded for the *in vitro* rooted shoots. There was a high survival rate for shoots transferred to direct soil immediately after the IBA pulsing period in *ex vitro* conditions for acclimatisation in the greenhouse. The greatest plant loss occurred at 28 days when plants were moved out of the enclosed crackpot trays (high humidity) into lower humidity levels of the greenhouse. The greatest shoot loss was observed in the first 28 days for shoots transferred to the humidity tent in March. Some plantlets did not acclimatise and survive every time environmental conditions were changed (transfer to lower humidity; e.g. from closed trays, to open trays, from greenhouse to shade house); the greatest mortality rate was scored for the first trial. After a total of 6 repeats (Table 3.2) it was evident that with each change of environmental conditions total plantlet mortality increased, as some failed to acclimatise and thrive. After 112 days all surviving plantlets were re-potted and the following mortality rates (140 days) were further recorded. Following re-potting, most of the
surviving plantlets displayed new shoot growth: either branching or shoot elongation, which occurred at the end of winter and in early spring. After 252 days more than 70% of the plants (*ex vitro* root growth) had survived (Figure 3.11; Table 3.2).

![Figure 3.11](image.png)

**Figure 3.11** Periodic assessment of *ex vitro* grown roots and *in vitro* grown roots of GRD1 (180), acclimatisation and survival (initial experiment) was assessed every 28 days after the initial transfer to soil. All plants had visible roots after 48 days. Plants were re-potted into bigger pots with fresh soil (peat, perlite, sand 1:1:1).

The experiment with *in vitro* pulsed shoots and *ex vitro* root growth combined with simultaneous acclimatisation was repeated with larger sample sizes and the results were recorded (as follows in Table 3.2). After 252 days more than 72% of the GRD1 plants survived simultaneous *ex vitro* root growth and acclimatisation.
Table 3.2: Results from repeat experiments of survival rates of 1080 GRD1 shoots (ex vitro root induced) in a total of 27 trays @40 shoots each) during 2008 & 2009.

<table>
<thead>
<tr>
<th>Period</th>
<th>Plants survived</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>28 days</td>
<td>1068</td>
<td>98.9%</td>
</tr>
<tr>
<td>56 days</td>
<td>1029</td>
<td>95.3%</td>
</tr>
<tr>
<td>84 days</td>
<td>987</td>
<td>91.4%</td>
</tr>
<tr>
<td>112 days</td>
<td>956</td>
<td>88.5%</td>
</tr>
<tr>
<td>140 days</td>
<td>845</td>
<td>78.2%</td>
</tr>
<tr>
<td>168 days</td>
<td>839</td>
<td>77.6%</td>
</tr>
<tr>
<td>196 days</td>
<td>827</td>
<td>76.6%</td>
</tr>
<tr>
<td>224 days</td>
<td>813</td>
<td>75.3%</td>
</tr>
<tr>
<td>252 days</td>
<td>786</td>
<td>72.8%</td>
</tr>
</tbody>
</table>

During the following 2 years after these experiments started most plants had died, even though they were initially thriving for the duration (252 days) of the experiment.

3.8 Effectiveness of improved protocols on new clones

3.8.1 Shoot growth and maintenance

The improved shoot multiplication was not successful for all new clones introduced into culture. All clones of VOV, VDF and VFR did not produce shoots that could be sub-cultured over time, or they eventually died. The most successful new clones for shoot multiplication were GF 1, 2, 3 and 4, GRD 5, 8, 9, 10, and 11; these all produced shoot material that was appropriate for subcultures and for root induction trials.
3.8.2 Root induction, growth and acclimatisation

Root induction trials with the most successful new clones commenced approximately after being in culture for over 1 year. The optimised root induction protocol was applied to shoots where root induction and acclimatisation would occur simultaneously. Shoots were pulsed on IBA/agar media and then transferred to crackpots with IVS, placed in the humidity tents in the greenhouse and monitored. Long-term survival rates were monitored.

