MICROPROPAGATION AND GENETIC TRANSFORMATION OF *VERTICORDIA GRANDIS*

BY

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Verticordia grandis

DECLARATION

The work presented in this thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference is made in the text.

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B. E. Stummer

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Summary

Verticordia grandis (Scarlet feather flower) is the subject of this study. It is a member of the family Myrtaceae and has a brilliant display of red flowers on long upright stems. The horticultural potential of *V. grandis* as a cut flower and for pot culture is recognised in the industry. However, supply is limited due to difficulties of propagating the plant. In particular, the induction of roots on explants *in vitro* has limited the successful commercial micropropagation of this species.

A system has been established for the micropropagation of *V. grandis*. Protocols were developed for the initiation, shoot proliferation and root induction of *V. grandis* explants, *in vitro*. The sterilisation treatment successfully decontaminated 82% of the explants. Shoot multiplication rates of at least 3 fold were obtained on the multiplication medium. In addition, the location of meristematic cells at the leaf petiole region indicated that shoot proliferation from leaf discs was possible. The root induction medium consistently produced 100% rooting of explants. Anatomical investigations confirmed that roots were connected to stem vascular elements. Therefore the methods developed were suitable for commercial production. These methods are essential requirements for nursery production and are also important prerequisites for transformation studies.

Rooted plantlets were successfully transferred to glasshouse conditions. Survival rates were influenced by both the plant genotype and the time of year of out-planting. Soil conditions critical for the successful pot culture of *V. grandis* included adequate drainage (air filled porosity of 20%), slightly acidic medium (pH 5.5-6) and low supplementary nutrient application. *V. grandis* plants responded favourably to high illumination and low humidity levels. A foliar 6-benzyladenine (BA) spray (210mg/l) in combination with apical bud removal increased the number of lateral shoots and were useful in improving the shape of the plants for commercial pot culture.

The micropropagation and out-planting protocols developed for *V. grandis* were utilised in *Agrobacterium* mediated transformation investigations. Initially, susceptibility trials using wild type *A. rhizogenes* strains were employed. Results obtained from these investigations indicated that wild type *A. rhizogenes* does not induce hairy root formation in *V. grandis* stem explants. However, *V. grandis* is susceptible to infection with *A. rhizogenes* as determined by gall formation and opine analysis.

A system has been established for the genetic transformation of *V. grandis*. This system utilises the method developed for plantlet regeneration from leaf discs. Leaf discs were inoculated with an *Agrobacterium* strain containing a marker gene for antibiotic resistance (NPT II gene) and a reporter gene (GUS) for screening of transformants. The transformation results were confirmed by PCR and Southern hybridisation. These results represent the first example of genetic engineering of a plant from the Myrtaceae.

This thesis describes, therefore, the establishment of *in vitro* organ culture techniques for the propagation of *V. grandis* and for the establishment and pot culture of plants in the glasshouse and demonstrates the application of the leaf disc regeneration method for the transformation and regeneration of *V. grandis*.

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CHAPTER 1 LITERATURE REVIEW AND PROJECT AIMS

1.1 INTRODUCTION

The uniqueness and beauty of the Australian flora justifies the recent global interest in native plants for the cut flower industry and for pot culture. The economic importance of ornamental horticulture has been estimated by the Australian Nurserymen's Association to have a total value of $550 million in retail plant sales and $15 million in exports. Furthermore, $7.6 million of this export value was in cut flowers (Possingham and Wren, 1988). Early demand for cut flowers and seed supplies was met by exploiting native reserves and crown land (Rye *et al.* 1980). Although such bush harvesting still continues (both illegally and by permit), the practice is decreasing because resources are limited. Environmental concerns are receiving greater attention and wildflower exporters are working in collaboration with nursery wholesalers and tissue culture companies to propagate and grow the plant material specifically to meet industry demands.

Verticordia is an outstanding genus of economic importance belonging to the Myrtaceae. It is an endemic genus of approximately 79 species (*Verticordia* reference collection 1988) which are found mainly in Western Australia. The species range of *Verticordia* extends into South Australia and two species are located in the Northern Territory and Queensland (*V. verticillata, V. cunninghannii*) (A.N.P.W. 1988). Members of the genus are perennial woody shrubs and are found in well drained sandy soils in low rainfall areas. Their floral display is impressive with loose to tight clusters of showy flowers in a range of colours.

V. grandis (Scarlet feather flower) which is the subject of this study has a brilliant display of red flowers on long (greater than 30cm) upright stems. It is in high demand for cut flower export and pot culture but propagation has proved difficult. Rooting from cuttings is poor, although there are reports (P. Moyle. pers. comm.) of a few rare clones that root well. There has only been one report of successful micropropagation of *V. grandis* (McComb *et al.* 1986). However, *in vitro* rooting of shoots limited the satisfactory production of plantlets. The percentage of explants that developed roots (rooting

percentage) did not exceed 58%. Although this percentage is greater than that achieved in conventional propagation methods via cuttings, it is still too low for commercial tissue culture production.

While low rooting percentages may be acceptable in research to determine the mechanisms underlying particular culture effects, successful commercial production depends on both high multiplication rates (2-4 times) and high rooting percentages (greater than 80%). From a commercial standpoint, the induction of roots on explants *in vitro* is an expensive, labour-consuming process, which can account for 35-75% of the total cost of production (Debergh and Maene 1981). Therefore the lower the rooting percentage of shoots *in vitro*, the higher the wastage rate and overall cost of production.

One aim of this study was to develop a micropropagation protocol for the commercial production of *V. grandis*. Two approaches were investigated. The primary approach was based on utilising commercially successful micropropagation methods (see above) to investigate factors which affect root induction of shoots *in vitro* from woody explant material (see below). A second approach was based on the idea of utilising *Agrobacterium rhizogenes* to induce root development (see below section 1.6).

The use of wild type *A. rhizogenes* as a root inducer of woody plants has previously been explored (Moore *et al.* 1979, Rugini 1984 and 1986, Strobel *et al.* 1988, van der Mark *et al.* 1990, Lambert and Tepfer 1991 and Rugini *et al.* 1991). Stem inoculations of almond and olive plants produced transformed root systems which led to increased root mass and shoot growth compared to untreated control plants (Rugini 1984 and 1986). More recently, transgenic kiwi (*Actinidia deliciosa* A. Chev.) plants, regenerated from leaf disc inoculations with *A. rhizogenes rol* genes, showed an increased rooting percentage of the regenerated shoots (Rugini *et al.* 1991). Positive alterations in the rooting ability of woody plants by transformation with *A. rhizogenes* may have horticultural value in relation to improving root morphogenesis of woody plants. In addition to stimulating root production of tissue cultured shoots, the genetic manipulation of plants can offer other benefits. For

example, 'desirable' genetic characteristics such as herbicide or insect resistance, manipulation of flower colour, shape and vase life may be introduced (see below).

A second aim of this study was to develop a transformation system utilising the natural ability of *Agrobacterium* to infect and transform plants. Theoretical and practical factors involved in *Agrobacterium* transformation of plant species are discussed below (Section 1.6).

1.2 MICROPROPAGATION OF WOODY PLANT SPECIES: Methods for the induction of roots on micropropagated shoots.

1.2.1 Introduction

Plant tissue culture is a generic term used to describe the methods involved in the culture of plants from various plant parts, for example, cells, organs, embryos or anthers. It is carried out under aseptic conditions, where nutrients and environmental conditions are controlled. Micropropagation for large scale production involves the regeneration and clonal propagation of whole plants *in vitro*. There are a number of important features of micropropagation which can be applied both commercially and in breeding programs: (1) rapid propagation of desirable clones, especially where traditional *in vivo* vegetative propagation methods are slow, difficult, or unprofitable or plant stock material is limited, (2) elimination of viruses and other systemic pathogens, (3) all year round propagation. The development of plantlets *in vitro* can be divided into 4 main stages. (Figure 1.1 illustrates the stages involved in the micropropagation process).

Stage I: Explant establishment. The aim of this stage is to establish sterile explants in culture. Factors which affect the success at this stage include; the plant species and choice of explant material, elimination of contaminants from the explant and the culture environment.

Stage II: Multiplication. The aim of this stage is rapid shoot proliferation. The most common method employed with woody plants is 'axillary shoot proliferation', where shoots develop from pre-existing meristematic regions (apical shoot, leaf and node axils: see Fig 1.1). Shoot proliferation can be measured by shoot multiplication rates (which refers to the number of new shoots produced at each subculture).

Stage III: Induction of roots. Shoots grown *in vitro* may be induced to produce roots at Stage III or can be rooted directly in the glasshouse (Stage IV see below) under non-sterile conditions (referred to as microcuttings).

Stage IV: Acclimatisation. Plantlets are transplanted from the aseptic environment to glasshouse conditions. This transition phase involves a period of acclimatisation as the plantlets must become autotrophic and build resistance to desiccation and pathogen attack (see below).

The induction of roots (Stage III: Fig 1.1) is frequently the stage that limits the successful micropropagation of many woody species, including Australian native plants such as *Verticordia grandis*. Table 1 summarises the culture conditions of some rooting studies for 10 Australian woody plant species. The rooting percentages range from 40 to 100%. All have several cultural conditions in common. These include auxin for root induction. 1-Indole-3-butyric acid (IBA) was most frequently used in isolation at concentrations ranging from 5-10μM or in combination with naphthalene acetic acid (NAA) (IBA ranging from 0.5-4.9μM, NAA 0.5-2μM). For *Eucalyptus tereticonis* a combination of 4.9μM IBA, 5.5μM IAA and 5.3μM NAA was used. The pH of the media range from 4-5.5. Most species (7 out of 10) were given a dark pre-treatment of 1-2 weeks. The type of basal medium and the use of an elongation medium was less consistent between the studies. Table 1 highlights the variation among species in response to the culture conditions. This applies to hormone application, light conditions, media pH and basal or elongation media (the interaction of these factors is also variable between species).

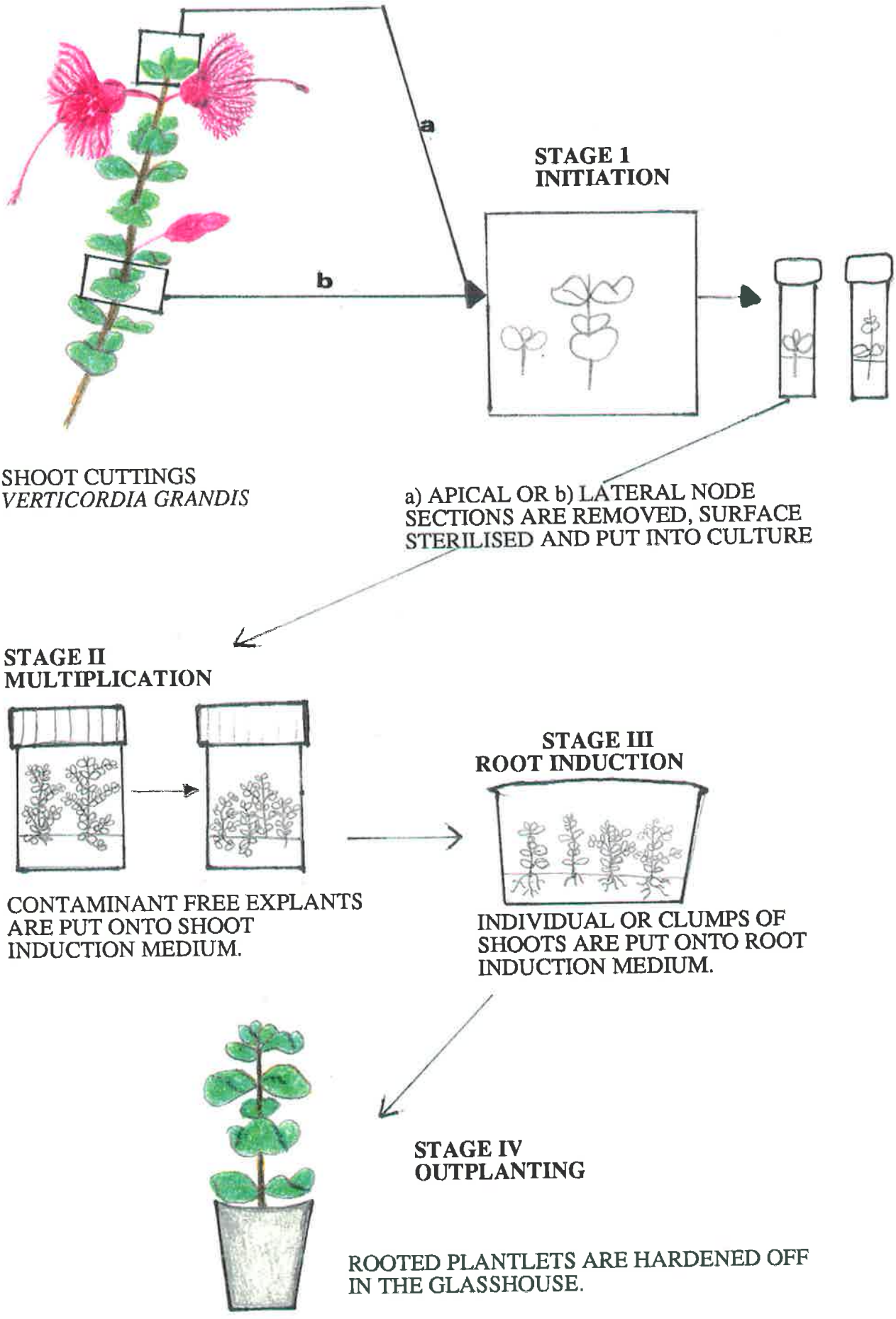


FIGURE 1.1: STEPS INVOLVED IN THE MICROPROPAGATION PROCESS.

Table 1: Summary of *in vitro* rooting studies on some woody native Australian plant species.

SPECIES	BASAL MEDIUM	HORMONE *µm*	pH	ETIOLATION	ROOT ELONGATION	ROOTING SUCCESS	REFERENCE
E. citriodora	Whites	NAA10.7	5.5	2 wks	1.Whites liq. basal 2.MS	50%	Gupta *et al.* (1981).
E. ficifolia	*deFossard low mineral,suc 2%	IBA 5	5.5			80%	Gorst *et al.* (1983)
Verticordia grandis	MS low micros suc 3%	IBA 10	5.7	stage 2, 2wks		58%	McComb *et al.* (1986)
S.acuminatum	Whites	IBA 5	4	Yes		40%	Barlass *et al* .(1981)
E. marginata	1/2 MS,suc 2%	IBA 10	5.5	7 days		52%	Bennet andMcComb (1986)
E. tereticonis sm	1/2MS liqiud suc. 2%	IBA 4.9,IAA 5.5 NAA 5.3			1.1/2MS+ 2. MS liquid	55%	Sankar Rao (1988)
Correa decumbens	MS	NAA 2, IBA 2	4	2 wks		95%	Williams *et al.* (1985)
Dampiera diversifolia	deFossard	NAA 0.5 IBA 0.5				92%	"
Grevillea biternora Meissner	MS	IBA 0.5 NAA 0.5				70%	"
Prostanthera striatiflora	MS	IBA 0.5 NAA 0.5.	4	2 wks		100%	"

Abbreviations: MS=Murashige and Skoog (1962), Suc=sucrose, Whites=White (1943), deFossard=deFossard (1976), NAA=Naphthaleneacetic acid, IBA=Indolebutyric Acid, NOA=Naphthoxyacetic Acid. *E=Eucalyptus, S=Santalum.* +=charcoal, *=riboflavin.

Williams (1989) has reviewed the *in vitro* culture of 10 genera of Australian species. In these examples there was considerable variation in the response of different species to similar culture conditions. Most of the attempts to determine conditions which result in root induction and growth have been empirical in nature and have not led to a clear understanding of underlying mechanisms. A number of common factors appear to be critical for root induction. These include: the source and condition of the plant material (juvenile vs mature woody material), the role of plant growth regulators, dark treatment, the pH of the medium, the length of time in culture, and genetic effects. These factors are discussed in the following sections.

1.2.2 Juvenility

Cultures derived from juvenile plant material often produce a higher rooting percentage of micropropagated shoots than mature woody material. For example, higher rooting percentages were obtained from juvenile (seedling) plant material than from material taken from mature tissue for Eucalyptus (de Fossard 1974) and Santalum (Barlass *et al.* 1981). Similarly, rooting of mature woody cuttings has proved difficult. Hartmann and Kester (1986) reported a number of experiments with *Malus sp, Quercus sp, Eucalyptus spp.* and *Pinus radiata,* where the rooting ability of cuttings from these plants decreased with the age of the stock plant. Thus it seems that some changes which affect rooting ability occur in the transition from juvenile to adult tissue. Dhahwan *et al.* (1979) working with *E. grandis* described three root inhibiting growth regulators called G-inhibitors. Low concentrations were found in juvenile leaves, while high concentrations were extracted from mature leaves. The rooting of mung bean cuttings was actually promoted by low concentrations of this inhibitor, whilst high concentrations inhibited rooting (Dhahwan *et al.* 1979). It is not known whether similar inhibitory substances occur in other species in which the production of roots is difficult.

Anatomical differences have also been found between juvenile and adult tissue. Developmental events which take place prior to root production were found to be different in cuttings taken from juvenile and mature plants (Lovell and White 1986). A thick layer

of sclerenchyma between the phloem and cortex forms in some mature stems, which is absent in the juvenile stem. The sclerenchyma is exterior to the point of origin of adventitious roots and therefore may act as a physical barrier to the emergence of root primordia (Hartman and Kester 1986).

To overcome the difficulties in rooting of cuttings of mature tissue, horticultural practices have evolved which maintain plants in an actively growing condition. Hedging treatments (removal of terminal shoots or spraying stock plants with cytokinins to induce the production of lateral shoots: see below Section 1.4) maintain shoots in the juvenile phase. For example, hedging of *Pinus radiata* ensures an abundant supply of young shoots which maintains the rooting potential of cuttings taken from these new shoots (Libby *et al.* 1972). Juvenile shoot growth may be beneficial for root induction due to anatomical differences, the lack of inhibitory chemicals, or a combination of factors not yet discovered.

Distinct from hedging is the practice of basal wounding. Root production on stem cuttings, such as junipers, can be promoted by wounding the base of the cutting (Hartmann and Kester 1986). Wounding stimulates the production of callus along the margins of the wound and subsequent production of root primordia. Evidence suggests that wounded cuttings absorb more water and permit greater absorption of applied growth regulators. Also the sclerenchyma layer (see above) may be disrupted which would permit the outward penetration of the developing roots (Breakbane 1969).

Micropropagation can also be an effective way to rejuvenate plant tissue and improve rooting of explants. Drew and Miller (1989) provide one example of the successful production of rejuvenated tissue *in vitro* from mature sources of papaya plants. A multiplication medium (Stage II) was chosen which produced numerous actively growing lateral shoots. High rooting percentages (90%) were obtained with the actively growing tip section of the lateral shoots. Only 20% rooting efficiency was obtained when slow growing woody shoots were used. The production of actively growing shoots at the multiplication stage is an important prerequisite for success at the rooting stage.

1.2.3 Role of plant growth regulators

Generally it is accepted that cytokinins are involved in shoot proliferation and auxins in root formation. Auxin is a principle endogenous promoter of root primordium initiation. Exogenously applied auxins are required for initiation of adventitious roots on stem cuttings and shoots grown *in vitro*. Table 1 shows the composition of rooting media (with respect to growth regulator type and concentration) utilised in a number of studies with woody Australian native plants. The media generally contained high levels of auxin. 1-Indole-3-butyric acid (IBA) is often the most successful auxin for root induction. However there can be a great diversity in the *in vitro* response to both the concentration and the type of auxin in media (Williams *et al.* 1985).

Two stages are involved in the formation of adventitious roots, (1) root initiation and (2) root growth. Although increasing the concentration of auxin in the culture medium tends to increase rooting, it can also promote excessive callus, rather than root growth (Bunn *et al.* 1989 and McComb *et al.* 1986). For this reason, some plants are given a short exposure (1-2 weeks) to a medium with high auxin concentration, followed by a period on elongation medium without hormones (Sankara Rao 1988 and Gupta *et al* 1981, see Table 1).

Endogenous cytokinin levels can accumulate with continuous subculture on a multiplication medium high in cytokinin, producing inhibitory carry-over effects for subsequent rooting (Williams 1989). In such instances, it can be beneficial to transfer plants to a pre-rooting medium, without hormones. However this is not always the case. Gasper (1989) reported that for Rhododendron and Kalmia micropropagation, low levels of cytokinin in the rooting medium promoted rooting of explants when compared to rooting medium with auxin alone.

1.2.4 Effect of dark pre-treatment

The effect of a dark pre-treatment of shoots has long been known to increase rooting (Hansen and Eriksen 1974). Methods have involved placing the plant stock material under shading or in the dark. Cuttings taken from these plants have a higher rooting percentage than cuttings taken from plants grown in the light (Hartmann and Kester 1986). Darkness during the first week of the rooting phase is often essential for root induction of woody species *in vitro* (Rugini *et al.* 1993). For micropropagated shoots, the most common method is to put Stage III shoots immediately in the dark (usually for 1-2 weeks) and then return them to normal photo period (16h light). Table 1 shows data from a number of reports in which a dark pre-treatment was used to induce rooting of woody plants *in vitro*. The length of the dark pre-treatment ranged from one to two weeks. McComb *et al.* (1986) adopted the method used for stem cuttings (see above). The mechanisms involved are poorly understood. Suggestions have been made that light inhibits root induction by increasing the carbohydrate content in plants (Hansen and Erikson 1974). This is consistent with reports of increased rooting percentages of shoots grown *in vitro* on media with low (1-3%) sucrose concentrations (Williams 1989, Cheng *et al.* 1992). Studies of *Hibiscus rosa-sinensis* demonstrated that etiolated shoots (the effect of a dark treatment) reduced the amount of structural tissue and increased parenchyma in these stems (Herman and Hess 1963).

1.2.5 pH of the medium

The pH level of the rooting medium can be an important consideration in the induction of roots of woody species *in vitro*. Plant tissue culture media are usually adjusted before autoclaving to values of between 5.5 and 6. Lower pH levels prevent the gelling agent from solidifying. This problem can be overcome by increasing the agar concentration of the media from 0.8 to 1.0%. *Prosthanthera striatiflora* explants produced roots at 57% at pH 4, compared to only 14% rooting at pH 5. Furthermore, when low pH (4) and a dark period were combined, up to 100% of plantlets produced roots (Williams *et al.* 1985).

Barlass *et al.* (1981) also utilised a combined dark period and low pH (4) to obtain 40% rooting of *Santalum* shoots *in vitro*.

1.2.6 Length of time in culture

The length of time in culture has been linked with rooting ability. *Eucalyptus citriodora* shoots did not produce any roots on root induction medium until after at least three subcultures on multiplication medium (Gupta *et al.* 1981). Hammerschlag *et al.* (1987) obtained a two fold increase in rooting of peach cultivars *in vitro* after 19-21 months in culture. Similar observations have been reported for apple rootstock's (James and Thurbon 1980). The authors suggest that increased rooting over time was related to a relocation of cytokinin to the upper growing parts of the plants away from the shoot bases.