The results from Figure 3.12 show that only an average of 51% of plantlets survived acclimatisation in the long term (GRD3 & GRD4 did not survive). The majority of plantlets showed roots at the bottom of the crackpots by 84 days and initial survival rates were relatively high during this period. At 112 days ~79% of the total plantlets were re-potted, but in the remaining weeks of the experiment many plantlets did not survive. After 1½ years post ex vitro transfer most of these plantlets had not survived and during this time very few displayed visible shoot growth or vigour.
Figure 3.12: Periodical assessment of acclimatization and ex vitro survival over 252 days of 11 clones of *V. grandis* (40 shoots of each clone). Plants were assessed every 28 days and all surviving plants had visible roots 84 days after transfer to soil.
4. DISCUSSION

4.1 Collection and survival of new clones initiated into culture

Micropropagation has become an invaluable tool in the plant propagation industry; e.g. ornamental horticulture and agriculture (Johnson, 1996; De Jeu & Cadic, 2000; DeKlerk, 2002; Sarasan, et al., 2006). This propagation method presents a viable option to achieve commercial success for the production of disease free plants in large quantities (Bandyopadhyay, et al., 1999) and in general is based on the principle of establishing and maintaining healthy micro-shoots in culture for multiplication so that these produce rooted micro-cuttings and plantlets (DeKlerk, 2002; Liu & Bao, 2003). There are no effective vegetative propagation techniques for reproducing numerous mature plants that can equal this.

New clones of *V. grandis* and other verticordia species were introduced into culture to test if optimised shoot multiplication and root induction media could be successfully applied to these. The introduction of new clones was only partially successful (Table 3.1), even though the source plants for cutting materials were carefully selected from wild verticordia populations in natural habitats around Eneabba. Cuttings from different individual explants are likely to behave differently under culture conditions (Geneve, 1989; Anthony, McLean & Lawrie, 2000). Most of the clones that did not survive were contaminated either by exogenous and/or endogenous microorganisms which proved to be detrimental (Idczak, & Brielmaier-Liebetanz, 2003). The surviving clones were sub-cultured for over 1 year and numerous viable cultures were produced for further experiments.
4.2 Changes to shoot multiplication protocol

Some research suggests that high BAP concentrations could inhibit shoot elongation (Lall, Mandegaran & Roberts, 2006), as well as stunt the growth of shoots and reduce the number of shoots produced (Martin, 2003). This was evident when using the original shoot multiplication protocol (McComb, et al., 1986); in the clones GRD1 and in GRD3 shoots were not consistently elongated, but shoots of 2-5cm in length were needed for further experiments. The results (Figure 3.2) substantiated the research that suggests that axillary bud formation and shoot elongation could be promoted by using very low concentrations of BAP; e.g. 0.2µM to 0.5µM (Chalupa, 1987, Lall, et al., 2006).

BAP and kinetin are generally considered as anti-senescence hormones and are known to reduce the apical meristem dominance and stimulate both axillary and adventitious shoots formation from meristematic tissues, resulting in shoot multiplication. Like auxins, cytokinins can produce a paradoxical effect whereby they produce an effect or its opposite depending on concentration (Madhulatha, Anbalagan, Jayachandran, & Sakthivel, 2004). The results confirmed that a reduced BAP concentration of 0.25µM improved shoot elongation for both clones (GRD1 & GRD3) and the new shoots of GRD3 appeared much more vigorous and healthier (more uniform shoot heights, leaf size and number of axillary shoots) in growth compared to most explant shoots before the experiment (variable shoot heights, leaf sizes and number of axillary shoots). Shoot length can determine the efficiency of root formation, as longer shoots are in a better physiological condition for root induction (Lin, Bergann & Stomp, 1995; Thengane, Bhosle, Deodhar, Pawar & Kulkarni, 2006; Xu, et al., 2008). The longer shoots were easier to transplant
and the uniform lengths facilitated better comparisons for all subsequent experiments. Consequently, the protocol was altered for the BAP concentration and all shoot multiplication media used the reduced BAP concentration of 0.25µM.

### 4.3 Changes to root induction media composition

The success of micropropagation largely depends on achieving root induction and then *ex vitro* acclimatisation for all *in vitro* grown plantlets, but acclimatisation of rooted *in vitro* cultured plantlets have many morphological limitations; e.g. unusual tissue organisation, incomplete vascular connections between shoot and root resulting in low hydraulic conductivity and limited transport of nutrients (Romano & Loucao, 2003). *In vitro* cultured plantlets have nutrients and water readily available and this may explain the anatomical and morphological differences in development of tissues; e.g. leaves have an undeveloped cuticle, lack of secondary xylem and phloem tissues that enhance conduction and minimal vascular differentiation in adventitious roots (Metivier, *et al.*, 2007).

One of the most commonly used auxins for root induction includes IBA and the effect depends on the plant species, the concentration and the purpose of application (De Klerk, *et al.*, 1999; Ibrahim & Debergh, 2001). Different auxins, concentrations or combinations, may have to be applied depending on rooting treatments (*in vitro* or *ex vitro*) and on plant species (DeKlerk, *et al.*, 1997). The application methods vary greatly, depending on the culture methods; for *in vitro* root induction the auxin is added to the culture medium, for *ex vitro* root induction the stems can be dipped in a highly concentrated auxin formula (Ibrahim & Debergh, 2001). The original root
induction protocol (McComb, et al., 1986), which required etiolation of shoots prior to root induction, produced inconsistent root induction and poor root formation with GRD1 clones.

4.3.1 Optimisation of duration of IBA pulsing

IBA is often the preferred auxin for root induction (Epstein & Muller, 1993), as plant tissues can rapidly oxidise other auxins such as IAA (DeKlerk, et al., 1997; Hausmann, 2003). Plants utilise different auxins at different uptake rates (Nissen & Sutter, 1990; DeKlerk, et al., 1997; Simon & Petrasek, 2011), which could explain the variation of root numbers formed (Figure 3.3). Plants are generally not very sensitive to auxins during the first 24 hours, but after this time has lapsed cells become more competent and begin to respond. Root initiation usually occurs between 72 and 96 hours as cells become activated, respond and result in root formation (De Klerk, et al., 1999). A variation of sensitivity to auxins has been observed in clones of the same plant and this can affect the transport of auxins to the plant cells and affect rooting response (Kim, et al., 1998). Rooting response (number of primary roots) can depend on the duration of IBA pulsing (Glocke, Delaporte, Collins & Sedgley, 2006) and the highest mean of root numbers was recorded for 6 days of pulsing on IBA, and the mean number of roots (Figure 3.3) declined rapidly thereafter. The results clearly showed that auxin pulsing is only necessary for a short period of time; so all the subsequent root induction experiments used a 6-day IBA pulsing duration.

The spontaneous root formation in GRD3 on shoot multiplication media occurred repeatedly with some shoots, but always in isolated shoots of a culture vessel, and did not occur again when the same shoot was sub-cultured. A possible explanation for this could be that plants contain the
natural, most abundant and endogenous auxin IAA. IAA is synthesised by the plant itself and in these particular shoots it may concentrate locally at the base of the shoot cutting causing cell enlargement, cell division (callus formation) and eventually root formation (Nordstrom, Jacobs & Eliasson, 1991; Epstein & Muller, 1993; Hobbie, 1998). However, the results with GRD3 were very inconsistent, so all further experiments used only GRD1 clones.

4.3.2 IBA concentration

IBA can either promote the formation of adventitious roots, or it can inhibit this process; this variation depends on the IBA concentration and plant species (Muller, 2000). Variation in IBA concentrations can result in great variation of root response (Glocke, et al., 2006) and high IBA concentrations can increase the rooting rate (Xu, et al., 2008). These different rooting responses (Figure 3.4) can be explained with the increased concentration of IBA allowing for faster action on the differentiating cells to become competent and enhance the cell division of the first root initials (Smith & Thorpe, 1975). It was observed that very high concentrations of IBA (160 µM IBA in Figure 3.4) had an inhibitory effect on root formation (DeKlerk, et al., 1997; Wynne & McDonald, 2002).

4.3.3 Auxin combinations for *in vitro* root induction

In contemporary plant propagation research it has become evident that endogenous plant auxins interact with exogenous auxins resulting in fast and slow responses (Woodward & Bartel, 2005). Different auxin combinations have been successful for root induction in various woody plant
species, including medicinal woody plants, rose, apple and others (Marks & Simpson, 2000; Martin, 2003; Martin, 2003/4; Pati, et al., 2006; Nandagopal & Kumari, 2007). Treatments containing NAA (80µM, 54µM, 40µM and 26µM, Figure 3.5) produced large calluses (not measured) at the shoot base and means of 0.76 – 1.73 roots/shoot. This could be because NAA remains longer in the tissue as it is more persistent than IBA, hence the callus formation, which may have blocked the root meristemoids from developing any further (De Klerk, et al., 1997).