1.2.7 Genotypic variability

The induction of roots on cuttings and micropropagated shoots is frequently determined by the genotype of the plant. Using a number of lines of *Eucalyptus marginata* shoots initiated from individual mature trees, McComb and Bennet (1986) found differences for the various lines in the percentage of shoots which produced roots (10-90%). Coleman and Ernst (1989) also reported genotypic differences in shoot proliferation and root induction of *Populus deltoides* in response to the same culture conditions. These studies and others (eg, Hammerschlag *et al.* 1987, Neiderwieser and Staden 1990, Abe and Futsuhara 1986) demonstrate the importance of the explant genotype for successful micropropagation. Differential responses of species to culture conditions have been attributed to different levels of endogenous hormones within different genotypes (Hempel 1979, Abe and Futsuhara 1986). The identification and screening of useful genotypes for shoot proliferation and root induction is necessary for successful commercial micropropagation (see above, Section 1.2.1).

1.3 OUT-PLANTING

Stage IV (see Section 1.2.1), the transfer of plantlets from *in vitro* conditions to the glass house is a critical stage which determines the overall success of a microporpagation protocol. In many cases this stage is often not reported. When it is reported, survival percentages of plantlets is often low. Sankaro Rao (1988) was able to obtain a rooting percentage (55%) of *Eucalyptus tereticornis* shoots *in vitro* however, only 10% survived after transfer to glasshouse conditions. Gupta *et al.* (1981) reported 40% survival of *E. citriodora* plantlets at Stage IV (see Table 1). Although this may be acceptable for scientific investigations, successful commercial production depends on wastage rates below 10%.

Successful acclimatisation depends on a number of environmental conditions in the initial period after transplanting. The soil mix should be neutral or slightly acidic pH with good drainage and aeration to allow new roots to develop and grow quickly. It must also be free of soil contaminants. In addition, the maintenance of high relative humidity (60-100%) protects the plant from desiccation, and low light conditions are required. Hygiene practices are necessary to protect the plantlets from various pathogens until some natural resistance has developed.

1.4 POT CULTURE: INDUCTION OF LATERAL SHOOTS

Plants are grown in containers for a number of reasons. It enables growth out of their normal climatic zone and to allows special cultural conditions to be bestowed on difficult-to-grow plants. The container should have adequate drainage, yet provide sufficient water retention to maintain steady growth (Torres 1989). Often special forms of plant growth are required. For sale as pot plants, the growth form should be compact. The centuries-old practice for producing lateral bud growth involves removing terminal buds to induce branching.

A feature of development in many plants is the dominant influence of the apical bud meristem over the lateral, axillary buds. A growing apical bud will inhibit the development of other lateral shoot buds (Phillips 1975). The mechanisms involved are complex. An active apical bud is both a source of growth regulators and also a sink for nutrients and water. Interruption of auxin transport (removal of the apical bud) also stimulates lateral shoot growth by removing the "correlative inhibition" signal from the apical bud (see Phillips (1975) for a comprehensive review). Early work recognised that lateral shoot growth can be induced by the application of cytokinin and gibberellin. Horticulturally, foliar sprays such as 6-benzyl amino purine (BA) have been widely used to stimulate lateral branching in a variety of plants (Henny and Fooshee 1985). "Pinching" or "topping" (manual removal of the apical growing shoot) is another method employed to induce the development of one or more lateral shoots (Imamura and Higaki 1988). The combination of both these techniques has stimulated branching in ornamental (Jeffcoat 1977; Henny and Fooshee 1985; Imamurs and Higaki 1988), flowering (Ohkawa 1979) and fruit (Kender and Carpenter 1972) crops.

1.5 SUMMARY

Empirical investigations with woody plant species suggest that the underlying mechanisms involved in root production are poorly understood. This is illustrated by the variability of response shown by different plant species to similar culture conditions. There are a number of culture conditions that need to be considered for the rooting of woody plants. These include the condition and age of the plant material, media conditions (mineral levels, sucrose, auxin type and concentration and pH), environmental conditions (eg light, dark treatments) and the plant genotype. At this stage it is not possible to outline specific *in vitro* rooting requirements for different plant species but rather an empirical approach has to be explored.

McComb *et al.*(1986) have shown that micropropagation is possible for *Verticordia*. Good shoot multiplication was obtained with kinetin in combination with indole-acetic-acid, but rooting of these shoots was limited. McGuire (1989) in collaboration with honours students investigated the ability of cytokinin type and concentration to induce lateral shoots *in vitro* in this genus. BA was found to be the most successful cytokinin for shoot production. No rooting percentages of the shoots were reported. There have been no further reports of investigations of root induction in this genus. As a first approach to improve the rooting percentages of *V. grandis* explants *in vitro*, a dark pre-treatment in combination with other factors, such as pH of the medium, sucrose levels, and plant genotype will be investigated further in Chapter 3.

An alternative approach to overcoming the difficulties of root production of *V. grandis* may lie in the utilisation of *Agrobacterium rhizogenes* to induce hairy roots via genetic transformation. To date there have been no reports of genetic transformation of *Verticordia* or, indeed of any plants within the Myrtaceae. The application of *A. rhizogenes* for root induction and as a tool for genetic manipulation with 'desirable' genes will be considered in the following section.

1.6 *AGROBACTERIUM* INFECTION AND GENETIC TRANSFORMATION

Genetic transformation can be defined as the transfer of foreign genes into a new genetic background. In nature, the soil bacteria *Agrobacteria tumefaciens* (Smith and Townsend 1907) and *A. rhizogenes* (Riker 1930) are the causal agents of crown gall and hairy root disease in plants, respectively [(For reviews see Gelvin (1990), Hooykaas (1988), Binns and Thomashow (1988), Klee *et al.* (1987), Peterson *et al.* (1989), Birot *et al.* (1987), Depicker *et al.* (1985)]. Transformation is based on the ability of *Agrobacterium* to insert DNA into the genome of host plants, which includes most dicots and also some monocots (De-Cleene 1984). The host range is dependent upon both the type of Ti or Ri plasmid

carried by the *Agrobacterium* strain (see below) (Nester *et al.* 1984) and the type of wound response induced in the plants (Schafer *et al.* 1987).

Systems for the transfer of foreign DNA into plant cells range from simple infection methods, eg wounding and co-cultivating plant tissue (eg stems, leaf disks, hypocotyls and embryos) with *Agrobacterium,* to more sophisticated methods such as direct gene transfer by electroporation or micro injection of plasmid DNA into plant protoplasts (Neuhaus *et al.* 1987). Recently, the biolistic technique has been used for the delivery of DNA into intact cells and tissues. This process utilises a particle gun which projects DNA-bearing particles through cell walls and membranes (Klein *et al.* 1990). However, the most commonly used method relies on the natural ability of *Agrobacterium* to infect and transfer part of its plasmid DNA into a plant cell (see Fig 1.2). This system of transfer is dependent both upon the susceptibility of the plants to infection by *Agrobacterium* and upon a system of plant regeneration for the recovery of transformants. As mentioned above, the regeneration of shoots and induction of roots, *in vitro* are more difficult for woody plants than for herbaceous plants. Therefore, the method of gene transfer which is chosen is governed by the plant species and its regenerative response *in vitro* (Webb and Morris 1992).

1.6.1 *Agrobacterium:* Biology

The ability of *Agrobacterium* to transform cells is determined by large plasmids termed Ti for tumor inducing and Ri for root inducing. The sizes of the Ti and Ri plasmids range from about 180-250 kb. The infection process requires wounding of the plant tissue. Wounded cells release phenolic compounds which act as chemical signals to induce virulence (vir) gene expression in the bacterial cell (Depicker *et al.* 1983) see Fig 1.2. Following infection the T-DNA, a small region of the Ti or Ri plasmid, is transferred and integrated into the plant genome (Chilton *et al.* 1977). The size of the T-DNA region ranges from 23-25 kb, and is dependent upon the plasmid type (Peterson *et al.* 1989). The T-DNA region is flanked by almost perfect direct repeats, termed the left and right borders.

The T-DNA region contains all the genes necessary to induce the morphological changes in the transformed plant. The root morphological changes incited by the Ri plasmids of *Agrobacterium rhizogenes* are investigated in this study (see below). A characteristic of transformed galls and hairy roots in culture is their ability to grow on hormone-free medium. Oncogenic (onc) genes, are responsible for the synthesis of growth hormones which induce the characteristic tumors and hairy roots. Depending on the *Agrobacterium* strain the onc genes code for a number of enzymes involved in the biosynthesis of an auxin, indole acetic acid (IAA) and a cytokinin [for reviews see Peterson *et al.* (1989), Cardarelli *et al.* (1985) and Binns and Thomashow (1988)]. The T-DNA also contains genes which code for enzymes that synthesise disease specific compounds called opines. Opines provide the bacteria with a source of carbohydrate and nitrogen not normally synthesised by plant tissue. Classification of the *Agrobacteria* is based on the specific opines they produce.

1.6.2 *Agrobacterium rhizogenes*

Plants susceptible to infection by *A. rhizogenes* develop roots (hairy roots) at the inoculation site (Elliot 1951). Characteristically hairy roots grow profusely on hormone free medium (Tepfer 1984), see oncoengic genes above. Three types of Ri plasmids have been identified. These are distinguished by the opines they encode and the structure of their T region. For agropine type Ri plasmids the T region is split into two parts of unequal size, the TL-DNA and the TR-DNA (see Fig 1.3). The mannopine and cucumopine Ri plasmids contain only a single T-DNA region. The mechanisms which elicit the hairy root morphology appear to be different for the two types of plasmids, and are related to the stable integration of the whole T-DNA region or for agropine type plasmids only one region, either the TL or TR-DNA may be integrated (Peterson *et al.* 1989). For example, following agropine Ri transformation of potato roots with prolonged root culture and shoot regeneration deletions of the TL and TR-DNA insertions occurred (Hänischten Cate 1990). Furthermore, the detection of opines in regenerated shoot lines was correlated with the presence of the TR-DNA, loss of opine synthesis was caused by deletion of TR-DNA.

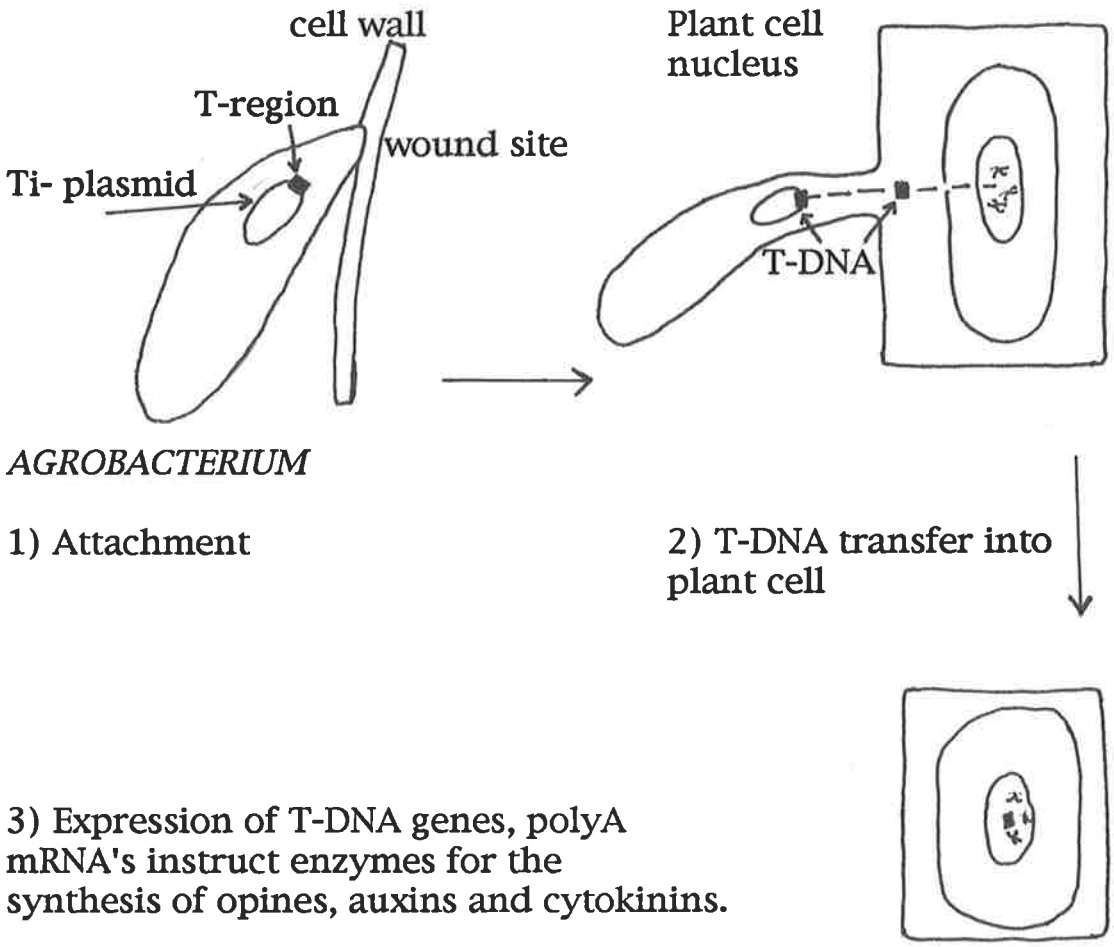


Fig 1.2: A simplified diagram of the infection and integration of *Agrobacterium* into a plant cell.

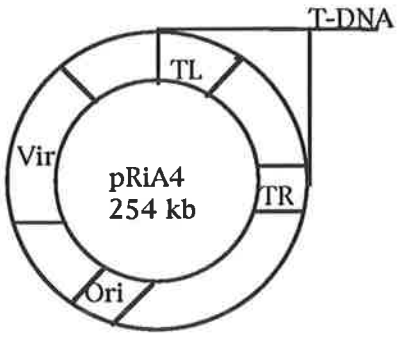


FIG: 1.3: A simplified map of an agropine Ri plasmid, showing the two regions of the T-DNA, the TL and TR, the virulence region and the origin of replication (ori) (Peterson *et al.* 1989).

1.6.3 The Ri phenotype

The hairy-root phenotype is similar in many plant species. Typically, roots show high growth rate, reduced apical dominance and plagiotropism. The root morphology incited by the Ri plasmids are of particular interest in this study (see Chapter 5). Examples of the use of wild type *A. rhizogenes* strains for root induction on a number of woody plants have been reported in apple (Lambert and Tepfer 1991) and kiwi (Rugini *et al.* 1991) plants. Transformed roots are often capable of regeneration into transformed plants. Birot *et al.* (1987) provide a list of over 50 herbaceous plants which have been regenerated from transformed hairy-roots. Indeed for some species, shoots can spontaneously regenerate from hairy-roots on hormone free medium (Phelep *et al. 1991*). Shoots regenerated from transformed hairy roots display altered phenotypes. Shoot characteristics include wrinkled leaves, reduced shoot apical dominance, altered flower morphology and reduced pollen and seed production (Tepfer 1984).

1.6.4 Genes involved in hairy root formation

The expression of Ri T-DNA genes appears to be highly variable, and often dependant on the plant species under study (Ooms *et al.* 1986). The T-DNA of Ri plasmids code for at least three genes that each can induce root formation, and that together cause hairy root formation in transformed plant tissue. The TL-regions of agropine Ri plasmids are strongly homologous to the single T-region of mannopine and cucumopine Ri plasmids. Both the TL and TR region induce roots on transformed tissue but the mechanisms are different (Peterson *et al.* 1989). Roots induced by the entire agropine plasmid T-DNA or the single T-region of mannopine and cucumopine plasmids exhibit the typical phenotype of hairy roots (see above Ri phenotype) whereas, roots caused by TR-DNA alone exhibit a phenotype similar to normal roots. The TR region of agropine strains contains sequences exhibiting homology to the auxin genes of *A. tumefaciens* T-DNA (Peterson *et al.* 1989). TR induced roots (similar to crown galls) are the result of phytohormone production, due to the *aux* genes, and therefore may contain chimeric tissue (Schröder *et al.* 1984).

Mannopine and cucumopine Ri plasmids do not contain *aux* genes (Cardarelli *et al.* 1987 a). Of interest, the *aux* genes in the TR-DNA were shown to be responsible for extended virulence on carrot discs transformed with agropine type *A. rhizogenes* compared to mannopine and cucumopine types (Cardarelli *et al.* 1987 a). Auxin provided by the TR-DNA triggers differentiation of auxin-responsive TL-DNA genes. Similarly, the same effect can be obtained with plant auxins in the case of hairy roots induced by mannopine and cucumopine strains (Cardarelli *et al.* 1985).

Four loci involved in hairy root induction and defined according to the tumor morphology observed were identified by White *et al.* (1985). The loci are in the TL-DNA region of agropine-type Ri plasmids and named root loci (*rol*) A, B, C, D. Subsequent DNA sequence and transcript analysis of this region found that the *rol* genes corresponded to specific open reading frames (Slightom *et al.* 1986). Cardarelli and associates (1987 b) demonstrated that the *rol* B locus on its own was capable of inducing the hairy root phenotype on wounded tobacco stems. However, leaf explants regenerated from the hairy roots did not produce roots on hormone-free medium, unlike transformants with construct *rol* A, B, C. Furthermore, both the addition of auxin and the presence of T-DNA in carrot cells was required for hairy-root induction on the carrot discs. Morphological changes in belladonna plants transformed with a CaMV 35S-*rol C* gene of the Ri plasmid were attributed to the integration of the *rol* C and were also related to the degree of expression of this gene (Kurioka *et al.* 1992). The induction of roots by inoculation of apple stem cuttings with a mannopine *A. rhizogenes* strain was found to be due to the transfer of the *rol* C gene (Lambert and Tepfer 1991). The action of the *rol* D gene appears to be less thoroughly investigated. Hairy root cells have been shown to be more sensitive to the effects of auxin (Shen *et al.* 1988). Also, higher concentrations of auxin were found in leaves regenerated from hairy roots compared to normal plants (Spano *et al.* 1988). These results and others (Cardarelli *et al.* 1987 a,b) suggests that the products of the *rol* genes probably alter the sensitivity of plant cells to auxin (see Gelvin (1990) for a review of the role of *rol* genes in the hairy root syndrome).

1.6.5 Target cells for genetic transformation

The type of cells, tissues and organs targeted for *Agrobacterium* infection can affect transformation frequencies in different host species. For example, soybean cotyledons inoculated with A. *rhizogenes* produced a greater hairy root response than infected hypocotyls (Rech *et al.* 1988). In contrast, transformed *Brassica campestris* plants were regenerated from hypocotyl explants at a frequency of 7-13% compared to only 0.02% regeneration of cotyledon explants (Mukhopadhyay *et al.* 1992). The differences in transformation efficiencies were related to anatomical studies on the mode of regeneration in the two types of explants.

Earlier evidence of *Agrobacterium* tissue specificity was reported by Moore *et al.* (1979). They showed that the presence of a pericycle region of the primary root (in carrots) was essential for the induction of roots by *A. rhizogenes*. In plants the pericycle region is the location where secondary roots arise and has meristematic potential. Juniper and elm stem cuttings which lacked a pericycle region and were infected with *A. rhizogenes* failed to form roots (Unpublished results quoted in Jayes and Strobel 1981). Apple rootstock's infected *in vitro* with *A. rhizogenes* produced roots at the wound site, but infected scions did not form any roots (James 1987). Furthermore, genotypic variation of susceptibility to *Agrobacterium* infection has been demonstrated.

The oncogenic genes (see above) of wild type *A. tumefaciens* strains can be disruptive to normal growth. This is reflected in the limited number of reports of shoot regeneration from crown gall tissue. For the successful regeneration of transformed shoots it is important to avoid chimeric growth (McGranahan *et al.* 1990). However, galls can consist of both transformed and untransformed cells (James 1987). An advantage of the *A. rhizogenes* transformation system is that the hairy roots are composed entirely of transformed cells (Moore *et al.* 1979). Therefore this distinctive phenotype becomes a easily recognisable marker to screen for transformants. Another advantage of Ri-compared to Ti plasmid-mediated transformation is that the presence of the exogenous Ri T-DNA

segment does not prevent the regeneration of whole fertile plants from hairy roots (Han *et al.* 1993). However, not all plants respond to infection with wild type *A. rhizogenes* by producing hairy roots, some produce tumors or shoots or even a combination of all three (Peterson *et al.* 1989). For example, Tepfer (1984) found that depending on the plant genotype, inoculations of tobacco leaves with *A. rhizogenes* produced numerous leaves and only occasionally roots.

Selection, growth and regeneration of transformed cells is critical for the successful recovery of genetically transformed plants. The construction of vector plasmid systems with *Agrobacterium* enables the integration of marker genes into plant tissue. The gene inserts should allow for selection of transformed cells, preferably without the undesirable tumorous growth induced by the onc genes of Ti plasmids.

1.6.6 Disarmed vector systems

Ti plasmids have been used extensively as vectors to introduce foreign DNA into plants. To ensure the recovery of normal plants, the plasmids are disarmed by removing the oncogenes responsible for the synthesis of growth hormones and replacing them with selectable or screenable markers. Two types of vector systems are possible, cointegrating and binary vectors (Ahuja 1987). Cointegrating vector systems contain a region of genetic homology between the Ti plasmid and the vector plasmid, while the binary vector systems rely on a mutual co-existence of two plasmids in the *Agrobacterium*. With the latter method, a foreign gene is inserted into a disarmed T-DNA plasmid that can replicate in *E. coli* and in the *Agrobacterium*. Transfer of the T-DNA to the plant is obtained by the plasmid in *Agrobacterium* which contains the vir genes. In addition, the T-DNA border sequence must contain 25-bp direct repeat sequences (Depicter *et al.* 1986). However, binary vectors tend to be less stable and can be lost from the *Agrobacterium*.

1.6.7 Marker genes

The most common marker systems are single dominant genes encoding suitable resistance to a selective agent which does not disrupt plant regeneration, but allows for selection of transformants. The selection agent must inhibit growth of non transformed tissue without being too toxic to them. Dying cells can release phenolic compounds which inhibit the growth of surrounding cells (Webb and Morris 1992). The most commonly employed selection marker system is the neomycin phosphotransferase (NTP-II) gene, which confers resistance to aminoglycosides such as kanamycin. The enzyme NPT-II detoxifies aminoglycosides which affect translational activities of cells. Cells carrying this gene have a selective advantage in the presence of kanamycin (Ahuja 1987). This results in faster growth of transformed cells relative to non-transformed cells and consequent selection for the former. However, in some plants the growth of non-transformed cells is not limited by kanamycin. Consequently a number of researchers have reported difficulties with kanamycin selection. McGranahan *et al.* (1988) found kanamycin resistant walnut embryos could only be reliably selected after continued subculture for several months on kanamycin medium. The induction of hairy roots on potato leaf discs was found to be a better selection criterion than kanamycin resistance screening, using the binary vector pBI121 in conjunction with the Ri plasmid AM8703 (Viser *et al.* 1989).