IAA did not prove to be that effective for root induction; treatments with 80µM and 40µM IAA (Figure 3.5) only produced a mean of less than 1 root/shoot. A possible explanation for this occurring could be the fact IAA is an endogeneous auxin synthesised by plants and degrades rapidly, so that only very small amounts of free auxin can be taken up by the target cells. IBA, IAA (endogeneous auxins) and NAA (synthetic auxin) are conjugates where free auxin can be released for preferential uptake (Van der Krieken, Breteler, Visser, Mavrdour, 1993). The endogeneous auxins are less stable and IAA photo-oxidises more rapidly than IBA; IAA oxidises by 50% and IBA by 10% in 24 hours, whereas NAA is a very stable, synthetic auxin that does not oxidise (Nissen & Sutter, 1990; Epstein & Muller, 1993; Hausman, 2003; Simon & Petrasek, 2011). Treatments containing 40µM IBA combined with 40µM of IAA or NAA (Figure 3.5) produced means of 3.43 and 3.85 roots/shoot. These results indicated that IBA was the more active auxin for root induction and formation. IBA seemed to have a superior effect on root induction and formation, which could be due to the preferential uptake and metabolisation of IBA by the shoot tissues (Muller, 2000), but this was not further tested. The high concentration of exogenous IBA applied to the wounded shoots for 6 days is synthesised by the receptor cells targeted; initially wounding related compounds and auxin activate the cells at the cut site. This is followed by cell division (after 24 hours) and primordial growth can begin to develop from
meristemoids after 96 hours with outgrowth from the stem occurring after 120 hours (De Klerk, et al., 1999). This could explain the observed results as only the control treatment was significantly different from the other treatments, and consequently all further experiments for root induction only used 80 µM of IBA.

### 4.4 Different substrates

Roots are vital to any plant as they serve a multitude of functions; anchorage and support, as well as being the supply channel for water and nutrients to the shoot. Tissue culture techniques have utilised the universal application of gelling agents as the most favourable anchorage and support system for the plant cultures. Gelling agents have long been the preferred substrate for root induction and formation, but the limitations of agar and gelrite gelling agents for root formation have also been investigated (DeBergh, 1983; Williams & Taji, 1987; Kumar, et al., 2003; Peira-Netto, Petkowicz, Cruz-Silva, Gazzoni, Mello & Silveira, 2007). Root induction in porous substrates has also been trialed as porous substrates are thought to alleviate some of the physiological deficiencies that limit acclimatisation; one of the limitations in the physiology of *in vitro* cultured plants is that the roots do not function properly due to reduced numbers or lack of root hairs (Jay-Allemand, Capelli & Cornu, 1992; Lin, et al., 1995; Gangopadhyay, Das, Mitra, Poddar, Modak & Mukherjee, 2002; Mohan, Soccol, Quoirin & Panday, 2004; Gangpadhyay, Bandypadhyay, Gangopadhyay & Mukherjee, 2004 & Gangopadhyay, Roy & Mukherjee, 2009). Porous substrates could overcome these short-comings and increase the survival rate during acclimatization (Barrett-Lenard & Dracup, 1988; Rossetto, Dixon & Bunn, 1992; Hoarau &
Cornic, 1998; Zobayed, Zobayed, Kubota, Kozai & Kasgawa, 2000; Gangopadhyay, et al., 2004; Gangopadhyay, et al., 2009).

Most roots grown in black sand, sterilised soil, rock wool, soil jiffies and coarse white sand (Fig 3.6) were positively gravitropic; the downward growth of the roots resulted from the additional rooting space that these substrates provided (4-7cms depth depending on substrate) compared to ~2cm depth that agar provided. Surprisingly, root formation was not statistically different in the 2 white sands, sterile soil and soil jiffies – it was expected that root formation/numbers in sterile soil and soil jiffies would be greater in these more aerated substrates.

Generally, roots can sense their environment (air, humidity gradients, nutrients, substrate and water) that they touch and respond physiologically with growth accordingly; this response is known as thigmotropism (touch), but the exact mechanism is not yet fully identified (Perrin, Young, Murthy, Harrison, Wang, Will, & Masson, 2005; Gilroy, 2008; Gangopadhy, et al., 2009). Experimentation with different substrates clearly showed different growth responses and results from this experiment were not conclusive; e.g. destruction of roots when separating (rock wool and soil jiffies), thus results may be incorrect as soil jiffies are designed for direct transfer into soil where it biodegrades and rock wool is commonly used as a substrate for hydroponic cultures. For in vitro root induction experiments these were too cumbersome and probably did not provide adequate rooting space for 5 shoots. The different root growth responses were evident in the relatively poor results for both white sands compared to black sand and lower means for root numbers in sterile soil compared to agar and black sand (Fig 3.6).
4.4.1 Comparison of Agar, black sand and IVS

Root physiology, length and surface area/distribution (root architecture) are vital parameters for plant growth and survival, but this is difficult to quantify with living plants (Lynch, 1995; Davies & Jacobs, 2005; Jacobs, Salifu & Seifert, 2005). Root numbers alone, where short and long roots are counted as equal, are not an ideal parameter when comparing plant root systems. Therefore it is better to measure more than just one parameter in order to get more comprehensive data on root systems by analysing the means of root length and surface area, as well as shoot and root biomass for differences in substrate affects (Melhuish, 1968). The alternative in vitro rooting substrate being researched by Newell (et al., 2005) in vitro – soil medium (IVS) and other porous substrates (Barrett-Lenard & Dracup, 1988; Rossetto, et al., 1992; Zobayed, et al., 2000; Gangopadhyay, et al., 2002; Mohan, et al., 2004; Gangpadhyay, et al., 2004) are aerobic and less dense than solidified agar media.

Root formation in the alternative substrates of black sand and sterile soil (Figs 3.7 & 3.8) was compared with agar-solidified media. The results showed the improved root performance (root surface area and length) observed for sterile soil, which was statistically different to the other treatments (Figure 3.7). Sterile soil is not a substrate that is uniform in density or particle size (it consisted of peat and perlite 1:3), so different root sizes can occupy the substrate pores, which could explain the increased root numbers, root length and surface area (Pierret, Moran & Doussan, 2005). This could be due to the different mechanical resistance of the substrates and the stimulation that this provides to the root and could explain the variation in root morphology of shoots grown in sterile soil (Gangopadhy, et al., 2009). It is hypothesised that medium
hypoxia and the build-up of ethylene and CO₂ observed in agar substrates can limit root initiation and elongation. Using a porous substrate could reduce these effects, as the porous media could allow for greater diffusion of inhibiting effects of ethylene and CO₂ (Newell, et al., 2003). However, most of these root growth patterns observed are the short-term effects in response to physiological stimuli and cannot be regarded as root growth patterns, but rather as spontaneous root growth in individual shoots (Grabov, Ashley, Rigas, Hatzopoulos, Dolan & Vicente-Agullo, 2005). This experiment showed that roots could display a high level of morphological plasticity in response to the different substrates that they were grown in (Figure 3.8).

4.5 Comparison of sterile soil with different nutrients

The previous experiment achieved good root induction on GRD1 clones using an aerated, porous substrate of sterile soil instead of an agar medium. The limitations of agar-based media have been discussed (Newell, 2005; Gangopadhyya, et al., 2009) and as a result, some research has been done on ex vitro rooting of in vitro cultured plantlets (Kim, et al., 1998; Martin, 2003; Xu, et al., 2008; Gangopadhyya, et al., 2009). The process of ex vitro root growth usually entails that the in vitro grown shoots receive exogenous auxin treatment before being transferred for acclimatisation and root growth to ex vitro conditions. This method is often applied as a cost and time reducing strategy (Martin, 2003; Thomas & Schiefelbein, 2005; Feyissa, et al., 2007). The experiment showed that root induction and growth was possible (Figure 3.9) without added nutrients in the substrate, as the pulsing on IBA media induces root formation. The immediate application of IBA after wounding did not seem to adversely affect root formation and the results from the sterile soil treatments with no nutrient supplements were comparable (no statistical
difference) to the other treatments for mean root length and numbers (Fig 3.7). This suggested that roots could be induced with a high concentration of IBA pulsing (*in vitro*) and these results were comparable to results from experiments by De Klerk, *et al.*, (1999) where cuttings were exposed to very high auxin concentrations *ex vitro* before being planted out in to soil directly. For the verticordia shoots in this experiment the adventitious roots formed spontaneously as a direct result of exogenous auxin application. There was no statistical difference in root length or surface area between treatments with just DDI and DDI with sucrose. It has been suggested that starch accumulation is a prerequisite for adventitious tissue regeneration and root formation (Jasik & De Klerk, 1997). Sucrose from the media can be synthesised to starch and has a regulatory effect on adventitious organ formation; starch grains accumulate in the target cells that then produce adventitious roots, but then degrade after cell division has occurred (Capellades, Lemeus & de Bergh, 1997; De Klerk, *et al.*, 1999, Hazarika, 2003). However, in this study the sucrose concentration was not apparent to have an effect on adventitious root formation by increasing sensitivity to IBA (Calamar & De Klerk, 2002), as the shoots in the treatment without sucrose had comparable root formation. The IBA concentration of the pulsing media was high enough for free auxin to reach the target cells and to stimulate rooting response (Figure 3.9). Root induction and growth occurred in sterile soil without nutrients and a carbon source, so this suggested that the *in vitro* root induction process could be eliminated to save time and costs.
4.6  *Ex vitro* root formation and acclimatization