Some of these problems can be overcome by linking the kanamycin selection system with a marker gene such as betaglucuronidase (GUS) gene. The GUS gene system permits the detection of gene expression in transformed plants by simple histochemical and flurometric assays (Jefferson *et al.* 1987). Histochemical staining with 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc) as a substrate for GUS can be applied to demonstrate the localisation of GUS in transformed cells and tissue. For a review of the GUS fusion system for marker genes in plants see Jefferson *et al.* (1987).

McGranahan *et al.* (1990) developed an efficient transformation system for the regeneration of walnut embryos *in vitro* using a vector containing both the NPT-II and

GUS genes. This facilitated early screening and selection of transformants. The introduction of GUS allowed the positive identification of transformed embryos as early as 5-6 weeks after inoculation. There was a problem with background GUS activity due to the presence of bacteria, however the GUS activity of the bacterial and plant tissue could still be distinguished, as the blue colour caused by the bacterium was diffuse and lighter than that of the transformed embryos. A number of investigators have also reported 'false positive' results or 'background activities' with GUS assays see Hu *et al.* (1990). These 'false positives' may be due to intrinsic GUS-like activities (Hu *et al.* 1990), which appear to be related to the pH at which the assay was performed (Hodal *et al.* 1992). And are also linked to the presence of endophytic bacteria which can express GUS (Tör *et al. 1992*). These findings reinforce the need for appropriate controls to confirm that GUS activities are due to transformation.

1.7 APPLICATIONS

The altered root system induced by *A. rhizogenes* may confer an increased drought tolerance on plants (Moore *et al.* 1979). Moore and Millikan (unpublished results in Moore *et al. 1979*) claimed that apple trees exhibiting the hairy root syndrome survived the drought period of 1975-'76 better than control plants. This appears to be the only report of utilising the hairy root syndrome for drought tolerance in woody plant species. However, the use of wild type A. *rhizogenes* to induce root development of woody plants has recently been explored (see Section 1.1).

Although woody plants do exhibit susceptibility to *Agrobacterium* infection, there have been only a few reports of regeneration of transformed woody plants. These include *Malus* (James *et al.* 1987); *Populus* (Fillalti *et al.* 1987); *Jugans* (McGranahan *et al.* 1988); *Rubus* sp (Graham *et al.* 1990); *Allocasuarina* (Phelph *et al.* 1991); *Robina* (Han *et al.* 1993) and *Citrus* (Vardi *et al.* 1990). Transformed roots have been reported in *Rosa canina* rootstock's (van der Mark *et al.* 1990). Grape shoots regenerated from inoculated meristems have been shown to contain both transformed and untransformed tissue (Baribault *et al.* 1990). Transformed callus has been obtained from other species eg,

douglas-fir (Dandekar *et al.* 1987); *Alnus* and *Betula* (Mackay *et al.* 1988) however, the transgenic plants were not successfully regenerated. There are several possible reasons for the limited transformation success of woody plants and include; the host range of *Agrobacterium*, the genotypic variation of susceptibility to *Agrobacterium* infection and, probably most importantly, the difficulty of shoot regeneration systems. The ability of cells to regenerate into plants in culture varies not only with the cells and tissues themselves but also with the plant species, cultivar and genotype independent from cultural conditions. For transformation and successful regeneration of transgenic plants, both *Agrobacterium* susceptibility trials and well defined *in vitro* culture conditions are necessary.

The application of *Agrobacterium* mediated gene transfer techniques remain dependent on the shoot regeneration system presently available for a given plant species and genotype. Further time spent developing these methods will prove beneficial. With improved tissue culture methods, regeneration of transformants should become more frequent. Similarly, gene fusion techniques also allow for the development of vector systems which can provide more efficient screening and selection of transformants.

Many woody plant species are of great economic importance for forestry (eg, *Eucalyptus, Pinus*), horticulture (eg fruit and nut) and as ornamental (pot plants and cut flowers). Gene transfer techniques applied to these plants have potential use for improvement programs. Genetic engineering of resistance to a variety of pests, diseases and herbicides is the focus of a great deal of current research in crop plant biotechnology. For example plant damage caused by some insect larvae can be suppressed in transgenic plants expressing the *Bacillus thuringiensis* toxin gene. This gene produces an active insecticidal protein which protects against *Lepidopteran* insect attack (Mol *et al.* 1989). Recently, Perlak *et al.*(1990) transformed cotton plants with the protein genes of *Bacillus thuringiensus* var *Kurstaki* HD-1 (*cryIA*b) and HD-73 (*cryIA*c) and found these plants to be resistant to two Lepidopteran insects, *Trichoplusiani sp* and *Spodoptera exiqua*. This type of approach could be useful for a wide range of plant species sensitive to insect attack. Other

applications of genetic engineering for the ornamental plant industry are possible. Some important traits for flower breeding are flower colour, shape, and vase life. An anti-sense chalcone synthase gene is available which has the potential to alter the flower colour of plants of floricultural value. Expression of this gene in transgenic petunia plants, resulted in a high frequency of plants with an altered flower pigmentation (van der Krol *et al.* 1988). By manipulating endogenous genes involved in flower pigmentation and senescence, new, longer lasting plant varieties may be possible.

1.8 PROJECT AIMS

This project has two objectives. Firstly to establish a micropropagation system for *Verticordia grandis* which can be successfully applied to commercial production, and secondly to use the *in vitro* regeneration system as a tool to enable genetic transformation of *V. grandis* and regeneration of transformed plants.

Experiments were carried out to provide answers to the following questions:
What cultural conditions are required for successful micropropagation of *V. grandis*?
What methods of *in vitro* regeneration are possible eg. axillary, adventitious shoots?
What factors affect root induction of *V. grandis* explants *in vitro*?
What conditions are required for successful acclimatisation to the glasshouse?
What cultural conditions are required for pot culture production of *V. grandis*?
Is *V. grandis* susceptible to infection by *Agrobacterium*?
Does *A. rhizogenes* induce hairy roots in *V. grandis* explants?
Can an efficient transformation system be established?
Which *Agrobacterium* vectors and selectable markers would be most useful to select for the recovery of transformants?
Which cell types are infected? Is it possible to infect meristematic cells and regenerate transformed plants?

CHAPTER 2 MATERIALS AND METHODS

2.1 TISSUE CULTURE TECHNIQUES

2.1.1 Plant material

The plant used in all investigations was *Verticordia grandis* (Myrtaceae). Two clones of *V. grandis* were used. Clone (865) was supplied as micropropagated shoots from Phytotech Australia Pty. Ltd. It had been in culture for approximately 2 years. During this time its rooting performance was poor. A second Clone (866) was obtained as stem cuttings and was supplied by Mrs P. Moyle from Western Australia. It has been used as the main source of cutting material for propagation. Fig. 2.1 depicts stem cuttings of *V. grandis* Clone 866. Cuttings were transported from Perth by air freight, wrapped in moist tissue. On arrival, the material was kept refrigerated prior to initiation into culture. Due to the immediate availability of large numbers of Clone 865 shoots *in vitro,* initially this clone was used for both tissue culture and transformation experiments. As soon as Clone 866 was initiated into culture and a sufficient number or shoots were multiplied, this clone was included in both the tissue culture and transformation experiments.

2.1.2 Initiation of *V. grandis* Clone 866

Stem segments 6-10cm in length, with the flower buds removed, were excised and the cut ends were sealed with paraffin wax. The explants were washed by constant agitation in 0.1% available chloride as sodium hypochlorite containing a drop of Tween 20 for 3 hrs. The explants were then cut into sections 1cm in length and consisting of a single node and were placed into a fresh solution of 0.1% sodium hypochlorite for different time periods (see Results Section 3.1). Sections were then removed and inserted vertically into basal media described by Murashige and Skoog 1962 (MS medium, see Appendix A, Table 1 for details). Fungal and bacterial contamination was detected visually and occurred in 46% of cultures. These cultures were discarded. Results section 3.1 shows the contamination rate for cultures sterilised for the various time periods.

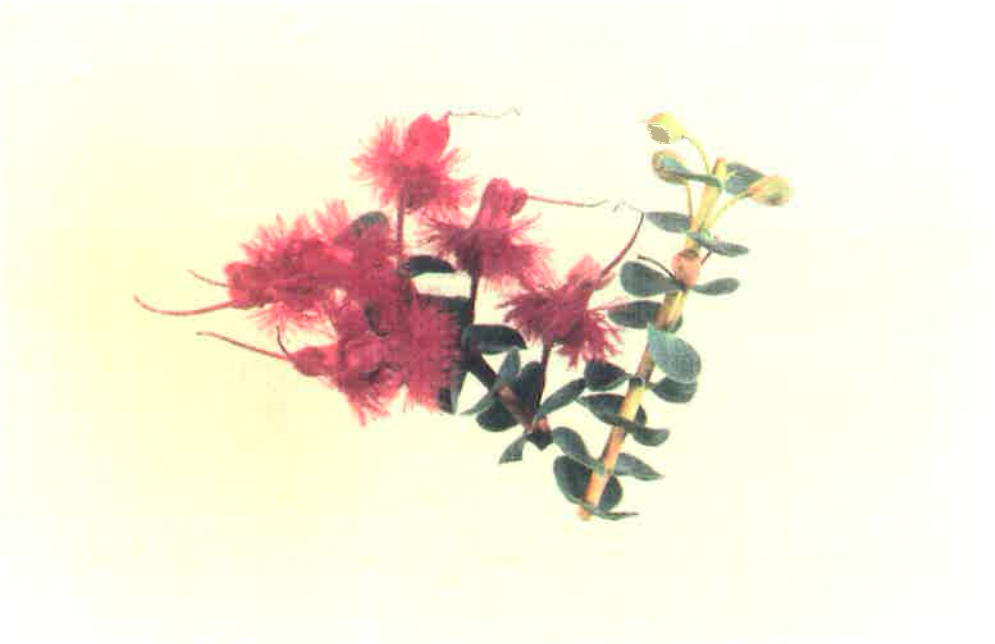


Figure 2.1. *Verticordia grandis* cuttings from material grown in W.A. Clone 866.

2.1.3 Media and tissue culture conditions

All media with added plant growth regulators were dispensed in either 50ml aliquot's in 8.5x7cm screw cap polycarbonate jars for multiplication, or 75ml aliquot's in 6.5x9.5cm polycarbonate snap sealed tubs, for root induction. The pH of the medium was adjusted prior to the addition of agar and autoclaved at 121°C for 20 min. All cultures, unless stated otherwise, were maintained at 23 ± 2°C, under cool white fluorescent lights (approx.35-50μE/s/m^2) 16hr day/8hr dark regime. Explants were subcultured at 3-5 week intervals.

2.1.4 Shoot multiplication media

The basal medium consisted of MS mineral salts and vitamins (Appendix A: Table 1). Unless otherwise stated, other components (3% sucrose, 0.8% Davis agar and pH 5.7) remained constant.

Separate experiments were carried out to evaluate the effects of hormone additions of the cytokinins benzyladenine (BA) and kinetin (KIN) and the auxin indole-3-butyric acid (IBA) on shoot proliferation. The multiplication media developed by McComb (1986), (MS basal with the addition of 0.5 μM IBA and 20μM KIN) was also tested. Each jar of media contained 10 explants.

The medium adopted for shoot proliferation, as a result of earlier experiments (see above and Results Section 3.3.2) was MS mineral salts and vitamins with the following additions; 3% sucrose and 4.5μM BA. Media were solidified with Davis agar at 0.8%. The pH of the media was adjusted to 5.7 with sodium hydroxide prior to autoclaving for 20min at 121°C.

2.1.5 Shoot regeneration from leaf discs

For shoot regeneration from leaf discs, whole leaves were removed from explants on multiplication medium (described above) and were placed on MS medium with the addition of one of the following phytohormones; BA (4.5 or 9μM), BA (4.5μM) and IAA (1.5μM), KIN (20μM) and IBA (0.5μM) (McComb 1986), and a phytohormone control. Each treatment was replicated 3 times with at least 5 leaves per replication. Leaves (leaf

discs), with the basal side facing the media, were cultured in 100mm sterile polystyrene Petri dishes (each dish contained 20 ml of medium) and were sealed with parafilm. Four weeks after the leaves were placed on the culture medium, the percentage of leaf discs forming shoots and the mean number of shoots per leaf disc were determined for each treatment. The shoots were then transferred to multiplication medium (described above).

2.1.6 Root induction media

Media containing half-strength (1/2MS) minerals supplemented with either 1.5% or 3% sucrose (as stated) and MS vitamins was used for all root induction experiments. Care was taken to use only actively growing axillary shoots 2-3cm in length, avoiding woody material. Most treatments contained 3 replications with at least 30 explants per replication. After 6 weeks in culture, the number of rooted explants was recorded and rooting percentages were determined.

2.1.7 Pre-treatment

Comparisons were made using explants from various pre-treatment multiplication media. Shoots were excised from cultures growing on multiplication medium with the addition of either; 4.5μM BA, 20μM Kin and 0.5μM NAA, or a basal control with no added phytohormones. The auxins, IAA and IBA at concentrations of 0 and 10μM each were added to the root induction medium with 3% sucrose (described above) to determine which auxin was more effective for root induction of micropropagated explants. In addition, explants were exposed to a 2 week dark period prior to exposure to the standard 16hr light/ 8hr dark cycle. A control treatment without a dark period was included.

2.1.8 Dark treatment

For ease of handling during out-planting an experiment was designed to test the length of time necessary for explant exposure to darkness to maintain high rooting percentages. Explants of Clone 866 were placed on root induction medium 1/2MS minerals supplemented with 3% sucrose, MS vitamins and 10μM IBA. The pH of the medium was

adjusted to 5.7. Each treatment contained 2 replications with 20 explants per replication. The culture jar with explants were exposed to 0, 1, or 2 weeks of darkness after which the cultures were returned to the normal photo period cycle as described above. After 6 weeks in culture, the number of rooted explants was recorded and rooting percentages were determined.

2.1.9 Auxin, pH and sucrose concentration

Subsequent experiments also tested the effect of sucrose concentration (1.5% and 3%), reduced pH of the medium (media with the pH adjusted to 4 instead of 5.7), and different concentrations of IBA of 0, 10 and 50μM and combinations of auxins at 10μM IBA plus 5.4μM NAA for improved root production. All treatments were exposed to a 2 week dark period.

2.1.10 Genotype effects

The effect of the genotype on root formation on explants of the two *Vericordia* Clones, 865 and 866, was determined. The root induction medium 1/2MS minerals supplemented with MS vitamins with the following additions was tested; sucrose concentration of 1.5% or 3%, IBA at concentrations of 0, 10 and 50μM and 10μM IBA in combination with 5.4μM NAA was added to the medium. The pH of the medium was adjusted to 4 or 5.7 and all treatments were exposed to a two week dark period.

2.1.11 Explants from different sources

Comparisons were made between the type of explant used for root induction. Three types of shoot material was tested. 1) axillary shoots with the growing tip intact, 2) as above without a growing tip (internode section) and, 3) small clumps of whole shoots (main shoot with 2-4 axillary shoots intact). Twenty explants of each type replicated 5 times were placed onto root induction medium 1/2MS minerals supplemented with MS vitamins, 3% sucrose and 10μM IBA, with a 2 week dark period. The percentage of explants which developed roots was recorded after 6 weeks.

2.2 TRANSFER OF PLANTLETS TO SOIL

2.2.1 Glasshouse conditions

Verticordia plants were grown in a glasshouse of the following construction;

A brick base to 1.1 metres, topped by 4.5mm prismatic acrylic to a height of 1.2 metres for the outer panels. Panels between compartments are 3mm clear glass starting at 1.2 metres high with an outer panel rising to 2.2 metres at the central corridor. The roof is of prismatic acrylic. Supporting struts are 35mm aluminium. The compartment (3m x 2.2m) was of western aspect. Plants were grown on benches 92mm high and 50mm wide. The construction permitted an average percentage of light transmission of 30% during the months from October through to March (70% white knitted shade cloth was permanently down during these months) and 50% between April and September.

The temperature of the glasshouse was maintained between a minimum of 15°C and 30°C maximum. The mean temperature range varied according to the season. Light levels measured as $\mu E/s/m^2$ also varied according to the season. Appendix B (Table 1) shows the average minimum, maximum and mean temperature of 4 months throughout the year. Corresponding light intensities are also presented.

2.2.2 Hardening off Conditions

Plantlets were removed from tubs and as much agar as possible was removed without damaging the roots. Humidity levels were kept high by frequently spraying the foliage with water from a fine spray bottle. Plantlets were planted into 64 plug cell trays containing a soil mixture ratio (5:2:1) of Composted pine bark*: Isolite: Peat moss*. Nutrients added to the mix were Slow release Osmocot® (18:2.6:10) at $2kg/m^3$ and Fe EDTA at $30g/m^3$, the pH of the medium was adjusted to 5.5 with lime, (* fine grade). The trays were well watered with a mixture of Previcure® and Benlate®.** Once planted each tray was placed under a humidity tent (see Fig 2.2 A) in a glasshouse (The tray was covered with a gas-permeable, clear plastic film under shade cloth). The humidity levels

** 1ml/l of Previcure®, 50mg/l of Benlate®.

were gradually reduced over a period of 6 weeks by gradual removal of the plastic film. The shade cloth was removed after 4 weeks to increase light levels. Once roots emerged from the bottom of the trays and new shoot growth had occurred, plants were repotted (see Fig 2.2 B). *V. grandis* plants were always watered at the soil level to avoid water sitting on and around the plant.

2.2.3 Effect of soil mix on growth

Four soil mixes were used to compare plant growth rates (height in cm and the number of lateral shoots) of *V. grandis* pot plants. Plants 6 weeks after transfer to the glasshouse were repotted into 15cm black plastic pots. Plant height and number of new lateral shoots was measured fortnightly. The number of flower buds per plant was also recorded. The following soil types were compared;

1) Out-planting soil (see above),

2) Composted pine bark: coarse sand (5:2),

3) Loam: bush sand: coarse sand:: peat (2:1:1:1:1),

4) Coarse sand: bush sand: composted pine bark: peat: (1:1:1:1:1).

A total of 24 plants were randomly allocated to each mix. Each group was split into half. Group A received an additional complete liquid fertiliser (Kendon® Liquid manure- see Appendix A: Table 2 for the nutrient analysis of this fertiliser), applied at either one of two application rates, group A received a dilution of 1 part to 800 parts of water, while group B received an application of 1 part to 400 parts of water.

The air filled porosity of each soil mix was determined by the method described in (Handreck and Black 1984) for potting mixes.

Figure 2.2 Plant-out conditions for *Verticordia grandis* tissue cultures.

A: High humidity levels were maintained with a plastic cover looped over the plants. Shadecloth was used to reduce light levels during the first four weeks.

B: After six weeks plants are hardened off and ready to pot on.


A


B

2.2.4 Methods of lateral shoot induction

In this study a number of experiments were designed to test the effectiveness of BA (and the method of application) in increasing the number of lateral shoots in *V. grandis*. The effect of apical tip removal in isolation or together with BA application was also tested. The two methods of BA application were, spraying plant foliage (to run off) or a soil drench. Individual plants received 10 ml of BA solution regardless of application method. Untreated control plants received the same application of water.

In the first experiment 80 *V. grandis* plants 6-8 weeks since transfer to glass house conditions, were potted into 10cm black plastic pots containing soil Mix 1 (see Section 2.2.1). Prior to treatment, plant height from the plant base to the highest point reached with the foliage held vertically was measured. Ten plants received 1 of 8 treatments consisting of an untreated control or BA application at 50, 210, and 1000 mg/l by either application method.

In the second experiment *V. grandis* pot plants of various ages (length of time since transfer to glasshouse conditions) of 8, 22, and 30 weeks were treated with the most effective BA concentration and method of application for increasing the number of lateral shoots, as determined above. Ten plants of each age group received 1 of 7 treatments consisting of an untreated control, a standard BA application of 210mg/l applied by the spray method and with or without removal of the apical growing tip.

In all experiments, plants were watered thoroughly 12 hrs prior to treatment and not watered again until at least 48hr after treatment. Experimental units were arranged in a randomised block design in a glasshouse under natural photo period with a temperature range of 13-30°C. The number of new lateral shoots and the height of plants was measured fortnightly. Final data was collected 16 weeks after treatment. Mean number of shoots are presented in the Tables along with the standard error of the mean. See Section 2.10 for statistical analysis.

2.2.4 Effect of light

Plants were grown in a glasshouse with light condition as described above (Section 2.2.1). Twenty six percent additional light (average light intensity of 481 $\mu E/s/m^2$) was provided during mid July with two 400 watt high pressure sodium lamps applied for 16 hr during normal daytime which extended the actual daylenght to 16 hr/day. Thirty plants (twenty two weeks after transfer to glasshouse conditions ex tissue culture) were placed under the lights or left under normal conditions. The height, number of new lateral shoots and number of flower buds was measured fortnightly. Final measurements were recorded after 13 weeks.

2.3 TECHNIQUES FOR ANATOMICAL STUDIES OF *V. GRANDIS*

2.3.1 Plant material

Stem and leaf segments approximately 5mm in length were excised from *V. grandis* clones 865 and 866 growing on multiplication medium. Five (mm) segments of the stem/root connection were excised from rooted explants of Clone 865 and 866. This material was used for embedding and sectioning to examine and compare the anatomy of both clones.

2.3.2 Fixation and embedding

The tissue was dissected over ice in a drop of fixative and immediately transferred to the fixative solution. The fixative used was, (3% glutaraldehyde in 0.025M phosphate buffer pH 7.0 +0.5% caffeine) and was applied under vacuum for a minimum of 24hr. The tissue was then dehydrated in two changes of: Methoxy ethanol: Ethanol: Propanol: Butanol, each lasting for 2hr (ratio of 1:1:1:1).

The fixed material was infiltrated and embedded in a Glycol methacrylate (GMA) monomer mix consisting of:

93ml GMA,
7ml Polyethylene glycol 400 and
0.6gm benzoyl peroxide.

The solution was mixed for 2hr at room temperature. The mixture was then combined at a ratio of 1:1 with butanol. The tissue was infiltrated for 2hr, at room temperature and transferred to a fresh solution of GMA for 2 days. The final step was repeated once more with fresh GMA. Next, the tissue was embedded into gelatine capsules (Pharmaceutical) with fresh GMA. The capsules were left to polymerise for 2 days at 60°C.

2.3.3 Sectioning

GMA blocks were prepared as follows:

Excess GMA was cut away and the block face was filed to ensure that the plant material was correctly orientated in relation to the direction of sectioning. Sections were cut with a Sorvall JB4 microtome using a glass knife. Individual sections were collected with forceps, floated onto a drop of water on a microscope slide and straightened using a dissecting needle. Slides were dried overnight at 60°C.