For acclimatisation from *in vitro* to *ex vitro* to be successful physiological changes to correct the abnormalities in leaf morphology, photosynthetic capacity and water relations must occur. Improved physiological functions of *in vitro* plantlets should facilitate rapid acclimatisation to *ex vitro* conditions and thereby increase plantlet survival rates (Rossetto, *et al*., 1992, Bonal & Monteuuis, 1997; Hazarika, 2003; Martin, 2003; Feyissa, *et al*., 2007). Thomas & Schiefelbein (2005) compared *in vitro* with *ex vitro* physiological rooting responses and examined if there were any significant differences in gene expression pattern. They discovered that gene expression was identical between *in vitro* and *ex vitro* root induction.

For both treatments soil (*in vitro* = sterile soil) was used (Figures 3.10), which is more aerated than an agar medium, and this had positive effects on root induction and development, as all stages of rooting in plants require oxygen (Newell, 2006). Medium hypoxia is assumed by Newell (2006) to occur in agar rooting medium and this could limit not only root induction but also root elongation, as compared to the root growth patterns observed in IVS (Newell, *et al*., 2005). Another advantage of using a sterile soil media is that it has better water holding capacity over agar media and therefore can prevent medium hypoxia. In this experiment the biomass of shoots and roots for *in vitro* and *ex vitro* soil had no statistical difference (Fig 3.10), and neither visible shoot growth nor wilting was observed for the duration of the experiment. These results indicated that possibly the increased aeration of a soil media could allow for greater root growth, because aerated media allows for greater diffusion of ethylene caused by *in vitro* pulsing with auxins, which enhances ethylene synthesis (Newell, *et al*., 2005). Agar medium does not readily allow for rapid gas diffusion, hence the accumulation of ethylene at the basal part of the stem. This accumulation can inhibit root induction, growth and elongation (De
Klerk, 2002). Ex vitro rooting would eliminate the process of transfer and reduce the risk of root destruction (Gangopadhya, et al., 2009).

Combined in vitro shoot multiplication with ex vitro root induction and acclimatisation has been trialled to enhance survival of plantlets, as well as a cost and time reducing strategy (McClelland, et al., 1990; Kim, et al., 1998; Martin, 2003; Thomas & Schiefelbein, 2005; Feyissa, et al., 2007; Xu, et al., 2008). Newell (2006) alleged that agar medium can suppress rooting performance in many Australian plant species, so the rooting potential of these are difficult to be accurately determined. He concluded that survival rates dramatically increased when the root induction phase for many Australian woody plant species occurred ex vitro and simultaneously with acclimatisation on IVS without de-flasking and transferring, when compared to the conventional root induction on in vitro agar medium followed by transfer to ex vitro substrates and conditions. For this experiment the in vitro shoots grown in sterile soil were re-potted into crackpots at the beginning of this experiment. The positive results (Figure 3.10) demonstrated that it was possible to multiply shoots in vitro and then to root and acclimatise these shoots ex vitro simultaneously. This process of combining Stage 3 and 4 of micropropagation excludes disturbing and breaking roots during transfer, using soil in aseptic laboratory conditions can be avoided and it is a time and money saving strategy. Ultimately the effectiveness and success of this relies on the survival of the plantlets (Martin, 2003).