2.3.4 Staining

Sections were stained with Periodic acid-Schiff's stain and Toluidine Blue O. Initially a saturated solution of 2,4-dinitrophenylhydrazine (0.5g in 100ml 15% acetic acid) was prepared by gentle mixing for 1hr at room temperature. The solution was then filtered through Whatman paper. The staining procedure is described as follows:

Slides were placed in the solution (described above) for 30min and were then rinsed for 1hr in running water;

The slides were then emersed in 0.1% periodic acid for 30min and rinsed in running water for 5min;

Next, the slides were placed in Schiff's reagent (BHD) for 1hr after which they were transferred to a solution consisting of (5ml of 10% sodium metabisulphite, 5ml 1N HCl and 90ml water) for 2min. This last step was repeated twice with fresh solutions (3 changes in all), followed with a rinse in distilled water;

The slides were then placed in a solution consisting of (0.05% toluidine blue O) in benzoate buffer pH4.5 (0.29g sodium benzoate, 0.25g benzoic acid, 200ml water). The benzoate buffer was prepared by mixing the ingredients for 30min and then left to stand overnight. The next day the solution was filtered.

The slides were placed in this solution for 5min and rinsed in water until the colour was washed out of the plastic. Finally the slides were mounted in dammar in xylene.

2.3.5 Photography

Photographs were taken with an Olympus OM2 camera attached to a Zeiss compound microscope (Standard lab. 16). Kodak ASA 200 film was used.

2.4 GENERAL TECHNIQUES AND MEDIA USED FOR *AGROBACTERIUM* CULTURE

2.4.1 Strains and plasmids

Bacterial strains used in this study were obtained from the Department of Crop Protection and are listed in Table 2.1.

Table 2.1: *Agrobacterium* strains and plasmids used in this study.

Strain	Plasmid	Observations
A. *A. rhizogenes*		
K565 (Biovar 2)	pArA4(pArA4a), pRiA4(pArA4b)	Catabolic and anabolic, for agropine, mannopine, mannopinic acid, and agrocinopinic acid
K568 (Biovar 2)	data not available	Catabolic and anabolic for mannopine and mannopinic acid
K596 (Biovar 1)	data not available	Catabolic and anabolic for agropine
K597 (Biovar 1)	pRi2655	Catabolic and anabolic for cucumopine
B *A. Tumefaciens*		
LBA4404	pBI121	ß-glucoronidase, neomycin phosphotransferase
AGL 1	pKiwi 105	Mannopine synthase, ß-glucoronidase, neomycin phosphotransferase
LBA4404 *	pGV3850: pLGVneo2103	Nopaline synthase, neomycin phosphotransferase

*Information from Hain *et al.* (1985). The culture collection held in the Department of Crop Protection, Waite Campus University of Adelaide.

2.4.2 Bacterial culture media

A. rhizogenes strains were cultured at 28°C in YM broth medium (see Appendix A: Table 3.A) for 2 days. *A. tumefaciens* strain LBA4404 was cultured at 28°C in YEB (see Appendix A: Table 3.B) also for 2 days. Bacterial strains with the plasmid pBI121 were grown on the appropriate medium with antibiotic selection (rifampicin and kanamycin) The antibiotics were filter sterilised and were added to the medium just prior to pouring the plates, at a concentration of 50mg/l. The cointergrate pGV3850:pLGV2103 was also selected with these antibiotics, with the addition of 50mg/l of carbenicillin.

2.4.3 Introduction of plasmids into wild-type *A. rhizogenes* strains

Two plasmids were introduced into the 4 wild-type *A. rhizogenes* strains (see Table 2.1), by the following procedure.

Overnight cultures of *A. rhizogenes* were grown to an OD 660 0.5-0.6 in 200ml YM, spun at 3000g for 20 min, washed in 10ml TE, and resuspended in 20 ml YM. Transformation of *Agrobacterium* was performed as follows:

500µl of the bacteria was added to 1ul plasmid DNA (1µg), placed on ice for 5min, followed by 5min in liquid nitrogen and finally at 37°C for 5min;

To this, 1ml of YM medium was added and the cultures were incubated on a shaker for 2-4hr at 28°C;

200µl of the bacteria was plated on YM medium with appropriate antibiotics (see section 2.4.2) and incubated for a further 2 days;

Individual colonies were restreaked and the integrity of the plasmids were verified by an *Agrobacterium* Plasmid Mini Prep (for restrictable plasmids).

2.4.4 Storage of isolates

All strains were maintained as glycerol stocks at (-80°C). In addition, stab cultures of the wild-type *A. rhizogenes* strains were maintained in YM slops at 4°C.

2.5 TECHNIQUES FOR INOCULATION OF *V. GRANDIS* WITH STRAINS OF *AGROBACTERIUM*

2.5.1 Stem inoculations with wild-type *A. rhizogenes* strains

Wild-type *A. rhizogenes* (see Table 2.1) was grown as described (see Section 2.4.2). In the first experiment one hundred *V. grandis* explants per treatment, were excised from cultures on multiplication medium and were inoculated with one of the 4 strains by one of two methods:

1) as the shoots were excised the cut end was dipped into the bacterial suspension for 5 seconds and then planted onto MS basal medium for 2 days. To control bacterial growth explants were then transferred to MS medium with 500mg/l Claforan® (cefotaxamin);

2) explants were inoculation by stabbing the plant stem with sterile hypodermic needles 0.55x55mm attached to 1ml syringes. One (ml) of the bacterial suspension was extracted and a drop was injected into the plant stem on or just above the second node from the base of the plant. Explants were then planted into media as described above.

This experiment was replicated 4 times. Susceptibility to infection was scored fortnightly by gall and root formation. Final data was collected after 8 weeks, at the same time the galls and roots were harvested for opine analysis (see Section 2.6.1).

2.5.2 Leaf disc inoculations

Wild-type *A. rhizogenes* strains were grown as previously mentioned (Section 2.4.2). The leaf disc regeneration method described in Section 2.1.5 was applied. Leaves were

removed from explants growing on multiplication medium. One hundred leaves per treatment were inoculated by stabbing (with a hypodermic needle containing the inoculum) the leaf petiole area (meristematic region). Leaves were blotted briefly on sterile paper and were transferred to MS basal medium for 2 days. To control bacterial growth, leaf discs were then transferred to multiplication media (MS+4.5µM BA) with 500mg/l cefotaximine. As shoots, galls and or roots emerged they were transferred to fresh medium. The final number of shoots, galls or roots produced was recorded after 8 weeks. The experiment was replicated 3 times with strains K596 and K565, and only once with strains K568, and K597. Shoots from the last experiment were subcultured twice in order to obtain shoots large enough for root initiation. Rooted plants were out-planted to the glasshouse as described in Section 2.2.2.

2.5.3 The toxicity of antibiotics to *V. grandis*

A kill curve experiment was designed to test the level of toxicity of various *V. grandis* tissue to a number of antibiotics used as selectable marker genes in plant transformation experiments. The antibiotics kanamycin (0, 25, 50, 100, 250, 500mg/l), phosphinotricin (0, 0.01, 0.1, 1mg/l), methatexate (0, 0.5, 1, 2mg/l) and hygromycin (0, 25, 50, 100mg/l) were added to media at the varying concentrations. Plant material, namely, leaf discs, and shoot explants were tested for both ability to grow shoots or roots on the various media. After 6 weeks explants were scored for shoot or root growth.

2.5.4 Kanamycin selection

Leaf disc inoculation experiments with *Agrobacterium* strains containing either the plasmid pBI121, pKiwi, or pGV3850:pLGVneo2103 (pGV3850) (see Table 2.1) were selected for shoot growth on MS medium supplemented with 4.5µm BA and 50mg/l kanamycin. Once shoots emerged they were transferred to MS medium supplemented with 4.5µM BA and 250 mg/l kanamycin. Root growth was selected on 1/2MS medium supplemented with 10µM IBA and 25mg/l kanamycin.

2.6 TECHNIQUES TO TEST FOR TRANSFORMANTS

2.6.1 Opine assay

The presence of agropine, agropinic acid, mannopine, mannopinic acid, and cucumopine* in shoot and gall tissue was established by high voltage paper electophoresis (HVPE) and silver staining according to Tate (1981). *Staining with Pauly reagent (Ames and Mitchell, 1952)

Gall and shoot material was extracted in 70% ethanol by maceration with a glass rod in a 1.5 ml centrifuge tube. If necessary liquid N_2 was added to aid maceration. The extracts were centrifuged and 2µl was spotted on Whatman No. 1 filter paper. HVPE was performed using the following solutions: 0.75M formic Acid/1M acetic acid, pH 1.7; Citric acid buffer, pH 3.6 and 0.2M NH_4CO_3/ 0.2MBoric acid/ 0.2M ammonium, pH 9.2.

2.6.2 ß-glucuronidase assay (GUS and MUG)

The procedure described by Jefferson (1987) was followed with some modifications. Leaf stem and root tissue was excised from rooted explants on selection medium (see Section 2.5.4) in a laminar flow hood. Tissue was roughly cut into small segments 1-5mm in length, and placed directly in 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) solution, (1mM X-gluc in 50mM sodium phosphate buffer, pH 7.0). These were then placed under vacuum (15mm) prior to incubation overnight at 37°C in a humidified chamber. Sections were then fixed and cleared of chlorophyll by incubation for 30min in a solution containing 5% formaldehyde, 5% acetic acid and 20% ethanol, followed by 30min in 50% ethanol, 30min in 70% ethanol and 10min in 100% ethanol and 2 washes in distilled water. Material was visually checked for blue colouration and stored in 50% Gycerol. Free hand sections were cut between two pieces of foam styrene with a razor blade.

Qualitative fluorometric GUS assay was performed as described by Jefferson (1987). Fifty (mg) of fresh tissue from plants rooting on selection medium was weighed out. The tissue was mixed with 50µl extraction buffer consisting of; 10mM ß-mercaptoethanol, 10mM

DETA, 0.1% sodium lauryl sarcosine and 0.1% Triton x-100, diluted with 50mM sodium phosphate buffer pH 7.0 to 1ml volume. The tissue was macerated and centrifuged for 5min. Forty (µl) of the supernatant was removed and put into a clean eppendorf tube with 40µl of reaction buffer, (20µl of a 50Xstock of Ferricyanide, 50ul of a 20mg/l X-gluc stock), brought to 1ml with extraction buffer. The solution was incubated for 1-1.5h at 37°C and the reaction was stopped with 1% 0.2M stop buffer (Na_2C0_3). The blue fluorescence was observed visually with a long-wave UV light box.

2.6.3 Isolation of endophytic bacteria

Plant tissue was tested for the presence of endophytic bacteria within *V. grandis* tissue which could cause false positive localisation of GUS expression (Tör *et al.* 1992). Plant tissue (10 clones) from material previously positive for GUS expression was examined for the presence of endogenous bacteria by finely cutting and immersing between 0.08 and 0.44g of tissue into 5 ml YEB, or YM liquid medium (see Section 2.4.2) and incubating for 2 days at 28°C on an orbital shaker. The 5 mls was then diluted to 1ml aliquot's, centrifuged and resuspended in 100µl YEB media. This 100µl was then spread onto agar plates and incubated at 28°C for 2 weeks. Control *V. grandis* tissue and the *Agrobacteria* vectors were included as controls.

2.7 ISOLATION OF DNA FROM *V. GRANDIS*

2.7.1 Small scale isolation of total DNA

DNA was extracted from *V. grandis* leaf tissue and tobacco plants as control tissue. A number of DNA extraction methods were tested with *V. grandis* tissue. Methods included the phenol/chloroform technique of Guidet *et al.* (1991) for DNA extraction of cereals, the CTAB method of Doyle and Doyle (1988), QIAGEN-tips (Phoenix scientific manual, and supplier) and CTAB method of Bernatsky (personal communications) with some modifications, see below [see Appendix C for DNA extraction solutions]. Low quantities

of DNA of varying quality was extracted using these methods. The main problem was contaminating polysaccharides which affected the DNA purity, inhibited the activity of restriction endonucleases and interfered with concentration of the DNA samples (see below).

The most successful technique finally employed was a method closely resembling the CTAB method of Bernatsky (pers. comm.) [see Appendix C. 3]. Modifications were investigated which included an additional step to remove polysaccharides with the inclusion of a high salt (3M NaCl) precipitation, as suggested by Fang *et al.* (1992). This method is outlined below:

Approximately 1gm of young leaves were ground to a fine powder in liquid nitrogen. To this, 5ml of extraction buffer (see Appendix C. 3) was added in a mortar, mixed and allowed to thaw. The slurry was then poured into 50ml capped centrifuge tubes with 5ml of lysis buffer (see Appendix C. 3) at room temperature. To this 1.65ml of 5% sarcosyl was added and gently mixed by inversion. Samples were then incubated at 60°C for 20min with occasional mixing by inversion. Next, 10ml of chloroform/isoamyl alcohol (24:1) was added and mixed to form an emulsion. Samples were then spun in a MSE bench centrifuge using a swing out rotor at 1000r.p.m. to separate the phases. The aqueous phase was carefully removed with a wide bore pipette and transferred to a clean 50ml centrifuge tube. To this 2/3 volumes of isopropanol was added and inverted to precipitate the DNA. If the DNA was visible it was spooled out with a glass rod, otherwise the tube was centrifuged at 500g for 5min. The DNA was then rinsed twice in a 2ml eppendorf tube with 10mM NH_4OAc, 76% EtOH. This was left at room temperature for 30min and then centrifuged at 12,000 r.p.m. for 15 min. The supernatant was discarded and the pellet washed twice with 1ml of 70% ethanol and briefly air dried in a vacuum centrifuge for 2 min. The DNA pellet was resuspended in 200-400µl of TE (depending on pellet size) at 4°C overnight.

2.7.2 Purification of *V. grandis* DNA

DNA pellets extracted from *V. grandis* leaf tissue were viscous and difficult to precipitate in order to concentrate the DNA samples. A number of methods were tested to remove the polysaccharides and included:

1) Purification using a manufacturer's "Geneclean" kit (Geneclean II Bio 101 Product No. 3106, USA) following the manufacturer's instructions. This method did remove the polysaccharides and aided digestion of samples with restriction endonucleases, but a large proportion of the DNA was also lost.

2) DNA purification via caesium chloride (CsCl) centrifugation. The method was performed according to Maniatis *et al.* (1982). DNA was resuspended in 7.0ml of TE buffer. CsCl (7.5g) was added and dissolved by gentle hand mixing. The solution was then transferred to an ultracentrifuge tube and 0.5ml of ethidium bromide (10mg/lstock) was added. After thorough mixing the sample was centrifuged at 40,000 r.p.m. for 40hrs (rotor 70.1 T., Beckman L2-65B). The DNA band was transferred to a 5ml tube and extracted with water-saturated butanol until no pink colour was visible. The DNA was transferred to dialysis tubing and dialysed against three changes of 2 litres TE buffer for 16 hrs. The DNA was then precipitated with ethanol and NaAc and resuspended in 50µl TE. This was very effective in removing the polysaccharides, however, as above only a small proportion of the DNA was recovered. This procedure is also time consuming for screening large numbers of samples.

A third method, adapted from Fang *et al.* (1992) was finally chosen. The DNA was resuspended as mentioned above in a larger volume of TE (1ml). To this 2M NaCl was added and mixed thoroughly by inversion. The DNA was then precipitated with 2 volumes cold 100% ethanol on ice for 1h and centrifuged at 12,000 r.p.m. for 10 min. The pellet was washed in 70% ethanol and resuspended in 200µl TE. DNA was stored at -20°C.

2.7.3 Digestion of DNA with restriction endonucleases

DNA (1-2μg) was digested in a reaction containing 10μl DNA and 2 units restriction enzyme (enzymes eg. Eco R1, Pst 1 Hind III, Dra 1, Bam H1 and buffers were supplied by Boehringer Mannheim). All reactions were incubated at 37°C for 6hrs.

2.7.4 Electrophoresis of DNA

The success of the restriction digestion was tested by agarose gel electrophoresis as described by Maniatis *et al.* (1982). Southern hybridisation was performed in 1.0% agarose gels using TAE buffer (0.04M Tris-acetate, 0.001M EDTA, pH 8.3) at a constant current of 20 mA run overnight. Ficol dye (0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficol) was added at 1/10 volume to DNA samples prior to gel loading. After electrophoresis, gels were stained with ethidium bromide (1μg/ml) and DNA bands were visualised over a UV transilluminator. Lambda fragments digested with Hind III were used as size markers. Photographs were taken using a Landpack camera fitted with a red filter.

2.8 SOUTHERN HYBRIDISATION PROCEDURES

2.8.1 Preparation of membranes

DNA was transferred to Hybond N^+membrane (Amersham) using the method first developed by Southern (1975). After staining as described above Section 2.7.4, the gel was placed in 200ml denaturing solution (0.5M NaOH, 1.5M NaCl) and shaken on a rocking platform for 30 min. The denaturing solution was decanted and 200ml neutralising solution (3M NaCl, 0.5M Tris-HCl, pH7.2) was added for a further 30 min. On a plastic tray a sponge then 4 sheets of Whatman 3MM filter paper all soaked in 20x SSC (3M NaCl, 0.3M Na citrate, pH 7.0) were placed covered with a screen. The gel was then placed upside down within the frame on the Whatman paper being careful to avoid trapping air bubbles. The membrane (pre-soaked in warm water, and rinsed in 20xSSC) was placed on

top of the gel. This was then covered with a further 2 sheets of 20xSSC soaked Whatman paper, followed by a 6cm stack of dry paper towels and glass plate. The gel was left to transfer for a minimum of 4 h.

The DNA was fixed to the membrane by the following method: Initially the membrane was rinsed in 5xSSC, blotted dry on Whatman filter paper and dried in a vacuum oven at 80°C. The membrane was then placed in sufficient 0.4M NaOH, just to cover, for 20min, followed by 5min in neutralising solution and then rinsed in 5xSSC and blotted dry. The membrane was stored in a sealed plastic bag at 4°C.

2.8.2 Preparation of labelled probes

Probes were prepared with an 'oligo' priming labelling kit (Rapid hybridisation system-Multiprime RPN1517; Amersham). To the labelling mix (12.5µl), 3µl of alpha-32P-dCTP (30 microcuries), together with 5µl template DNA (approx. 1µg), 12.5µl buffer and 1µl Klenow enzyme (1-2 units) were added and the solution was incubated in a 37°C water bath for 90min. Hybridisation probes were isolated from the plasmid pBI121 using "Geneclean" (Besitech). Plasmid DNA (pBI121 0.2 mg/ml) was digested with Eco RI and Hind III (as recommended by Boehringer Mannhein) to release a 3.1kb GUS fragment (Jefferson *et al.* 1987). The digestion of pBI121 with Pst I and Eco RI released a 1.9kb NPT II fragment. The GUS (3.1kb) and the NPT II fragments (1.9kb) were excised after ethidium bromide staining of the gel under a UV transilluminator. The fragments were purified by the "Geneclean" method previously described (see Section 2.7.3).

The unincorporated radio nucleotide DNA was separated from labelled DNA by a Sephadex G100 column. The probe was denatured and added to the hybridisation mix.

2.8.3 Hybridisation procedures

Membranes were pre hybridised in glass bottles for 4h at 65°C with a 20ml solution consisting of; 10ml nanopure (Np) H_2O, 6ml 5X HSB (3M NaCl, 0.1 M Pipes, 0.02M Na_2 EDTA, pH 6.8), 2ml 10x Denhardt's III (2% gelatin, 2ml Ficol, 2% polyvinyl pyrollidone,

10% sodium dodecyl sulfate, 5% tetrasodium pyrophosphate) and 2ml of freshly boiled carrier DNA (10mg/ml sonicated and denatured herring sperm DNA, Sigma). Hybridisation was performed overnight at 65°C in a hybridisation oven (Hybaid) with 10 ml of a solution containing 2ml Nanopure H_2O, 3ml 5xHSB, 1ml 10x Denhardt's III and 4ml dextran sulfate (25%w/v) plus 20µl of freshly boiled carrier DNA and the ^{32}P labelled probe. Following hybridisation, membranes were washed 3 times with 20 min washes at 65°C as follows: 1) 2xSSC, 0.1%SDS; 2) 0.5 xSSC, 0.1% SDS; 3) 0.2xSSC, 0.1% SDS. The membranes were exposed to X-ray films (Fuji) at -80°C for 5-7 days using intensifying screens. The film was developed in a G 153 X-ray Developer.

2.9 DNA AMPLIFICATION BY PCR

2.9.1 Primers

The oligonucleotide GUS primers were: 5'-CTG TAG AAA CCC CAA CCC GTG-3': 5'-CAT TAC GCT GCG ATG GAT CCC-3', yielding a fragment size of 514 base pairs (bp). The primers were synthesised on an applied Bio systems 381ADNA synthesiser and purified on an OPC cartridge supplied by the manufacturer.

2.9.2 PCR reaction

The polymerase chain reactions were carried out in a total volume of 25µl containing, 14.5µl H_20; 2.5µl 10x buffer (100mM Tris-HCl pH8.3, 500mM KCl, 15mM $MgCl_2$, 0.1%(w/v) gelatin.); 4µl dNTP (200µM each dNTP(dATP, dCTP, dGT and dTTP) in water; 2.6µl GUS primer 3'; 3.8µl primer 5' and 1unit T*aq* polymerase (Promega). The reaction mixture was overlayed with 60µl paraffin oil to prevent evaporation. The PCR was performed in an Intelligent Heating Block (Hybaid). The conditions set up in the program were as follows: Initial denaturing at 94°C for 4min, thirty two cycles each of denaturation at 94°C for 1min, annealing at 55°C for 2 min and an extension at 72°C for 2 min,. 72°C for 7min, then cooled for 1 min at 25°C. One fifth of the PCR products were

analysed on a 2% agarose gel and visualised under UV light following ethidium bromide staining. Confirmation of the identity of the PCR products was confirmed by Southern hybridisation to the GUS probe.

2.10 STATISTICAL ANALYSIS

Statistical analysis were performed for experiments in Chapter 4. These included 2 tests for determining significant differences between: the means of 2 samples (T-Test for independent samples); and between groups of samples (Analysis of Variance, ANOVA).

CHAPTER 3 MICROPROPAGATION OF *VERTICORDIA GRANDIS*

3.1 INTRODUCTION

The horticultural potential of *V. grandis* as a cut flower is recognised in the industry. Flower stems attract high prices, ranging from $3.00- $4.50 per stem wholesale (Aust. Hort. 1992). However, supply is still limited, due to difficulty of propagation. Seed set is often low, and germination is slow (Kullman undated). Root development from cuttings is poor. However, considerable genotypic variability exists within the species and some clones do root well (Moyle, personal communication). Furthermore, limited cultivation success has been reported outside areas in western Australia (W.A.) where the species is endemic and where specific conditions suit the growth of this species (see Chapter 4).

McComb *et al.* (1986) developed a successful protocol for micropropagation of *V. grandis*. However, the percentage of explants that developed roots did not exceed 58%. For commercial production, rooting percentages should be above 80%. This maintains production costs within a profitable margin (see Chapter 1).

The initiation and emergence of roots on explants grown *in vitro* (Stage III, see Fig 1.1) is critical for the successful micropropagation of *V. grandis* for commercial purposes. Root induction is often the limiting stage in the micropropagation of many woody species (Williams 1986). The ability of plant tissues to form roots depends on the complex interaction of both endogenous and exogenous factors. The role of auxins in root development is widely accepted as one of the main factors involved in root formation, and has previously been reviewed (Komissarov 1969, Németh 1986, Gaspar 1989). The role of exogenously applied auxin has no simple explanation. Studies indicate a relationship between the natural rooting ability of the plants, endogenous auxin levels and the accumulation of auxin (in the free form) at the cutting bases (Gaspar 1989). Media of low pH have also been reported to show effects similar to that of auxin on the growth of *Avena* coleoptiles (Rayle and Cleland 1973). The authors suggested that one possible effect of auxin is on cell wall loosening due to enhanced acidity. A study by Lee *et al.* (1977) found

that pre-treatment of stem cuttings with acid promoted rooting in *Bougainvillea, Hedera, Juglans* and others. Low pH of the root induction medium has also been shown to promote rooting of shoots grown *in vitro* (Williams *et al.* 1985).

Often greater rooting percentages of stem cuttings and shoots *in vitro* can be achieved with juvenile rather than woody plant material (Drew and Miller 1989). Physiological and anatomical changes occur in relation to the age of the plant material (ie. the transition from juvenile to mature tissue). Anatomically, bark and sclerenchyma develop in woody tissue which may act as a physical barrier for root emergence (Hartman and Kester 1986). However, the significance of the sclerenchyma in inhibiting root elongation is unresolved. There are numerous cases where roots overcome the apparent resistance of the sclerenchymatous barrier and emerge. For example, cuttings taken from one year old and eight year old wood of Iberian Oak (Quercus) plants had similar sclerenchyma development. Yet the one year old cuttings developed roots while the eight year old cuttings did not produce any roots (Komissarvo 1969). Therefore, physiological factors other than wood development are involved which affect root development in woody tissue.

Juvenile, actively growing shoot material is most suited for propagation of difficult-to-root woody plant material. Similarly, micropropagation methods can effectively rejuvenate plant tissue and consequently improve rooting of explants. Root initiation of papaya explants was enhanced when shoots were actively growing before transfer to the rooting medium (Drew and Miller 1989).

Multiplication of axillary shoots (organ culture) *in vitro* depends on the continued development of existing meristematic cells. This technique maintains clonal integrity as shoots develop from pre-existing meristems, without a callus phase. In contrast, tissue culture methods require the tissue explant to become meristematic through an intermediate callusing step and, having achieved this, to induce these cells to regenerate into new plants. The obvious method of choice for clonal propagation of *V. grandis* is 'axillary shoot proliferation'. Anatomical investigations are employed in this study to locate meristematic regions suitable for multiplication of *V. grandis* shoots *in vitro*. In addition, histological

evaluation of root development, in particular, stem root connections can provide information on the viability of root development of *V. grandis* explants, *in-vitro*.

In summary, the aim of this chapter was to develop suitable culture conditions and micropropagation methods for efficient multiplication and rooting of *V. grandis* shoots *in vitro*. These techniques are essential requirements for nursery production (Chapter 4) or to establish plantations for cut flower export. They are also important prerequisites for transformation studies (Chapter 5).

3.2 EXPERIMENTAL PROCEDURES

This study describes several plant and culture media factors that influence micropropagation and in particular, root development of *V. grandis* explants, *in vitro*. It also demonstrates that the genotype of the explant influences the success of the system. Histological investigations are employed to test whether the genotypic differences in response to cultural conditions can be explained in terms of histology. In addition, the location of meristematic regions highlights tissue which is suitable for regeneration.

Experiments are based on a systematic investigation of factors which affect the overall micropropagation process, these include:

1) the decontamination of nodal explants by surface sterilisation

2) the cytokinin type and concentration required for shoot multiplication

3) the effect of the pre-treatment, such as, the multiplication medium and a dark period on subsequent root induction

4) the effect of media conditions, eg, phytohormone (auxin type and concentration), pH and sucrose concentration on root induction *in vitro*

5) genotype effects

6) the effect of the type of explant used for root induction

7) histological investigation of the two *V. grandis* Clones 865 and 866 from both Stage II and Stage III media to determine if anatomical differences are evident between the clones.

Initially an existing Clone (865) in culture at Phytotech Aust. Pty. Ltd. was the only source of plant material available for experimentation. It was maintained on the medium outlined by McComb *et al.*(1986) see section 2.1. Following this protocol Phytotech were only successful in producing adequate multiplication rates, while root induction of explants was poor. A number of experiments were designed to develop protocols for the successful micropropagation of *V. grandis* suitable for commercial production (see Chapter 1). Meanwhile, an additional stock plant source was obtained from W.A (Clone 866) and was initiated into culture. Details of the tissue culture media and procedures are given in Section 2.1. Specific experimental procedures and results are presented below.

3.3 RESULTS

3.3.1 Surface sterilisation of Clone 866

Due to the limited amount of material supplied by P. Moyle W.A. see Fig 2.1, (from a stock plant which had proven to root well from stem cuttings), only two sterilising treatment times were tested. Nodal stem segments were used as explants. All explants received an initial treatment of 3hrs in 0.1% NaOHCl, after which, explants were immersed in 1% NaOHCl for either 30 or 45 min. The initiation method is explained in detail in Section 2.1.3. After sterilisation, explants were placed onto MS basal medium. Contamination was visually evident after 1-3 weeks. All remaining explants were screened for bacterial or fungal contamination by the method outlined in Section 2.1.3. All contaminated cultures were discarded.

The increased sterilisation time from 30-45min produced a higher percentage of successfully decontaminated explants, 13 compared to 82%, respectively. The sterile

explants from both treatments were then transferred to multiplication medium determined from cytokinin experiments with Clone 865 (see below). Explants were subcultured every 3-4 weeks for a period of 19 weeks. The total number of shoots and mean number of new shoots per explant was recorded at each subculture.

Table 3.1: Number of units (plants) and multiplication rate of *V. grandis* Clone 866 over 5 subcultures on the shoot proliferation medium MS supplemented with 4.5μM BA.

Time in weeks	Mean No. of plants	Multi rate
4	20	0
8	30	1.5
11	80	2.7
15	236	3
19	875	3.7

As evident from Table 3.1 in the first 8 weeks shoot proliferation was low (x1.5). After initiation into culture 4 subcultures were required to establish minimum multiplication rates (x3), after which, both multiplication rate and shoot quality were satisfactory.

3.3.2 Effect of cytokinin type and concentration on shoot proliferation of *V. grandis* Clone 865

All shoot explants used for this experiment were grown previously on McComb *et al.* (1986) multiplication medium. It is important to observe the effect of a chosen medium over a number of subcultures to determine if there are any undesirable effects of the phytohormones with time. Therefore, shoots were subcultured every 4 weeks for a period of 16 weeks, using 10 shoots per container. At each subculture the number of new shoots

(referred to as the multiplication rate, or XF, the net increase in the No. of shoots after each subculture) was calculated over 4 subcultures. Multiplication rates and shoot quality of Clone 865 on, 1) McComb *et al.*(1986) multiplication medium (MS supplemented with 20µM Kinetin and 0.5µM NAA) was compared with two other media. The alternative media did not contain auxin and BA at either 1 or 4.5µM was substituted for Kinetin, (Media 2 and 3, respectively).

TABLE 3.2: Proliferation of Clone 865. Number of shoots (N) and multiplication rate (M) of *V. grandis* grown on 3 media (see text) over 4 subcultures.

Time of subculture weeks	Media 1 N	M	2 N	M	3 N	M
1	10	-	10	-	10	-
4	35	3.5	30	3	53	5.3
8	90	2.6	81	2.7	280	5.3
12	210	2.3	194	2.4	888	3.2

As evident from Table 3.2 the number of shoots per subculture varied between the cytokinin treatments. Medium 3, containing 4.5µM BA produced the greatest number of new shoots after 12 weeks and also the highest multiplication rate compared to the two other treatments. However also of importance is the quality of shoots. Plant growth on Medium (1) was not uniform in height (data not shown). Characteristically explants growing on this medium contained one main shoot with small basal shoots emerging from a callus base (data not shown). A large proportion of these shoots were not suitable for rooting as most were too small (< 1.5cm). In comparison, both Media (2) and (3) produced

uniform shoot growth (data not shown). It was decided to use multiplication Medium 3 (MS supplemented with 4.5µM BA) as this gave consistently high shoot multiplication rates and compact uniform growth (see Figure 3.1: A). Multiplication rates and shoot quality were similar for both *V. grandis* clones on multiplication Medium 3). Figure 3 1: A and B, shows a comparison of shoot growth and quality between the two *Verticordia* clones subcultured at the same time on multiplication Medium 3.

3.3.3 Root production: The effect of pre-treatment

A Multiplication media

To test the effect of the pre-treatment multiplication media on subsequent root production, explants from *V. grandis* Clone 865 were excised from three multiplication media. 1) MS basal, as a control, 2) MS supplemented with 4.5µM BA and 3) MS supplemented with 20µM Kinetin and 0.5µM NAA (McComb *et al.* 1986), and were placed onto the following root induction media. The auxins IBA and IAA were both tested at 10µM along with a standard control (basal medium with no phytohormones). A two week dark period was compared to the standard photo period. Instead of using etiolated shoots (McComb *et al. 1986*) which are fragile and difficult to handle *in vitro* (personal experience), plants on root induction medium were placed under dark conditions for 2 weeks. Shoots were then returned to normal photo period conditions (for details see Section 2.1). Each treatment consisted of 3 replications with at least 20 explants per replication. The mean percentage of explants that produced roots and the standard error (SE) of the mean was recorded after 6 weeks.

The results (see Table 3.3) show that the multiplication media, does have an effect on the percentage of explants on root induction media which produce roots. Explants excised from multiplication Medium 2, had the highest rooting percentages (13%) for both auxin treatments irrespective of the dark treatment. The auxin IBA was more effective than IAA for root formation only in combination with a dark treatment (65% and 33%, respectively).

Overall, the dark treatment increased rooting percentages from 0 to 52% compared with no dark treatment. The highest rooting percentage of 65% was obtained from shoots excised from multiplication Medium 2) which were placed on root induction medium with the addition of 10μM IBA in combination with a two week dark treatment.

Table 3.3: Effect of pre-treatment, media composition and dark-pre-treatment on the production of roots by explants of *V. grandis* Clone 865 on three root induction media (see text). Means and standard errors of the means of 3 replicate tubs of 20 explants per treatment.

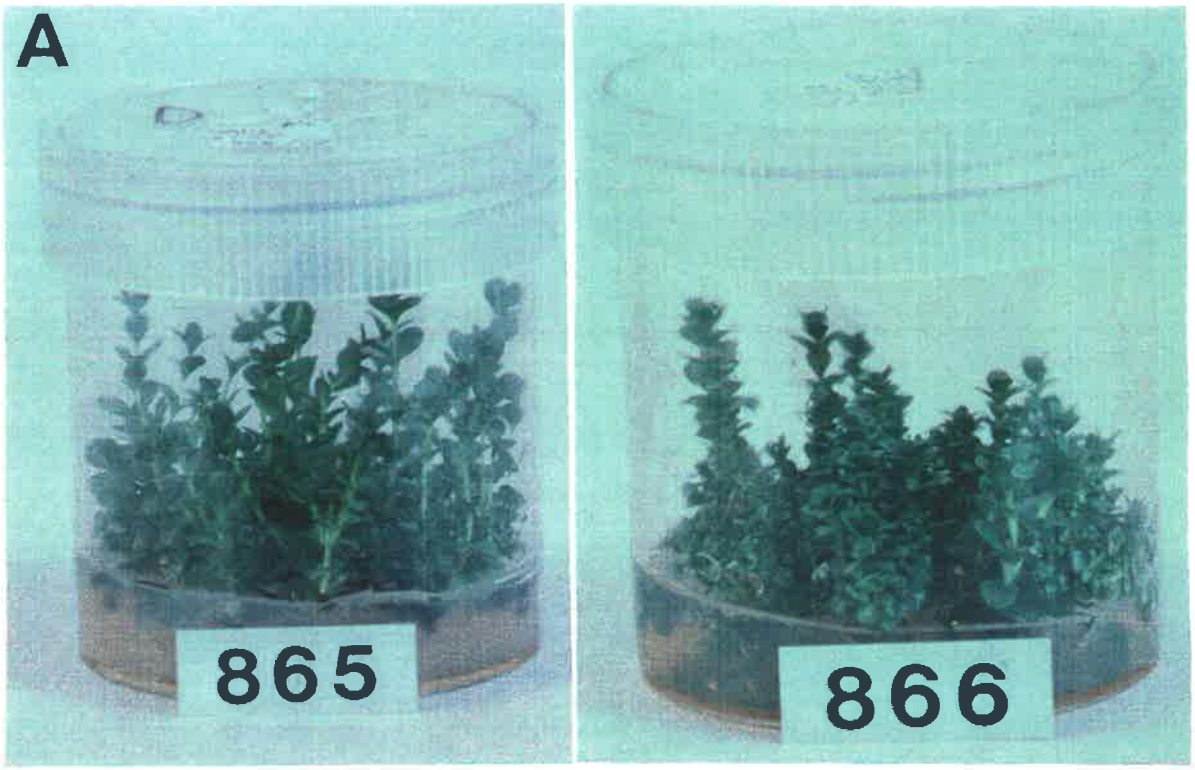
	% of Explants Rooted (SE)					
	Rooting Medium					
	Basal		10μM IBA		10μM IAA	
Pre-treatment	light	dark	light	dark	light	dark
Multiplication Media						
1	0(0)	3(0)	5(1)	28(1.5)	3(0)	28(0.5)
2	3(0)	3(0)	13(0.5)	65(1)	13(0.5)	33(0.5)
3	0(0)	5(0.7)	5(0)	5(0.7)	5(0)	30(2)

Figure 3.1 Micropropagation of *V. grandis*.

Examples of *V. grandis* Clones 865 and 866 growing in Stage II and III medium.

A: Proliferation of shoots on multiplication Medium 3 (MS basal medium supplemented with 4.5μM BA).

B: Rooted shoot explants from root induction medium (1/2MS basal medium supplemented with 10μM IBA).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865
866

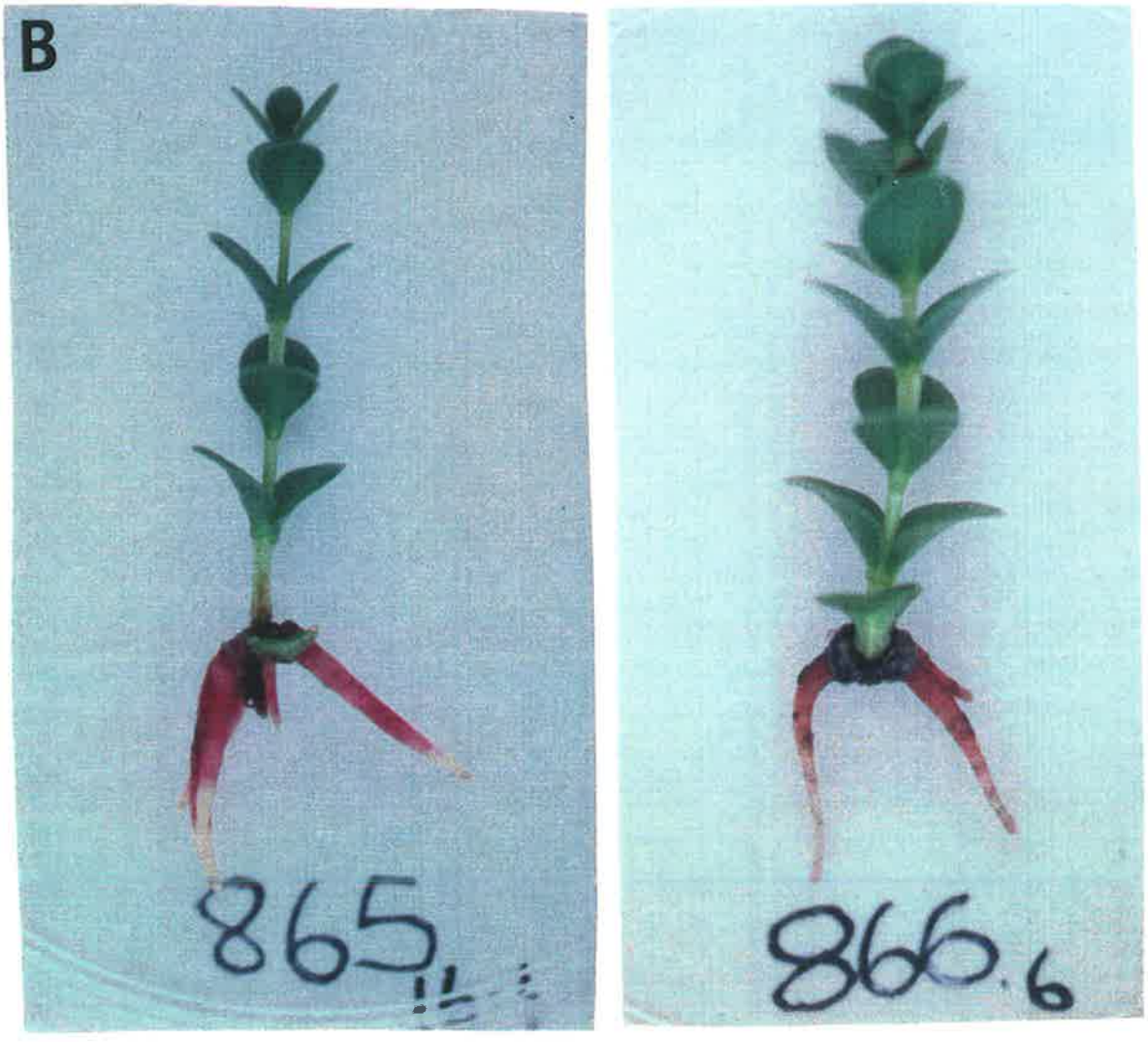

B
865
866.6

Table 3.4. Effect of the length of the dark-pre-treatment on the production of roots by explants of *V. grandis* Clone 866 on root induction medium, 1/2MS supplemented with 10μM IBA, pH 5.5 and 3% sucrose. Means and standard errors of the means of 3 replicate tubs of 20 explants per treatment.

Dark period(wks)	% of Explants rooted (SE)
0	50(0)
1	100(0)
2	100(0)

B Dark period

All rooted shoots produced on any of the media discussed above were of good quality. However, a two week dark treatment did produce an etiolated section in the stem which may cause handling problems at out-planting. To determine whether a full two week dark period is necessary for successful rooting of *V. grandis* Clone 866 explants, varying dark pre-treatment times of 0, 1 or 2 weeks were compared. Twenty explants from the multiplication medium MS supplemented with 4.5μM BA were placed onto the root induction medium 1/2MS supplemented with 10μM IBA and exposed the various dark-pre-treatment times, prior to exposure to the standard 16-hr light/ 8-hr dark cycle. After 6 weeks, the number of rooted shoots was recorded. Mean percentages and the standard error of the means (SE) are presented.

Table 3.4 shows that a 1 week dark period still produced 100% rooting of *V. grandis* clone 866 explants. Without a dark period rooting was reduced to only 50%. Shorter periods between zero to one week were not tested.

3.3.4 The effect of the following variables on root production of *V. grandis* Clone 865 explants, *in vitro*: A) pH of the medium; B) sucrose level and C) auxin type and D) Genotype effects.

Half strength MS medium supplemented with MS vitamins and 3% sucrose was the standard basal medium for root induction of *V. grandis* clone 865 explants. To this medium a number of variables were added to induce root production. These included:

A) reducing the medium pH from 5.5 to 4

B) reducing the sucrose concentration from 3% to 1.5

C) in combination with pH and the sucrose concentration, two concentrations of the auxin IBA (10 and 50μM), or 10μM IBA in combination with 5.4μM NAA were tested.

Treatments were applied to twenty explants, excised from multiplication medium (MS supplemented with 4.5μM BA). After 6 weeks, the mean and the standard errors of the mean (SE) of 2-5 replicate tubs was recorded. Shoot explants on the high auxin treatment (IBA 50 μM) were transferred to 1/2 strength MS basal media after the 2 week dark period to avoid excessive callus build up.

D) *V. grandis* Clone 866 explants were excised from multiplication medium MS supplemented with 4.5μM BA and placed on the root induction media variables described above with a standard pH of 5.5. After 6 weeks the mean percentage and (SE) of 5 replicate tubs of 20 explants per treatment was recorded.

Table 3.5: Effect of auxin and the level of sucrose and pH of the root induction media on the production of roots by explants of *V. grandis* Clones 865 and 866 (see text). Means and standard errors of the means of 2-5 replicated tubs of 20 explants per tub.

		% of explants producing roots		
		Clone 865		Clone 866
Root induction Medium	Sucrose conc.(%)	pH		
		4	5.5	5.5
Basal	1.5	20(1)	10(0.7)	45(5.7)
	3	33(0.5)	10(0)	52.5(0.4)
+10μM IBA	1.5	22(0.5))	46(4.3)	92.3(2.8)
	3	80(3.4)	43(7.7)	100(0)
+50μM IBA	1.5	78(1.7)	38(1.7)	n/t
	3	2.5(0)	87(0.54)	n/t
+(10μM IBA+ 5.4μM NAA)	1.5	35(0)	13(0)	71(11.14)
	3	3(1)	37(3.4)	97(0.82)

n/t = not tested

A Effect of pH (Clone 865)

The results (Table 3.5) show that reducing the pH to 4 in the standard rooting medium (1/2MS basal with 3% sucrose) increased the percentage of explants which produced roots (10 to 33%).

B Effect of sucrose (Clone 865)

Table 3.5 shows that lowering the sucrose concentration from 3 to 1.5% did not increase the rooting percentage (for the standard root induction medium, see above). However, in combination with reduced pH of 4, the rooting percentage increased from 20 to 33%.

C Effect of auxin (Clone 865)

The addition of auxin (Table 3.5) to the standard rooting medium increased the rooting percentage of clone 865 explants (from 10% to 87%). In fact, rooting percentages increased with increasing auxin concentrations of 10μM IBA to 50μM IBA, 43% to 87%, respectively. However the high auxin treatment together with low pH resulted in the lowest rooting percentages (2.5%). The combination auxin treatment produced moderate rooting percentages only in combination with low pH and low sucrose (35%) and with the standard basal medium (37%). The highest rooting percentage (87%) was obtained with the standard basal medium supplemented with 50μM IBA for two weeks followed by root elongation on 1/2MS basal medium. Callus development at the stem base was excessive for this treatment if plants were left on the high auxin medium longer than 2 weeks. Callus development was particularly excessive on treatments at pH 4. Callus development was also excessive in treatments with NAA and IBA in combination regardless of the sucrose concentration or pH. The treatment with standard sucrose of 3% supplemented with 10μM IBA at a pH of 4 produced the next highest rooting percentage (80%).

Figure 3.1 (A) shows an example of a rooted plant (Clone 865) from the following root induction medium, Stage III 1/2 strength MS basal supplemented with 3% sucrose, pH 5.5 and 10μM IBA.