4.7 Survival

Trials with ex vitro rooting and simultaneous acclimatisation have been considered promising for the micropropagation of many different plant species. This is due to the reduction of
establishment and production time, labour and associated costs, as ex vitro rooting can reduce the overall costs by 35% (McClelland, et al.; Kim, et al., 1998; Martin, 2003; Thomas & Schiefelbein, 2005; Feyissa, et al., 2007; Xu, et al., 2008). The appropriate substrate, with high water holding capacity, can be beneficial to acclimatisation as well as the reduction of light intensity in the greenhouse by using shade cloth (Viegas, Pereira, Ambrosano & Batista, 2005). The results (Fig 3.11) suggest that ex vitro root growth can occur simultaneously with acclimatisation as a time and cost saving strategy. The high long-term survival rate (Figure 3.11) could possibly be due to several factors; e.g. possible in vitro rejuvenation (Haapala, et al., 2004; Andreu & Marín, 2005) of plantlets due to the long time in culture (more than 2½ years) with constant sub-culturing, endo- and exogenous auxin levels (Ibrahim & Debergh, 2001) and favourable environmental conditions (DeBergh, 1991; Bonal & Monteuuis, 1997; Hazarika, 2003). The beneficial environmental ex vitro conditions included the medium substrate that possibly allowed for flushing of excess auxin, better gas exchange and water retention/drainage, additional to relative humidity and the quality/quantity of light (Gangopadhyya, et al., 2009).

The wilting and death (Figures 3.11 & 3.12, Table 3.2) of some plants was probably due to the combination of different factors during ex vitro acclimatisation; initially the lack of ventilation in the humidity tent (even though the crack pot trays had vented lids) and increase in ambient temperature due to the lack of ventilation. It could have been possible that these shoots could not adequately control plant water loss due to the limited stomata function (Gilly, Rohr & Chamel, 1997) combined with ambient humidity and temperature fluctuations. This may explain why shoots did not fully recover, acclimatise or survive in the first 56 days for the first trial.

Over time stomatal performance must have improved in the remaining plantlets, as these acclimatised and could control transpiration (this is only evident by survival and was not tested.
further), even though 100% survival was not achieved. Transpiration control and stomata function can usually improve between 8-12 weeks (Figs 3.11 & 3.12, Table 3.2). The autotrophic growth of new shoots and/or shoot elongation further confirmed this. The functioning roots also would have contributed to the water retention capacity as well as nutrient uptake required for further growth (Diaz-Perez, Sutter & Shackel, 1995).

The long-term survival of over 70% of in vitro cultured plantlets with ex vitro induced roots (Figs 3.11, Table 3.2) indicated that the abnormalities and limitations in shoot and root morphology and physiology were gradually overcome and these became functional during ex vitro acclimatisation (Pospislova, et al., 1999). In comparison the in vitro root induced shoots (Figure 3.11) did not perform as well, which could have been due to the additional stress of transplanting, due to root damage that occurred during this process, and sudden changes of ambient conditions (Hazarika, 2003). However, high mortality rates after the conclusion of this experiment suggest other factors negatively impacting on long-term survival, but due to time constraints this was not further investigated.

4.8 Effectiveness of improved protocols on new clones

One of the aims of this research was to test the effectiveness of the improved shoot and root protocols on other verticordia clones that were introduced into tissue culture. Not all new clones were successful for micropropagation (Chapter 3.8.1), but those that could be continuously sub-culture were trialled for ex vitro root formation combined with simultaneous acclimatisation (Chapter 3.8.2) and monitored for long-term survival (Figure 3.12) for comparison to GRD1.
4.8.1 Survival of new clones