D Genotype effects on root production

Table 3.5 results show that for the standard basal rooting medium the percentage of *V. grandis* Clone 866 explants that produced roots (52.2%) was 5 times higher than Clone 865 explants (10%). This demonstrates the variability of the two genotypes in response to *in vitro* culture conditions for root induction. Reducing the sucrose concentration did not improve the rooting percentages for Clone 866. As with Clone 865, treatments with both IBA and NAA also produced excessive callus for Clone 866. The standard basal medium supplemented with 10μM IBA produced 100% rooting of Clone 866 explants. This treatment produced healthy roots with no visible callus at the stem base. Figure 3.1: B shows a rooted explant of Clone 866.

3.3.5 Effects of different sources of explants (Clone 866)

To test the effect of the type of explant (of Clone 866) on rooting performance, 20 tips, internodes or shoot clumps all greater than (1.5 cm) in height were placed on the root induction medium (1/2MS standard basal supplemented with 10μM IBA) and were given a one week dark pre-treatment. Each treatment was replicated 5 times. After 6 weeks, the number of shoots that produced roots was recorded. The mean percentage and (SE) are presented.

Table 3.6. Effect of the origin of explants on root production of *V. grandis* Clone 866

Explant material	% of explants producing roots (SE)
Tips	100(0)
Internodes	100(0)
Clumps	90.4(0.4)

The rooting percentage of tip and internode explants (100%) was higher than that of clumped explants (90%) (Table 3.6). The clumped explants formed callus more readily than single shoot explants. Of interest, growth characteristics were different in relation to the type of explant used. Apical tip explants produced a single apically dominant shoot, internode explants produced occasional lateral shoots, whereas, the growth of clumped shoot explants remained compact.

3.3.6 Shoot proliferation from meristematic regions of leaf discs

Fifteen leaf discs were removed from *V. grandis* shoots growing on multiplication medium (MS supplemented with 4.5µM BA) and were placed onto MS medium containing one of the following additions: no hormones, 4.5 or 9µM BA, 4.5µM BA and 1.5 µM IAA, or 20µM Kinetin+ 0.5 µM IBA(McComb *et al.* 1986). The percentage of leaf discs that produced shoots and the mean number of shoots per disc was recorded after 6 weeks. The results presented in Table 3.7 show the means of 3 replicate Petri dishes of 5 leaf discs per treatment.

Table 3.7: Effect of media composition (see text) on shoot production by leaf explants of *V. grandis* after 6 weeks in culture.

Medium	% of leaf discs with shoots	Mean # of shoots per disc
Basal medium	0	0
+4.5µM BA	73	2.7
+9µM BA	13	4.6
+4.5µM BA+ 1.5µM IAA	20*	1.3
+20µM KIN + 0.5µM IBA**	0*	0

* brown, callus formation, ** McComb *et al.*(1986) multiplication medium.

Table 3.7 shows that shoot proliferation is possible from leaf discs. Leaf discs on basal medium did not produce any shoots. Basal medium with the addition of 4.5μM BA produced the highest percentage of leaf discs that developed shoots (73%). Increasing the concentration of BA (9μM) resulted in a lower percentage of leaf discs that produced shoots (13%) although the mean number of shoots per disc (4.6 vs 2.7) was higher. However these shoots were stunted and distorted and required a number of transfers (1-3) on MS basal medium without cytokinins, to induce shoot elongation.

Figure 3.2 (A) shows an example of a leaf disc after 4 weeks on multiplication medium MS+ 4.5μM BA. Figure 3.2 (B) shows shoot proliferation of the shoot depicted in (A) 4 weeks after transfer to fresh multiplication medium (total growing time of 8 weeks). The anatomy of the region from which these shoots were initiated was investigated (see Section 3.3.8) to determine if these shoots arose from predetermined meristematic cells (see below).

3.3.7 Histological studies:

A Anatomy of stem and leaf tissue. The location of meristematic regions

Techniques for all anatomical studies are outlined in Section 2.3. Leaf, stem, and stem/root regions of Clones 865 and 866 were examined.

Transverse and longitudinal sections of leaf and stem tissue revealed the basic structure of *V. grandis* explants *in vitro*. Figure 3.3 (A) shows a free hand unstained cross section of a stem with woody growth. Note the red pigments in the sclerenchyma* and xylem vessels. A definite ring of sclerenchyma is evident in this section and is related to the amount of woody tissue present. In comparison in Figure 3.3 (B) only a small ring of sclerenchyma can be seen. Figure 3.3 (B) shows a cross section of a stem and two lateral leaves. Both stem and leaf sections revealed the presence of numerous oil glands in the epidermal and outer parenchyma cells.

*Sclerenchyma cells were identified, in transverse sections of woody *V. grandis* stems, by a band of thick walled cells exteria to the phloem tissue.

Figure 3.2 Leaf disc regeneration of *V. grandis* (Clone 865) on multiplication medium (MS basal medium supplemented with 4.5μM BA)

Examples of shoot proliferation from leaf discs.

A: Shoots arising from the leaf petiole region of a leaf discs after 4 weeks on multiplication medium (see above).

B: Shoot proliferation of shoots depicted in (A) 6 weeks after transfer to fresh shoot multiplication medium (as above).


A


B
LEAF DISC
865

Figure 3.3 Anatomy of *V. grandis* (Clone 865 and 866) plants grown *in vitro* on multiplication medium (MS supplemented with 4.5µM BA). Bars indicate 100µm.

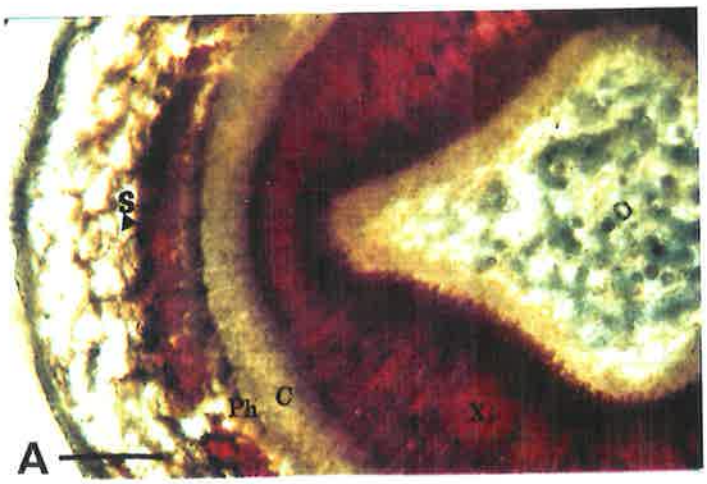
A: Free hand transverse section of a woody stem.

B: Transverse section of a stem showing two lateral leaves.

C: Longitudinal section of a stem showing an axillary bud.

D: Longitudinal section of a leaf showing meristematic cells located at the leaf petiole area.

a, axillary bud arising from meristematic cells; c, cambium; E, epidermis; g, oil gland; M, meristematic cells; Me, mesophyll cells; P, parenchyma cells; Ph, phloem; S, sclerenchyma; V, vascular elements; X, xylem.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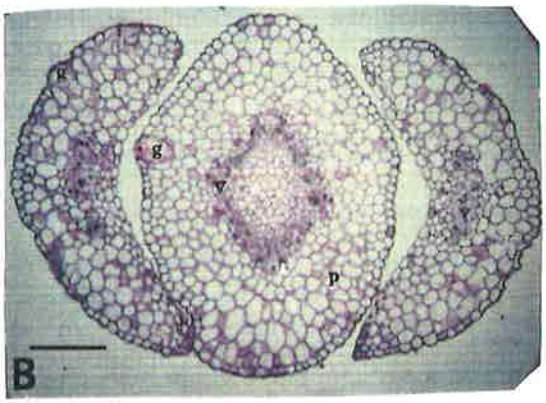
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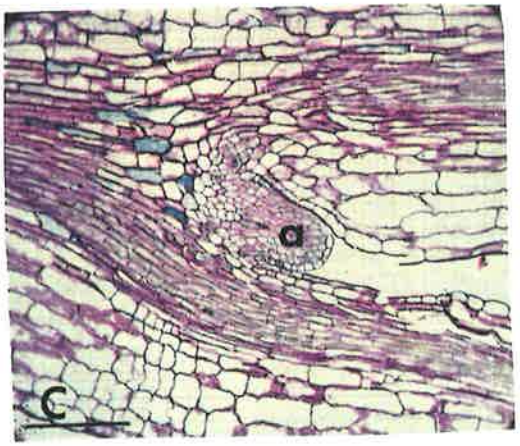
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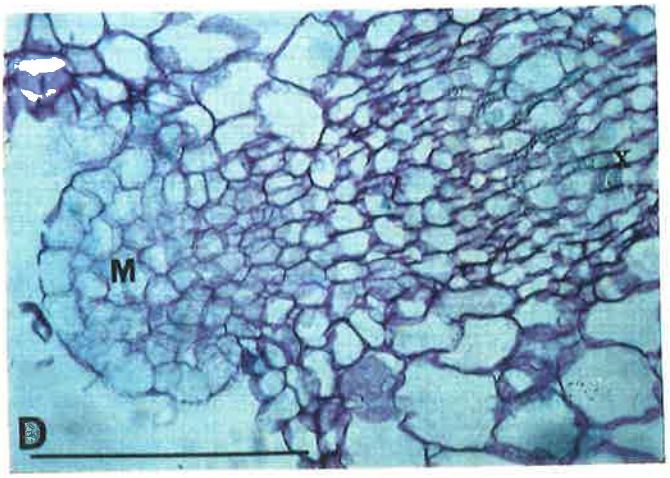

X
M
D

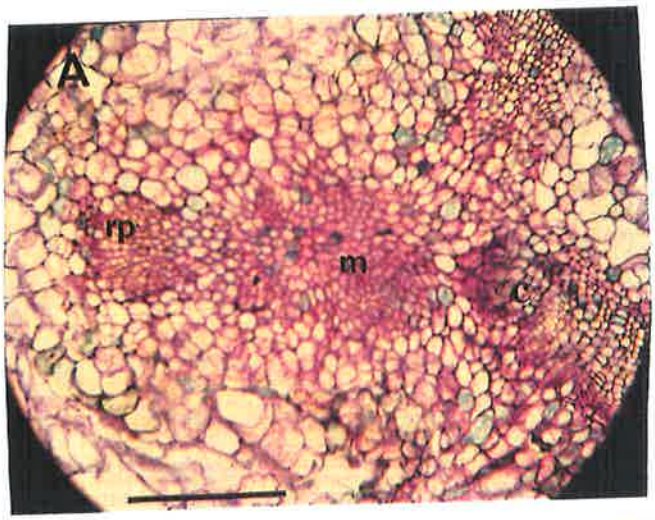
Figure 3.4 Anatomy of *V. grandis* (Clone 865 and 866) stem/root connections of explants removed from root induction medium (1/2MS supplemented with 10μM IBA), *in vitro*. Bars indicate 100μm.

A: Transverse section showing meristematic cells and root primordium differentiation.

B: Transverse section of the base of a rooted explant showing xylem differentiation from the stem into the root.

C: Longitudinal section of the base of a rooted nodal explant, showing root primordia, and continuous xylem connection from the stem into the root. White arrow (bottom right hand corner, points to neucrotic cells).

c, cambium; Ca, callus; M, meristematic cells; R, root; Rp, root primordia; V, vascular elements; X, xylem.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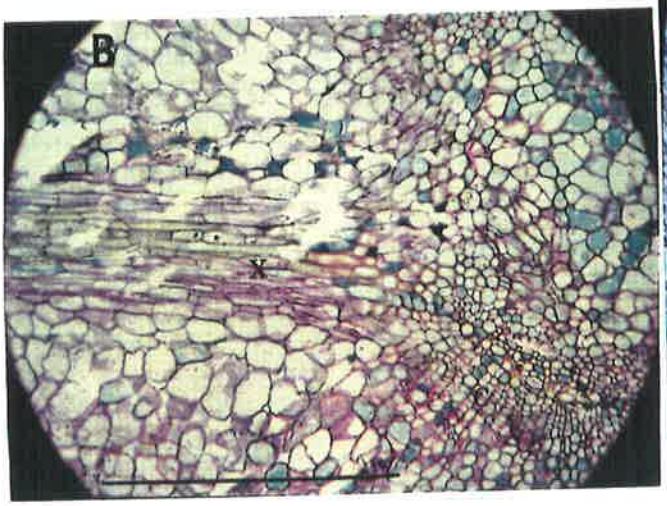
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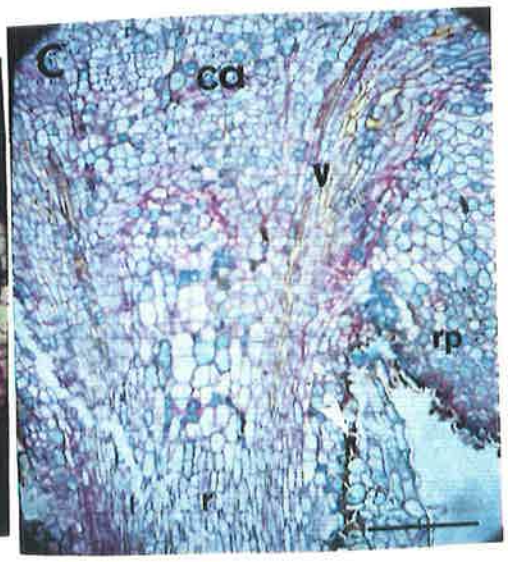
C
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V. grandis leaves contain a small petiole segment which clasps the stem. Axillary shoots arise from this region. Figure 3.3 (C) shows this area to contain meristematic cells showing an axillary bud. Figure 3.3 (D) shows meristematic cells in a longitudinal section of a leaf. Care was taken in removing leaves from the stem to avoid disrupting the petiole region. Meristematic cells can be seen at the leaf petiole area in Figure 3.3 (D). Overall there were no observable differences in stem and leaf structure between the two *V. grandis* Clones.

B Stem/Root connections

Sections of rooted explants revealed the formation of xylem connections between root and stem vascular tissue. These connections are evident in longitudinal sections see Figure 3.4 (B) and (C). Roots can be seen to emerge from the cambium/pericycle region shown in Figure 3.4 (A). Small areas of callus (C) and necrotic cells (white arrow) are also evident in the stem base of Figure 3.4 (C). Stem and root sections were taken from rooted plants 6 weeks in culture. By this time roots were well formed and growing vigorously. However this material was more difficult to fix and embed than leaf or stem tissue alone which resulted in many torn sections (data not shown).

3.4 DISCUSSION

The results discussed in this Chapter clearly indicate that commercial micropropagation of *V. grandis* is possible.

McComb *et al.* (1986) reported obtaining multiplication rates (mean number of shoots per main clump, per subculture) of between (x1.7-7.1) and a rooting percentage of 58%. Data obtained by Phytotech following this protocol, resulted in an average multiplication rate of (x3.2) over 6 consecutive subcultures and rooting percentages of between 20-40%. The reason for the low rooting percentages "Phytotech" originally achieved with Clone 865 may be a reflection of the poor shoot quality at Stage II. The multiplication stage was found to influence the subsequent root induction of papaya explants, *in vitro* (Drew and

Miller 1989). Importantly, the role of endogenous hormone levels in plants (particularly the interplay of auxin and cytokinin) is critical for organ formation (Skoog and Miller 1957). The multiplication medium chosen in this study (MS supplemented with 4.5μM BA) consistently produced good quality shoots suitable for root induction. This medium produced uniform shoot growth and consequently it was possible to harvest a high proportion of shoots for root induction. Smaller shoots were returned to multiplication medium for further growth. However, long term culture of explants on this multiplication medium produced an excessive number of small shoots which were not suitable for root induction. Although the excess shoot production does not pose problems for rapid multiplication planning, it affects the number of explants suitable for harvest for root induction. The excessive shoot proliferation is the result of a build up of endogenous cytokinin levels. An alternating subculture on the lower cytokinin medium (1μM BA) or medium without BA (MS basal) promoted shoot elongation. This step is often necessary to avoid an excessive build up of cytokinin levels (Williams 1989).

Media for shoot multiplication and root induction were developed for both clones of *V. grandis*. The pre-treatment including both the multiplication medium and a dark period were found to influence subsequent root induction. Explants excised from multiplication medium (MS supplemented with 4.5μM BA) produced the highest rooting percentages for all auxin treatments irrespective of the dark treatment (13-65%). This may be related to the quality of these shoots compared to shoots from the other media (shoots on the basal medium were tall and woody).

A dark pre-treatment of shoots was critical for high rooting percentages. This is consistent with other findings for difficult-to-root woody plants (Williams *et al.* 1985). The results obtained from multiplication media as pre-treatment for rooting, supported the need for juvenile, actively growing shoots as a prerequisite for successful rooting.

Rooting percentages for Clone 865 were consistently lower than Clone 866, given the same cultural conditions. Nevertheless, by manipulating media conditions (auxins, pH and sucrose levels) the results obtained indicate that it was possible to obtain high rooting

percentages (above 80%) of *V. grandis* Clone 865 explants, *in vitro* . However, the treatment (50μM IBA) which produced the highest rooting percentage of explants (87%) required two stages: 1) high auxin levels (50μM IBA) for 2 weeks to initiate roots; 2) root and shoot elongation on 1/2 strength MS basal media for a further 4 weeks. This procedure involved an extra handling stage (for root elongation) which would add to production costs in commercial systems.

The amount of exogenous auxin required for root induction is dependent on the plants endogenous levels (Gaspar 1976). This may be one possible explanation for the high auxin concentration required for root induction of *V. grandis* Clone 865 explants. Other factors, such as, medium pH and sucrose concentration were found to be not as critical for the root induction of *V. grandis* explants *in vitro*

The root induction experiments with *V. grandis* Clone 866 indicated that it was more suited to commercial micropropagation than Clone 865. Genotypic differences were found between the two *V. grandis* clones in response to culture conditions for root induction of explants *in vitro* . This is a common phenomenon and has previously been reported for Poplar, (Coleman and Ernst 1989); Eucalypts, (McComb and Bennet 1986); Peach, (Hammerschlag *et al.* 1987) and Rice, (Abe and Futsuhara 1986) plants. The fact that it was possible to obtain high rooting percentages for Clone 865 after numerous media manipulations, indicates that both genetic and environmental factors are involved in root formation.

Histological investigations revealed red pigments in the sclerenchyma and xylem vessels. Cresswell and Defossard (1974) also reported the occurrence of a similar "pink substance" within certain cells. These included, epidermal, vascular, cortical cells and were also associated with lateral root initiation. The authors suggested the pink substance may be an anthocyanin. There have been conflicting reports on the role of sclerenchyma in respect to structural resistance to root emergence of cuttings (Komissarrvo 1969). A definite ring of sclerenchyma was evident in both clones of *V. grandis* stem tissue and appeared to be related to the amount of woody tissue present. Consequently, actively growing shoot

explants (with limited amounts of sclerenchyma tissue) produced roots at a higher frequency than woody explant material. Therefore, these results suggest that there is a correlation between sclerenchyma development of woody tissue and root production of *V. grandis* explants. However, more detailed investigations are necessary to determine whether root initiation, structural resistance or other factors which cannot be explained in terms of histology, determine the ability of shoot explants to produce adventitious roots. As mentioned in Section 2.3. only one rooted plantlet of both clones was examined. For detailed examination of stem/root connections a larger sample is necessary. This would also enable detailed analysis of structural differences between the two clones. For outplanting success stem/root connections must be sound. Roots that arise from callus may be susceptible to fungal problems and slow *in vivo* establishment. The results obtained from this study indicated that stem/root connections of *V. grandis* explants were well developed to acquire and meet the water needs of the plant once transferred to glasshouse conditions (see Chapter 4).

The type of explant used for root initiation was not crucial for Clone 866. Apical tip shoots, and internode sections rooted equally well (100%). Multi shoot clumps did not root as well (90%). The choice of explant material put onto root initiation medium depends on the type of growth required for outplanting purposes. Single shoots facilitate apically dominant growth whereas explants with multiple shoots produce compact bushy growth. These results have shown that it is possible to induce roots on explants, *in vitro* for either type of growth requirement. Chapter 4 investigates the outplanting of *V. grandis* cultures, where these factors will be examined further.

In general, this study has developed an efficient tissue culture system for the initiation, shoot proliferation and rooting of *V. grandis* shoot explants. The protocol was released to "Phytotech", along with Clone 866, for full scale commercial production. The important aspects of the protocol can be summarised as follows: The choice of stock plant material with previous cultural information on rooting performance of cuttings. Plant tissue can be surface sterilised by immersion of cuttings in 0.1% sodium hypochlorite containing Tween

20 (0.01%)* for 3 hrs under constant agitation, followed by immersion in freshly prepared 1% sodium hypochlorite for 45min whilst stirring. MS basal medium with the addition of MS vitamins, 3% sucrose, 8g/l agar at pH 5.7 with 4.5µM BA is required for shoot proliferation. Healthy shoot explants greater than 1.5cm in height can be excised from multiplication medium and placed onto 1/2MS basal medium supplemented with MS vitamins, 3% sucrose, 8g/l agar at pH 5.7 with 10µM IBA, for root induction. A dark pre-treatment of one week is necessary, after which, containers are returned to the normal photo period conditions. Root production occurs within 6 weeks. Stem vascular tissue extended into the newly developed roots. Therefore rooted plantlets are adequately equipped to handle outplanting conditions.

In addition, a procedure was developed for shoot proliferation from leaf discs. Preformed meristematic cells were located at the leaf petiole region of *V. grandis* leaves. Shoot proliferation occurred from this region 4 weeks after leaf disc were placed on shoot induction medium (MS supplemented with 4.5µM BA). The leaf disc regeneration method would be appropriate for transformation studies (see Chapter 5).

CHAPTER 4 OUT-PLANTING AND POT CULTURE OF *VERTICORDIA GRANDIS*.

4.1 INTRODUCTION

The transfer of plants from tissue culture to soil and glasshouse conditions (Stage 4, acclimatisation) is a critical Stage in the success of the clonal micropropagation of plants. In many results this Stage is not investigated. Where reported the survival of plantlets is often low. For example, only 10% of *Eucalyptus tereticornis* (Sankaro Rao 1988) and 40% of *E. citridora* (Gupta *et al.* 1981) plants propagated by tissue culture survived when transferred to the glasshouse. There are several possible reasons for the low survival of plantlets. One factor can be related to the anatomy of plants grown *in vitro*. The culture vessel provides high humidity levels, while the media contains sucrose as the main carbon source. The intensity of illumination in most incubators is insufficient for much photosynthesis. These conditions are said to be conducive to heterotrophic growth. As a result, shoots grown *in vitro* have a different leaf structure to plants propagated by other means (eg, cuttings). Generally, the cuticle layer is thinner or absent *in vitro*, and the number of functional stomata is reduced. The survival potential of plants transferred from tissue culture to the glasshouse can be related to the plants inability to effectively photosynthesise and retain water. For example, the low survival of carnation plantlets regenerated *in vitro*, was attributed to their lack of an epicuticular wax layer, which resulted in excess water loss and hence desiccation on transfer to the glasshouse (Sutter and Langhans 1979). Therefore the acclimatisation to glasshouse conditions needs to be a gradual process from one of high humidity and relatively low light levels, to lower humidity and increased illumination. Other factors which influence the survival rate of plants transferred to glasshouse conditions include: the time of year, humidity and light levels, soil mix, quality of stock at out-planting and general glasshouse hygiene practices. The conditions adopted for outplanting of *V. grandis* are outlined in Section 2.2.2 also see Figure 2.2.