The success of *ex vitro* root induction for most new clones was confirmed by the fact that all plantlets had roots. Initial acclimatisation was also a success, but long-term survival was less successful (Figure 3.12). The stomatal function may have developed over time (8-12 weeks) in many plantlets, so the plantlets could control transpiration and water loss with the reduced humidity (Fila, *et al.*, 1998; Pospisilova, *et al.*, 1999), but functional roots were needed for nutrient uptake to facilitate growth and survival (Mulataya, *et al.*, 2002; Hazarika, 2003), however, evidence of autotrophic growth was insufficient. The poor long-term survival rates suggest that not all plantlets repaired their *in vitro* functional abnormalities, in particular, the lack of junction between the stem vascular tissue and the adventitious roots, as they failed to thrive and grow. The lack of functional roots (this was not tested, as plantlets had roots) could explain why these results do not compare to the results of acclimatisation and survival of GRD1 clones (Chapter 3.7). This may also be due to the genotypic and phenotypic variation that is a common occurrence observed in micropropagated plants (e.g. different internodes in GF clones, different leaf sizes in all clones), and these variations can pre-exist in the ex-plant material or result from *in vitro* conditions (Perez, *et al.*, 2011). There was inconclusive evidence with regards to *in vitro* rejuvenation (Haapala, *et al.*, 2004; Andreu & Marin, 2005) having occurred, even though most plantlets had root formation. The clones GF 1-4 originated from new shoot (possibly juvenile) growth (after fire destroyed the explant) and performed similarly to the clones taken from seasonal, fresh growth (Table 3.1), which is usually mature vegetative growth (Hackett & Murray, 1992).
5. CONCLUSION

This study confirmed that the improved shoot multiplication and root induction protocols produced overall good results with most *V. grandis* clones, but the best results were achieved with a single clone (GRD1) that had been in culture for over 5 years. It was possible to introduce new *V. grandis* clones into tissue culture, even though the success was limited for the other verticordia species; *V. ovalifolia*, *V. fragrans* and *V. densiflora*. The new shoot multiplication protocol attested to the effectiveness with good results for the new *V. grandis* clones.

The improved shoot multiplication protocol produced more uniform elongation for most clones, which was necessary for further experimentation; most new clones performed well on the lower concentration of 0.25 µM BAP, but elongation was greatly improved for the GRD3 clone. This resulted in having an endless supply of shoots from most clones that had more uniform lengths needed for further experimentation, as this allowed for better comparisons (Lin, *et al.*, 1995).

The optimal auxin for root induction was IBA and the best concentration was determined to be high at 80 µM, and the optimal *in vitro* auxin pulsing duration was found to be 6 days. This optimised root induction protocol worked well for most GRD clones. Recent studies by Newell (2006) hypothesized that Australian native plants perform better in an aerated medium compared to agar media. Experiments with different substrates, other than a gelled medium, were trialled on GRD1 and a soil mixture of peat and perlite (1:3) was found to support this with good results. Other studies (Kim, *et al.*, 1998; Hazarika, 2003) have suggested that species, which are problematic with *in vitro* root induction, often improve root formation when combined with *ex*
The effectiveness of combined root formation and growth, after *in vitro* IBA pulsing, and acclimatisation *ex vitro* was verified by high percentages of root formation and subsequent growth and survival. This process saved not only time, but eliminated the excessive handling of delicate shoots with roots, prevented breaking roots whilst de-flasking from *in vitro* media and transfer to *ex vitro* growth media (soil), and preparing soil in an aseptic laboratory was avoided as well. Simultaneous *ex vitro* root formation, after IBA pulsing, and acclimatisation was achieved and confirmed by some of the best survival rates in most experiments for *Verticordia grandis*, especially the GRD1 clone. The alternative trials with *ex vitro* auxin dipping (instead of *in vitro* auxin pulsing) prior to out-planting in the greenhouse for acclimatisation were not successful and resulted in 100% mortality of shoots. An illustration of how this approach is incorporated into a more standard micropropagation approach is illustrated in Figure 4.13 where the elimination of the need for roots prior to transfer to soil is illustrated.

Long-term survival was monitored for 252 days, which included re-potting plants after 112 days into larger containers and a peat – perlite – sand (1:1:1) mixture for added drainage. Plants that had roots induced *in vitro* and that were then transferred for acclimatisation to the greenhouse after 28 days had a high mortality rate (possible due to roots being damaged during transfer) and after 140 days there was 0% survival. In contrast, all *ex vitro* root grown and acclimatised plants had a high survival rate at over 72% after 252 days, when the experiments concluded.
Figure 4.13: The incorporation of ex vitro rooting process into the micropropagation process - adapted from George et al., 2007.

However, survival continued to decline after experiments had concluded at 252 days, and after more than 2 years there were no plants left. This indicates that more research into enhancing long-term plant survival and field performance would be required if commercial production for *Verticordia grandis* was to be pursued.
References