Culture conditions (soil mix, nutrient supply, temperature and light levels) all determine the growth rate and health of plants. However, there are no published specifications for cultivation of *V. grandis* plants. Early reports (Quick 1960 and Doney 1960) on the cultivation of the genus *Verticordia* state the difficulty in growing many of the species and in particular *V. grandis*. Reasons given are their intolerance to high humidity, and the need for excellent soil drainage. Details of soil and growth habit of *V. grandis* from areas in the south west province of Western Australia are presented by (Quick 1960). However it should be noted that *V. grandis* is not limited to the reported area and soil conditions. The soil profile consisted of a layer of light-textured yellow-grey sand 20 to 50 cm deep, underlaid by a layer of nodular laterite of similar depth, with a bottom layer of clay. Soil pH was acidic (4.5-5) and aspect was south to south-east on low gradients (4%). Quick (1960) also reported that plants in these areas were tolerant to high levels of sodium and potassium salts and concluded that the nature of the light sandy, open textured soil provided insulation against heat, sudden temperature change and loss of moisture. Personal communications with growers in W.A., suggested the following requirements for successful pot culture: adequate drainage, nutrient levels high in nitrogen and low phosphorus and a slightly acidic soil mix. Details of pot culture methods are given in Section 2.2.

For success as a commercially valuable plant, growth form and appearance is also important. For pot culture the plant should be attractive and have a compact growth form (Ben-Jaacov *et al.* 1988). This can often be achieved by inducing lateral shoot growth. Foliar sprays such as 6-benzyl adenine (BA) have been widely used to stimulate lateral branching in foliage and flowering plants (Henny and Fooshee 1985; Imamura and Higaki 1988) and in woody plants such as pines (Whitehill and Schwabe 1975). Pruning (removal of the stem-apices) is another method employed to induce the development of one or more lateral shoots (Imamura and Higaki 1988). Auxin produced in the stem apices travels down the stem and inhibits development of the axillary buds, removal by pruning of the stem apex removes the supply or source of auxin and, consequently, removes the inhibition of axillary buds thus "stimulating" them to develop (Phillips 1975). However,

"pruning" is generally time consuming and not very productive in terms of the number of new shoots produced. A combination of apical bub removal and cytokinin foliar spraying has successfully stimulated branching in a variety of horticultural plants. For example, lateral shoot production has been induced in ornamental (Jeffcoat 1977; Henny and Fooshee 1985; Imamurs and Higaki 1988), flowering (Ohkawa 1979), timber (Whitehill and Schwarb 1975) and fruit (Kender and Carpenter 1972) crops .

This study was primarily concerned with the development of techniques and conditions for the acclimatisation of *V. grandis* plantlets rooted *in vitro,* to glasshouse conditions. Another objective was to develop a pot culture method for long term growth of *V. grandis.* Experiments were based on a systematic investigation of factors which affect acclimatisation and pot culture of *V. grandis.* These include:

1) Acclimatisation of *V. grandis* to glasshouse conditions. Factors such as, the time of year of out-planting, the condition and genotype of outplanting material, humidity and light levels and hygiene practices were investigated.

2) For successful pot culture, media conditions such as, the effect of the soil mix, nutrient supply and light intensity were investigated.

3) The natural growth habit of *V. grandis* plants grown in pots is strongly influenced by apical dominance. Is it possible to induce lateral shoot growth in *V. grandis* pot plants? The following methods were investigated :1) BA application as a foliar or soil drench; 2) manual removal of the apical growing tip.

Details of the methods employed in this Chapter are presented in Section 2.2. Specific experimental details and results are presented below.

4.3 RESULTS

4.3.1 Transfer of plantlets to soil conditions.

A Root initiation of *V. grandis* microcuttings

An initial rooting experiment was performed with *V. grandis* microcuttings from Clones 865 and 866 (shoots harvested from Stage II) and with plantlets, from both Clones, which did not produce any roots on root induction media. Shoots were removed from the following pre-treatment conditions:

1) Multiplication medium MS supplemented with 4.5 µM BA;

2) Multiplication medium as for 1) but preconditioned for 2 weeks in the dark (to etiolate shoots);

3) Various root induction media (plantlets which failed to form any roots).

Thirty shoots from each treatment were removed and the bases dipped in a striking solution (absolute ethanol) consisting of IBA 5000µM and NAA 1500µM, prior to out-planting. Each treatment was replicated twice. Plantlets were monitored daily. The number of shoots which formed roots was recorded after 8 weeks. Out-planting conditions are described in detail in Section 2.2.2.

There was no root formation on any of the treatments. Shoots gradually deteriorated with time and no shoots survived longer than 8 weeks. These results indicated that it was not possible to induce root formation on *V. grandis* microcuttings.

B Survival rates of *V. grandis* Clones 865 and 866

Initial out-planting experiments were conducted with Clone 865 in mid winter. Plantlets which produced roots on root induction media were used in all experiments. Plants were labelled and grouped to keep records of plantlet performance in relation to the different root induction media at Stage III. Measurements of height and number of new shoots were

made weekly. A second out-planting experiment was conducted with Clone 866. Two planting periods were compared. The first was at the same time as Clone 865, mid winter with a total of 72 plantlets. The second was in early spring when a total of 56 plantlets were out-planted. For both experiments out-planting conditions were similar (see Section 2.2.2).

A total of 64 healthy rooted shoots of Clone 865 from various root induction media were out-planted. During the first six weeks the survival rate was good but thereafter shoot health deteriorated. By week 13, only 2 plants were actively growing, all other plants were woody with no signs of growth. Also in mid winter, 72 plants of Clone 866 with good root growth, were out-planted. In comparison to Clone 865, these plants acclimatised well and, as Figure 4.1 shows, after 6 weeks roots emerged from the bottom of the plug and there was new shoot growth. At this stage experiments were discontinued with Clone 865 due to the superior quality of Clone 866 *in vitro* (see Chapter 3) and at out-planting.

Table 4.1 shows the number and percentage of Clone 866 plantlets surviving after out-planting in winter (July) or spring (September). Results were recorded after 10 weeks. For the winter outplanting, plant losses occurred between weeks 8 and 10, reducing the overall survival rate from 100 to 86%. For the spring outplanting, only one plant died between weeks 4 and 6 resulting in an overall survival rate of 98%. The root induction media did not affect outplanting success.

For commercial nursery production, budgeted losses at outplanting range from 3-6%. The out-planting results for Clone 866 suggest that commercial production is viable. However as with any small scale experiment, viability needs to be confirmed with commercial production figures.

The outplanting experiments also indicated that a number of culture conditions were necessary to achieve these high outplanting rates. The humidity tent depicted in Figure 2.2 maintained high humidity levels and avoided leaving water sitting on and around the plants.

Figure 4.1: *V. grandis* clone 866, six weeks after transfer to glasshouse conditions.

Watering was by hand at the soil level. The humidity level was continually lowered by gradual removal of the tent cover over a one to four week period. Problems would be encounter with misting or fog systems as these systems produce a fine water layer on the plants. Hygiene was another critical factor. Fungal and bacterial contamination was controlled by weekly spraying with Benlate® and Previcure®. The greater survival percentage at the spring planting may be the result of variable glasshouse conditions with respect to the photo period, light intensity and temperature at the different out-planting times (see Appendix B Table 1) as all other conditions were constant.

Table 4.1: Plantlet survival rates of Clone 866.

Time of year planted	Number planted	Number (%) surviving after X weeks				
		2	4	6	8	10
Winter	72	72	72	72	72	62(86)
Spring	56	56	56	55	55	55(98)

4.3.2 The effect of the soil mix and nutrients on plant growth

A number of culture conditions are important for plant growth. These include: Media for adequate drainage and aeration (air-filled porosity of a mix), pH and conditions such as light, temperature and humidity. Four soil mixes with two levels of nutrient application (liquid feed fortnightly) were compared (see Section 2.2.3 for details of soil mixes and Appendix A for nutrient analysis). The medium pH and air-filled porosity was determined for each mix, these parameters and soil composition are presented in Table 4.2. Plant growth was measured fortnightly.

Table 4.2: Soil composition of the 4 soil mixes

Mix	Components	Air filled porosity %	pH
1	(5:2:1) Pine bark: Isolite: Peat	20	5.5
2	(5:2) Pine bark: coarse sand	16	5.8
3	(2:1:1:1:1) Loam: bush sand: coarse sand; pine bark: peat: isolite	10	5.3
4	(1:1:1:1:1) Coarse sand: bush sand: pine bark: peat: isolite	14	6.0

Parameters for growth were height (cm) and number of lateral shoots produced per plant. Twenty four plants were randomly allocated to each soil mix. Each group was then divided in two. Group A received the low nutrient application and group B the high rate (see Section 2.2.3 and Appendix B). The mean increase in plant height was compared between the treatments after 7 weeks. The nutrient treatment which produced the highest mean increase in growth was used for subsequent experiments and all plants then received this fertiliser application fortnightly. To evaluate differences in growth rates between the 4 soil mixes, the mean height (cm) was compared after 21 weeks.

Table 4.3: Influence of fertiliser and potting mix on plant growth.
12 plants were used for each treatment. The mean height increase in cm after 7 weeks is shown. Standard errors are given in brackets. *, ** indicates a significant difference between nutrient application rate within the soil mix (t-test *p=0.01, **p=0.001).

Soil Mix	Height (SE) NUTRIENT APPLICATION	
	LOW	HIGH
1	8.25(0.86)*	7.42(0.65)
2	5.5(0.53)**	4.25(0.71)
3	3.13(0.95)	2.9(0.71)
4	6.3(0.6)	6.23(0.62)

Table 4.4: Growth response of *V. grandis* in 4 soil mixes
24 plants were used for each mix except mix 3 where seven plants were used. The mean height (cm) after 21 weeks is shown. Standard errors are given in brackets. There was a significant difference in plant height between the soil mixes $LSD_{0.01}$=2.94

Soil Mix (Air filled porosity)	Mean Height in cm
1 (20%)	46.7(1.82) *c
2 (16%)	29.4(1.51) b
3 (10%)	24.1(1.95) a
4 (14%)	31.7(2.4) b

*Means with the same letter are not significantly different by LSD test (P=0.01)

Table 4.3 shows that for all the soil mixes, the low nutrient rate produced greater growth rates. Growth was significantly higher for Mix 1 and 3, Mix 2 and 4 show no significant difference in growth. Figure 4.2 shows plants grown in the 4 soil mixes at low fertiliser application. Visually, Mix 1 and 4 appear to produce the best growth. Mix 3 was clearly detrimental for plant growth, with 5 plants dying from each fertiliser treatment within 7 weeks. After 7 weeks all plants were given nutrients at the low rate.

The results shown in Table 4.4 supported those obtained in Table 4.3. A significant difference was found between the soil Mixes ($P<0.01$). Mix 1 produced significantly taller plants than all other soil mixes (LSD=2.94, values for the mean difference in height of each mix compared to Mix 1 are: Mix 2=17.3, Mix 3=22.6 and Mix 4=15). There was no significant growth differences between Mixes 2 and 4. The mortality rate for Mix 3 was high, with a total of 17 plants dying within the 21 week growth period. There was no plant death in the other soil mixes.

The air filled porosity of the soil mixes was related to plant growth rate. The higher the air filled porosity the greater the plant growth. This is shown in Table 4.4 where Mix 1, with 20% air filled porosity produced the highest growth (46.7 cm). Whereas, Mix 3 with the lowest air filled porosity (10%) produced the lowest growth rate (24.1 cm). Mix 2 and 4 with similar air filled porosity rates of 16 and 14 % produced similar growth rates 29.1 and 31.7., respectively. Mix 1 was chosen as the best potting Mix for growing *V. grandis* plants. The pH of the soil varied between 5.3 (Mix 3) and 6 (Mix 4). The growth response of the plants in the soil mixes suggests that a pH of between 5.5 and 6 is suitable for plant growth.

Figure 4.2: *V. grandis* plants growing in four soil mixes and liquid feed fortnightly at the low fertiliser rate (1 part in 800, see Appendix A). Growth was recorded after 7 weeks.

1 Mix 1 (5:2:1, composted pine bark: isolite: peat).

2 Mix 2 (5:2; composted pine bark: coarse sand).

3 Mix 3 (2:1:1:1:1; Loam: bush sand: coarse sand: isolite: peat).

4 Mix 4 (1:1:1:1:1; Coarse sand: bush sand: composted pine bark: peat: isolite).


1


2


3


4

4.2.3 Influence of light intensity on *V. grandis* growth

This experiment was designed to determine the effect of increased illumination during the winter growing months (August). The standard mean light intensity was 382 μEs/m/2. Twenty six percent additional light was provided by two 400 watt high pressure sodium lamps for 16/hr daylight (481 μEs/m/2.). Thirty, twenty two week old plants in 10cm pots were placed under the lights or left in a compartment with standard light levels (see Appendix B). The height (cm), number of lateral shoots and number of flower buds was measured fortnightly. Final measurements were taken after 13 weeks. Results presented show the mean number of lateral shoots, mean height (standard error of the means) and the percentage of plants with flower buds.

Table 4.5: The effect of providing supplementary light on *V. grandis* growth during Winter months.

For the light treatment, 30 plants were used to compare with 24 plants grown under standard conditions. * indicates significant differences between treatments for the light conditions (T-test, $p<0.01$). Standard errors are given in the brackets.

Supplementary Light	Mean height	Mean No. of shoots	Percentage of plants with flowers
Nil	20.1(1.11)	7.3(0.67)	8
Provided	25.8(1.4)*	10.4(0.74)*	27

As evident from Table 4.5 the higher light conditions produced significantly taller plants, with a greater number of lateral shoots ($p<0.01$). Also of interest is the higher percentage of plants with flower buds in the high light group. These results suggest that light intensity

is useful for increasing *V. grandis* growth during the slow growing months of winter. Light intensity also influences flower bud initiation.

4.3.4 Culture form

V. grandis plants grown from tissue culture show apical dominance. Figure 4.3 (A) shows the growth habit of plants with a single shoot (the four back pots) and plants with numerous basal shoots (3 front pots) after 8 weeks growth. Note that the overall growth rate is slower when the plant has numerous basal shoots (see Figure 4.3 (A), front row, third pot from the left). Plants with single shoots show strong apical dominance. If the apical tip is manually removed one or two lateral shoots develop along the stem (see Figure 4.3 (A), back row, first and third pot from the left.

A The effect of BA and method of application on lateral shoot production of *V. grandis*

This experiment was designed to test the effect of BA application on lateral shoot production of *V. grandis* plants. Two methods of BA application were compared, foliar spray and soil drench. Eighty *V. grandis* plants (with one shoot and no lateral shoots) were acclimatised to glasshouse conditions for 8 weeks and then transferred to 10cm pots. Ten plants received 1 of 4 treatments; an untreated control or BA application at 50, 210, and 1000mg/l, applied either as a soil drench or foliar spray, resulting in 8 treatments in all. The number of lateral shoots and the height of the plants was recorded fortnightly. Final measurements (mean number of shoots and the standard error of the mean) were recorded after 16 weeks.

Figure 4.3: Examples of *V. grandis* pot plants.

A: Examples of various types of growth of *V. grandis* plants 8 weeks ex tissue culture.

Front row: Growth habit of plants out-planted with numerous lateral shoots.

Back row: The growth habit of plants outplanted with a single shoot. Removal of the apical tip induces the growth of one to three new shoots (plants in pots first and second from the left).

B: Examples of plants given a 6-benzyladenine (BA) foliar spray at the following concentrations.

a: Control: b, 50mg/l, c, 2 examples of plants given 210mg/l, d, 1000mg/l.

C: Representative examples of plants given a BA treatment as a soil drench at various concentrations (0, 210 and 1000 mg/l).


A


B
a
b
c
c
d
SPRAY


C
20
CM
15
10
5

Table 4.6: Induction of lateral shoots by BA spray or soil drench.

Each treatment was applied to 10 plants. Data were collected after 16 weeks. At this time only 2 plants remained alive for the 1000mg/l spray treatment and only 9 for the 1000mg/l drench treatment. The mean number of lateral shoots are presented after 16 weeks. Standard errors are given in brackets.

BA concentration mg/l	Mean No. of shoots (SEM) Spray	Drench
0	2.6(0.16)*a	2.8(1.3) a
50	3.1(0.97) a	2.1(0.18) a
210	9.6(1.33) c	2.9(0.23) a
1000	6(N/T) b	2.8(0.32) a

*Means with the same letter are not significantly different by LSD test ($P<0.01$, LSD=3.14)

Analysis of variation showed a significant interaction between BA concentration and method of application ($P<0.01$ and $P<0.01$ respectively) (Table 4.6). A LSD test was used to test for significant mean differences between the method of application at each BA concentration. This showed significant differences between the two methods of application for all concentrations except the control and the 50mg/l treatment. The BA treatment, 210mg/l, applied as a foliar spray produced a significantly greater number of lateral shoots than all other treatments (mean differences between the number of lateral shoots of each treatment compared to this treatment are; spray control=7, spray 50mg/l=6.5, spray 1000mg/l=3.6, drench control=6.8, drench 50mg/l=7.5, drench 210mg/l=6.7 and drench 1000mg/l=6.8). Higher concentrations 1000mg/l initially produced a large number of

laterals (data not shown), however 8 out of the 10 treated plants died within 4 weeks (see Figure 4.3 (B) pot d)

The method of application was important. Soil drenching was significantly less effective than foliar spraying for BA concentrations of 210 and 1000mg/l (see Table 4.6 and Figure 4.3 (C)). No significant difference was found between any of the BA concentrations applied as a soil drench.

Figure 4.3 (B and C) shows examples of plants treated with BA as a foliar spray (B) and with a soil drench (C). There was some variation in shoot induction for treatment (B) pot (c)=210mg/l foliar spray. In addition, Fig. 4.3 (B) pot (d) shows that the 1000mg/l treatment was clearly detrimental to plant growth. The plant representing the soil drench treatment of 1000mg/l Fig. 4.3 (C.) pot (d) shows signs of leaf wilt and died shortly after.

B Comparisons of BA application and apical tip removal on lateral shoot production

In this experiment the influence of apical bud removal alone or in combination with BA spraying was observed in plants at various Stages of growth (ie, length of time, 8, 22 or 30 weeks, after out-planting). Ten plants of each age group received one of three treatments. The standard BA spray application was 210mg/l. Treatments consisted of apical tip removal alone or in combination with the BA spray, and a control treatment of neither spray nor tip removal. The mean number of lateral shoots was recorded after 16 weeks.

As evident from Table 4.7 the mean number of lateral shoots produced was greatest for the BA spray treatment in combination with apical tip removal. This was significant compared to apical tip removal alone ($P<0.01$), the control treatment ($P<0.01$) and was independent of the plant age ($P<0.01$). The optimum plant age for BA treatment in combination with apical tip removal was between 22-30 weeks.

Table 4.7: Lateral shoot induction by apical tip removal in combination with BA spraying.

Plants at 3 Stages of growth after 8, 22 and 30 weeks under glasshouse conditions, were treated with 210mg/l of BA applied as a foliar spray alone or in combination with apical tip removal. There were ten plants per treatment except for the spray and tip removal treatment for 22 week old plants, where six plants were used. The mean number of lateral shoots are presented. There was a significant difference between treatments for all plant ages except for the control (no spray/no nip) at 8 and 22 weeks, $LSD_{0.01}$=1.96. Standard errors are given in brackets

	Mean No. of shoots		
Age of plants at treatment	**NIP**		
	No spray	**Spray**	**No Spray/ No Nip**
8 wks	4(0.62)*b	7.5(0.15) c	1.8(0.4) a
22 wks	7.3(0.64) c	20.5(3.65) e	2.6(0.8) a
30 wks	18.8(2.48) d,e	22.7(2.53) f	5.9(0.19) c

*Means with the same letter are not significantly different as determined by LSD test (P=0.01).

Figure 4.4: Examples of *V. grandis* pot culture.

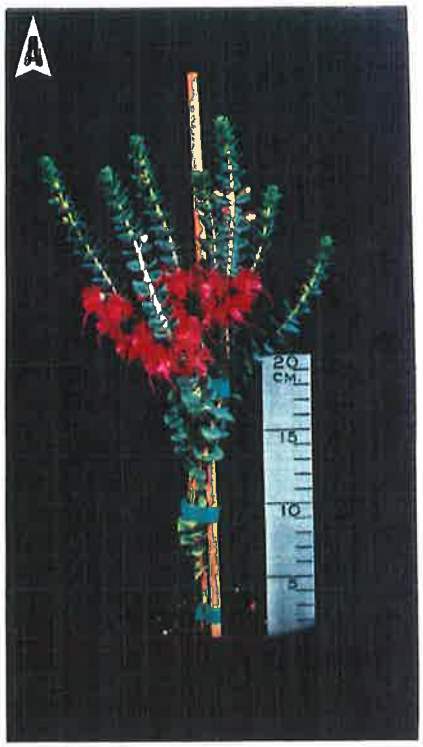
A: A plant 22 weeks after transfer to glasshouse conditions, grown in a 13cm pot.

B: A plant maintained in pot culture for 3 years.

C: Close up of *V. grandis* flowers. This is an example of a single flower stem suitable for cut flower arrangements.

D: An example of a multi flower stem.

E: A collection of plants showing the glasshouse compartment utilised for most of the experiments conducted throughout this study.


A
20
CM.
15
10
5


B
VERTICORDIA
GRANDIS


C


D


E

Plants have been maintained in pot culture now for 3 years. In that time they have been flowering consistently all year round with most profuse flowering between September and May. Figure 4.4 (C and D) shows examples of *V. grandis* growth at various stages in pot culture. (A) illustrates a plant after 22 weeks of growth in a 13cm pot, flower buds first emerged around 20 weeks. (B) shows an example of a plant in a 25cm pot, maintained in pot culture for 3 years. Plant growth form has been maintained by constant pruning to below the node of the last flower bud. This practice induces lateral shoots and maintains the compact growth form. Figure 4.4 (C and D) show close up examples of the flower form. (C) is an example of a flowering shoot which would be suitable for cut flower arrangements. (E) shows a collection of *V. grandis* pot plants growing in the glasshouse compartment utilised for most of the experiments discussed in this chapter.

4.4 DISCUSSION

The results described in this chapter outline a number of culture conditions necessary for the acclimatisation and pot culture of *V. grandis* plants in the glasshouse.

Root induction of microcuttings (unrooted plantlets ex Stage II, deflasked in the glasshouse and planted directly into the rooting medium, with or without an auxin dip) is possible for some species. Generally this method is only viable for species which produce roots easily from cuttings or layering techniques. Consequently, it was not surprising to find that this method was not applicable to *V. grandis* microcuttings. Root induction of plantlets *in vitro* was necessary for successful acclimatisation of *V. grandis* explants to glasshouse conditions. In addition, a number of culture conditions were also required. These included, initial high humidity levels without excess water sitting on or around the plants. The humidity tent provided these conditions. However, the high humidity levels were also conducive for fungal disease of *V. grandis* plants. Fungal contamination was controlled by a weekly spray program with Benlate® and Previcure®.

Furthermore, this study found that survival rates were influenced by both the plant genotype and the time of year of outplanting. It was not possible to obtain adequate survival rates of Clone 865 (only 3% of plantlets survived longer than 13 weeks). Whereas, Clone 866 acclimatised well to the soil conditions. Variation between species, but not within species, in success of transfer to soil conditions, has been previously reported (*Eucalyptus citriodora* and *E. diversicolor* were both easier to transfer than *E. marginata*, McComb and Bennet 1986). A number of factors may account for the difference in survival rates of Clone 865 and 866. This study has shown that root induction of Clone 865 explants was more difficult than Clone 866 (Chapter 3). Noticeably, Clone 865 plants became quite woody with no new shoot growth after transfer to soil. This suggests that the plant's water and nutrient demands were not adequately met. An adequate supply of nutritive and growth factors to the growing areas is essential for healthy growth. This can only be maintained if there are continuous vascular connections of the root and lateral buds to the main stem (Phillips 1975). However, anatomical evidence with both Clone 865 and 866 (Chapter 3) found sound vascular connections between the stem and root.

Another factor to consider, is the age of the plant material. This is known to affect the rooting ability of cuttings (Hartmann and Kester 1986, Komissarvo 1969) and may also influence plant development. Previous culture information for Clone 865 were not available. This clone had been in culture at Phytotech for at least 3 years before these experiments began. The survival rate of Clone 865 was good for the first six weeks. Thereafter plant growth deteriorated. In comparison, Clone 866 plants continued to grow after the initial six week acclimatisation period. These results highlight the importance of previous culture information on plant growth characteristics and suggest that a combination of genetic and environmental factors are important for the successful acclimatisation of *V. grandis* plants.

Environmental conditions which influenced *V. grandis* growth in pots included the soil mix, light intensity and nutrient supply. The soil mix was found to greatly effect the

growth rate of *V. grandis* plants. A significant difference in growth rate was found between the four soil mixes. Important factors included adequate drainage (air filled porosity of 20%), slightly acidic pH of the soil medium pH (5.5-6) and low nutrient levels. Light conditions influenced the growth of plants. Higher illumination during the low light winter months increased plant growth and flower bud formation. Characteristically, *V. grandis* plants grown in pots show apical dominance. For commercial pot culture, appearance is also important. Generally, the main criteria are compact, bushy growth.

In this study, foliar sprays of BA, alone or in combination with apical bud removal, increased branching of *V. grandis* plants. BA application at a concentration of 210mg/l was found to be most effective for increasing lateral shoot development. Shoot induction as a result of this application was also influenced by plant age. Plants treated between 22-30 weeks ex *in vitro,* produced the greatest number of lateral shoots. These results indicate that promotion of lateral shoot development can be induced in *V. grandis* plants. Consideration must be made for time of treatment as Ohkawa (1979) has previously reported for the promotion of axillary buds in BA treated rose plants.

Pruning, specifically the removal of the stem apices stimulates development of axillary buds. This study found that apical bud removal in combination with BA foliar spraying was more effective than pruning or spraying alone. The combination of these methods has been reported to be useful for inducing lateral shoot development in a number of ornamental crops (Jeffcoat 1977). For a continuous production of new laterals, multiple BA applications at various time intervals may be worth investigation.

This study has developed out-planting and pot culture protocols for the growth of *V. grandis* pot plants. Initially, culture information obtained from the plants natural growing conditions provided valuable information on growing requirements. For successful acclimatisation to the glasshouse, *V. grandis* plantlets need to have good root development and specific out-planting conditions are required, these include:

1) well aerated soil mix (at least 20% air filled porosity, pH 5.5-6) consisting of inert materials which are relatively free of possible contaminating microbes (eg. 5:2:1, composted pine bark:isolite:peat),

2) regular hygiene practices consisting of a weekly spray program of Benlate® and Previcure®,

3) Initial high humidity levels gradually decreasing at 1-4 weeks;

For successful pot culture the same soil conditions apply as above with the addition of a fortnightly supplementary nutrient feed of low levels of all major and minor elements (see Appendix A). The growth form of *V. grandis* pot plants can be manipulated by pruning (removal of the apical buds) and with foliar BA sprays. A foliar BA spray at a concentration of 210mg/l in combination with apical bub removal applied to 22-30 week old (ex *in vitro*) plants increased the number of lateral shoots. To maintain a compact growth form, spent flower buds should be continually pruned back to below the last bud.

In conclusion, these results represent the first report of out-planting and pot culture protocols for the successful growth of *V. grandis* pot plants.

CHAPTER 5 *AGROBACTERIUM* MEDIATED GENETIC MANIPULATION OF *VERTICORDIA GRANDIS*

5.1 INTRODUCTION

The most widely used methods of plant transformation are based on the natural ability of *Agrobacterium* to insert, Ti (tumor-inducing) or Ri (root-inducing) T-DNA (transferred DNA) into the genomes of many host plants (see Chapter 1). A large number of plant species have been reported to be susceptible to *Agrobacterium* infection, including a wide range of woody plant species (DeCleen and Deley 1976). However, shoot regeneration in woody plants is often limiting and consequently the recovery of transformants is difficult. Transformation of woody species and subsequent regeneration of transgenic plants has been reported for only a few genera including *Populus* (Fillalti *et al.* 1987), *Malus* (James *et al* 1987), *Rubus* (Graham *et al.* 1990), *Juglans* (McGranahan *et al.* 1988), *Citrus* (Vardi *et al.* 1990), *Allocasuarina* (Phelph *et al* 1991) and *Robinia* (Han *et al.* 1993). There have been no reports of transformation and subsequent regeneration of transgenic plants from the Myrtcaeae, although transient gene expression has been detected in electroporated protoplasts of *Eucalyptus citriodora* Hook (Manders *et al.* 1992).

Plants infected with *A. rhizogenes* develop roots (hairy roots) at the inoculation site (Elliot 1951). The use of wild type *A. rhizogenes* as a root inducer in woody plants has been considered in Chapter 1(Moore *et al.* 1979, Lambert and Tepfer 1991 and Rugini *et al.* 1991). However, some plant species do not produce roots upon infection with *A. rhizogenes* but react with tumor formation or a combination of both tumors and roots (Nester *et al.* 1984). It is possible to regenerate whole plants from unorganised tissues and transformed roots of a variety of herbaceous plant species (Birot *et al.* 1987). Recently, this has also been possible with two woody species. Transgenic *Allocasuarina verticillata* (Phelep *et al.* 1991) and Black locust (*Robinia pseudoacacia)* plants (Han *et al.* 1993) have been regenerated from *A. rhizogenes* induced hairy roots. The idea of utilising *A. rhizogenes* to induce rooting of *V. grandis* explants *in vitro* is explored in this study.

In addition to possible root inducing properties of *A. rhizogenes*, this study also explores the use of *Agrobacterium* to develop a transformation system with *V. grandis*. The following criteria are considered most important in achieving this aim:

1) *Agrobacterium* susceptibility experiments are necessary to determine whether *V. grandis* can be infected with virulent *Agrobacterium* strains. Symptoms of infection include root or gall formation. Confirmation of infection should be obtained by opine assays.

2) A suitable shoot regeneration system to produce transgenic plants. Targeted cells, tissues or organs, once infected, need to have the ability to regenerate.

3) The detection and analysis of transformants. Evidence of transformation should include both biochemical and molecular studies. The use of *Agrobacterium* gene transfer vectors which contain selectable marker genes, such as neomycin phosphotransferase (NPT II), and reporter genes, like ß-glucuronidase (GUS) have proved extremely useful for both the selection and detection of gene expression in transgenic plants (see below).

5.1.1 Selection systems

The selection system chosen should clearly identify transformants, so that a distinction can be made between transformed and untransformed tissue. In other words, the selective agent must inhibit the growth of untransformed tissue. Selectable marker genes available for this purpose include antibiotic and herbicide resistance. Chimeric marker genes are available which confer plant cell resistance to the antibiotics kanamycin, phosphinotricin, methatexate and hygromycin. The most widely used marker gene is NPT II from a bacterial transposon which encodes resistance to a range of aminoglycosides, such as kanamycin. Cells carrying this gene have a selective advantage in the presence of kanamycin (Ahuja 1987).

In addition to selection marker genes (like NPT II), which are useful to isolate transformed tissue, reporter genes like GUS permit the detection of gene expression in transformed

tissue by a simple assay. Expression of GUS can be detected histochemically and measured using fluorometric assays (Jefferson *et al.* 1987).

However, there are problems in using GUS. A variety of plant species express endogenous GUS activity (Hu *et al.* 1990, Hodal *et al.* 1992). Expression has been related to the pH of the assay buffer (Hodal *et al.* 1992) and has also been shown to be due to the presence of endophytic bacteria within plant tissue which express GUS (Tör *et al.* 1992).

The reporter gene ß-glucuronidase (GUS) constructed in the plasmid pBI121, is directed by the CaMV-35S promoter from the Cauliflower mosaic virus and is also transcribed by *Agrobacterium* (Van Wordragen *et al.* 1992). Therefore, no distinction can be made between bacterial and plant GUS activity. An alternative vector is pKiwi which gives negligible bacterial GUS expression due to the lack of bacterial ribosome-binding sites (Putterill and Gardner 1989). In this study the pKiwi vector was used in two experiments (see Section 5.2.8 A and B) to locate histochemical GUS expression and to detect the presence of endophytic bacteria within *V. grandis* tissue as compared to the pBI121 vector.

5.1.2 Gene transfer vectors

Two types of vector systems are possible to introduce foreign DNA to plants. Firstly, cointegrating vector systems contain a region of genetic homology between the Ti plasmid and the vector plasmid. Secondly, binary vector systems rely on a mutual co-existence of the Ti plasmid and the vector plasmid within the *Agrobacterium*. In this study, a number of wild type *A rhizogenes* and disarmed *A. tumefaciens* strains carrying the binary vector pBI121 or a cointegrating vector pGV3850 were employed to facilitate early selection and screening of transformants. The T-DNA of the binary vector plasmid pBI121 contains two marker genes, NPT II and GUS. The T-DNA of the cointergrating vector plasmid pGV3850 contains the NPT II marker gene (for a detailed map see Hain *et al.* 1985). A list of the *Agrobacterium* strains and plasmids used in this study is provided in Table 2.1 (see Section 2.4.1).

5.1.3 Transformation procedures

The genetic transformation system chosen for a given plant species is dependent on the regeneration system available for that species. A simple and effective method, which has successfully transformed a variety of plant species, is the leaf disc procedure (Horsch *et al.* 1985). Leaf discs are co-cultured on regeneration medium, generally for 2-3 days, with *Agrobacterium*. The leaf discs are then transferred to regeneration medium containing the appropriate antibiotic (see above). The method adopted for leaf disc transformation of *V. grandis* is described in detail in Section 2.5.2.

5.1.4 Molecular analysis of transformants: DNA quality

The integration of foreign genes into the plant genome was analysed by Southern hybridisation (Southern 1975) and PCR techniques. High quality DNA is necessary for the detection of genes with low copy numbers by Southern hybridisation techniques. This process can be hampered by the presence of contaminating carbohydrate polymers (polysaccharides) and polyphenols (Manning 1991) within the plant DNA. Polysaccharides in DNA samples form highly viscous solutions. These substances bind to nucleic acids and inhibit the activity of restriction endonucleases (Callahan *et al.* 1989). Also, the quantification of total DNA of contaminated DNA samples by UV absorption is unreliable (Newbury and Possingham 1977). Furthermore, these samples are difficult to concentrate by standard ethanol precipitation methods (Manning 1991). Most plant DNA isolation and purification methods do not effectively remove polysaccharides from DNA (Fang *et al.* 1992). Therefore, a number of protocols for DNA extraction were tested for their suitability for use with *V. grandis* leaf tissue (see Section 2.7).

5.1.5 Chapter aims

The overall aim of the work described in this Chapter is to establish a system for the genetic engineering of *V. grandis*. A number of stages are necessary in establishing a transformation system using *Agrobacterium*. These stages are outlined below:

1) The first step is to show that *Agrobacterium* will infect *V. grandis*. Susceptibility trials with wild-type *Agrobacterium* strains were used to screen for infection by gall and root formation. The use of *A. rhizogenes* as a root inducer of *V. grandis* stem explants was also examined.

2) A suitable regeneration system to produce transgenic plants. This involves targeting cells for transformation which would provide appropriate tissue for the successful regeneration of transformants. Initial experiments were conducted with Clone 865 due to the availability of shoot material of this clone. Sufficient shoot material of Clone 866 was only available for experimentation at a later date. It was then possible to compare transformation frequencies between the two clones (see below).

3) The use of selectable marker genes in *Agrobacterium* strains to aid in the selection and detection of transformants.

4) The analysis of putative transformants by opine assays, histochemical expression of GUS, PCR and Southern blot analysis to provide evidence of transformation.

There have been no reports of *Agrobacterium* infection of *Verticordia*, nor of the possibility of utilising *A. rhizogenes* to induce root formation of *V. grandis* explants *in vitro*. These factors will be examined as the first step in developing a transformation system with *V. grandis*.

5.2 RESULTS

5.2.1 Infection of *V. grandis* with *A. rhizogenes*

An inoculation experiment was performed to test the susceptibility of *V. grandis* (Clone 865) explants to *Agrobacterium* infection and also to determine whether *A. rhizogenes* will induce root growth on these explants. *V. grandis* stem explants grown *in vitro* were inoculated with one of four virulent *A. rhizogenes* strains, K565, K568, K596, K597. Two

methods of inoculation were tested, the base of freshly cut shoots was dipped in the inoculum or the stem was stabbed with a needle dipped in the inoculum (see Section 2.5.1 for a detailed description of the methods). For each treatment four hundred explants were used. *V. grandis* susceptibly to infection was judged on the basis of root or gall formation. Root and gall tissue was then assayed for opines.

Table 5.1 shows the response of *V. grandis* (Clone 865) explants to inoculation with one of four virulent *A. rhizogenes* strains. The results indicate that root formation on inoculated explants was not greater than for uninoculated controls. Furthermore, gall formation was only observed after *Agrobacterium* inoculation. Shoots inoculated by the stab method with strains K565 and K596 produced the greatest number of galls. Table 5.1 also shows the percentage of galls and roots which tested positive for the opines, agropine, agropinic acid, mannopine and cucumopine (see Section 2.6.1). Opines were detected in a large percentage (33-100%) of all galls and roots on inoculated plants, except K597. Figure 5.1 shows the electrophoretic analysis of *V. grandis* root and gall formations from explants inoculated with strain K596.

No roots developed on the control plants by the stab method and those that formed roots after inoculation with *A. rhizogenes* strains were phenotypically abnormal when compared to normal roots. Figure 5.2 shows representative examples of abnormal root formation on inoculated explants. Abnormal root characteristics included; aerial roots which grew aplagiotropically as shown in Figure 5.2 (A) (an explant inoculated with strain K565 by the dip method), (B) shows excessive callus formation on and around the inoculation site of an explant infected with strain K568 and (C) shows a short stunted root induced in an explant inoculated with strain K568, by the stem dip method). Attempts to regenerate shoots from excised galls and roots on a number of shoot induction media were unsuccessful.

These results indicate that *V. grandis* is susceptible to infection with the *A. rhizogenes* strains used in this experiment however, infection with these strains does not increase root production on *V. grandis* stem explants.

Table 5.1: Response of *V. grandis* Clone 865 to infection with 4 strains of *Agrobacterium rhizogenes.*

Explants were inoculated by one of two methods, (C) the freshly cut end of the stem was dipped into the inoculum, or (S) stem stabbed with a hypodermic needle. Results show the percentage of explants which produced roots and gall tissue and the percentage of these galls and roots which tested positive for an opine.

Strain	Total number tested		% of explants producing Roots	Galls	Opine result % tested positive
Control	400	(C)	4	0	0
		(S)	0	0	0
K568	400	(C)	3	4	G(50) R(n/t)
		(S)	0	5	G(75)
K565	400	(C)	1	1	0
		(S)	3	42	R(67) G(83)
K597	400	(C)	1	6	0
		(S)	0	6	0
K596	400	(C)	1	3	G(33), R(n/t)
		(S)	3	23	G(100), R(n/t)

G=Galls, R=Roots, n/t=not tested

Figure 5.1: Extract of *V. grandis* root and gall formations from stems inoculated with *A. rhizogenes* strain K596, seperated by HVPE. (Citric acid buffer, pH 3.6 at 3500v for 20 min) showed positive reactions to silver nitrate. N= neutral.

Lanes:	1-5:	*V. grandis* galls
	7-10	*V. grandis* roots
	11	*V. grandis* control tissue
	6&12	Agropinic acid standard
	13	Mannitol standard

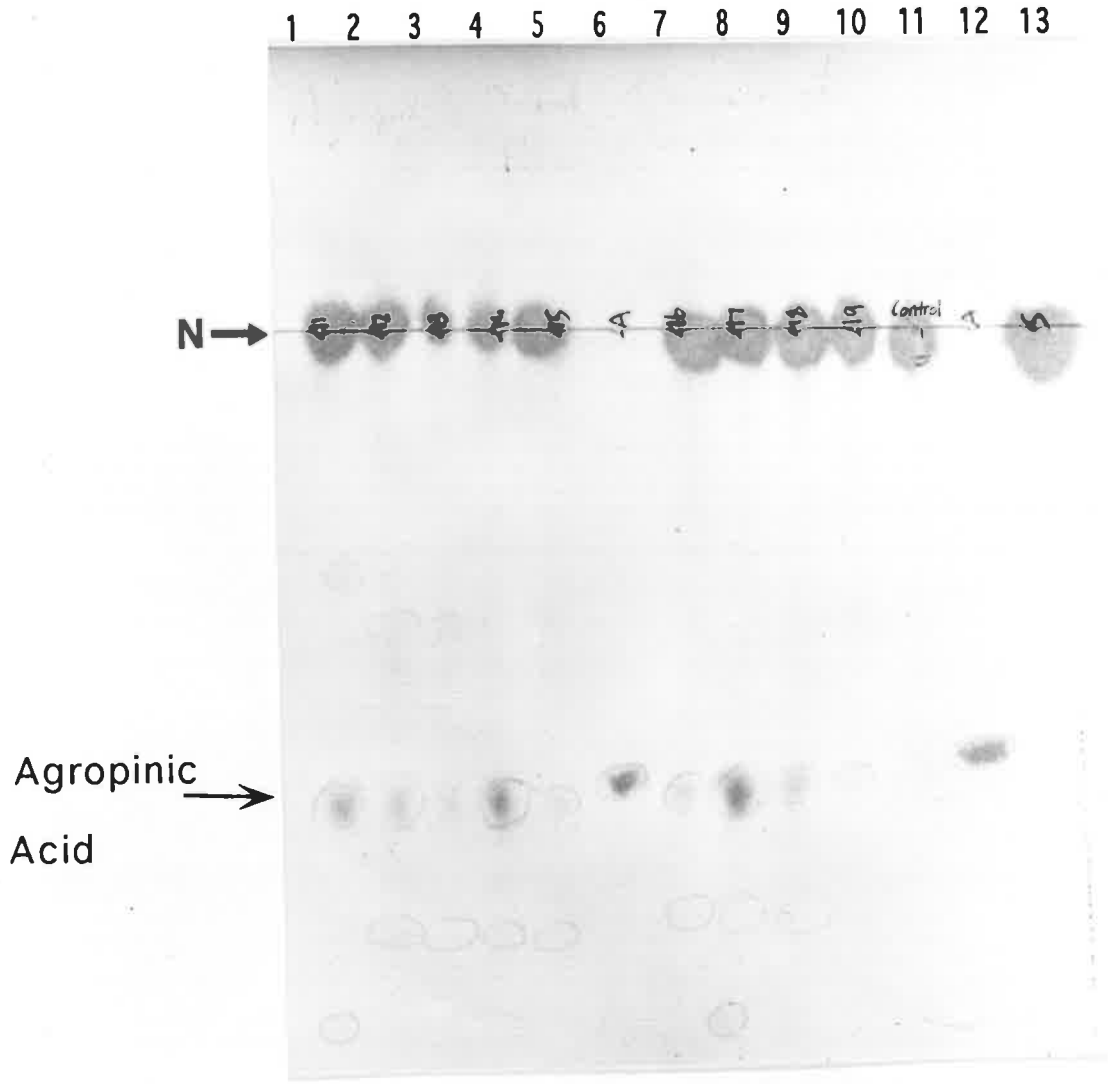

1 2 3 4 5 6 7 8 9 10 11 12 13
N
Control
Agropinic
Acid

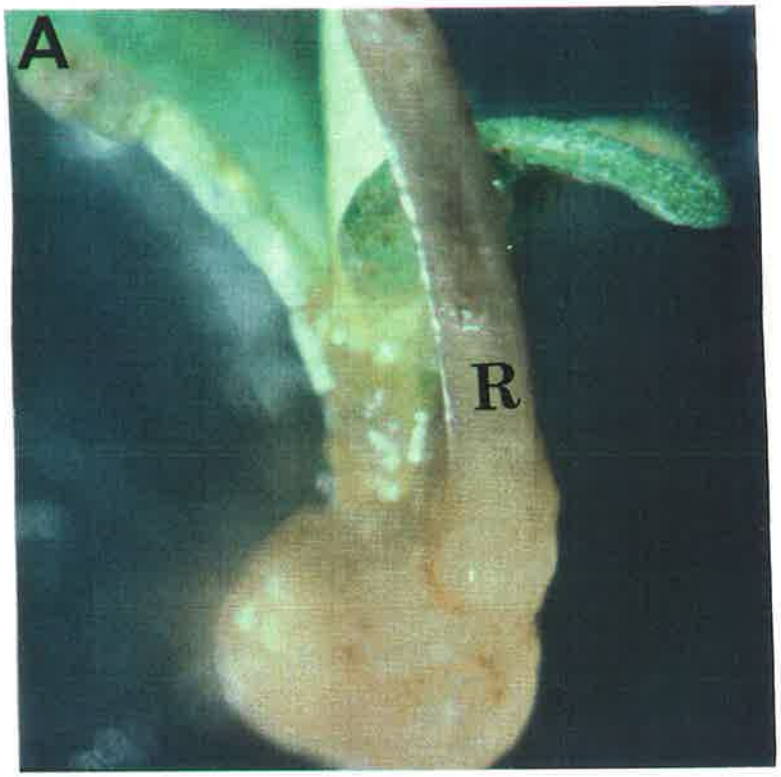
Figure 5.2: Response, after 8 weeks of *V. grandis* explants to stem inoculations with wild-type *A. rhizogenes* strains . Root formation on explants inoculated by either the stem dip or stem stab method. (1.6 x magnification)

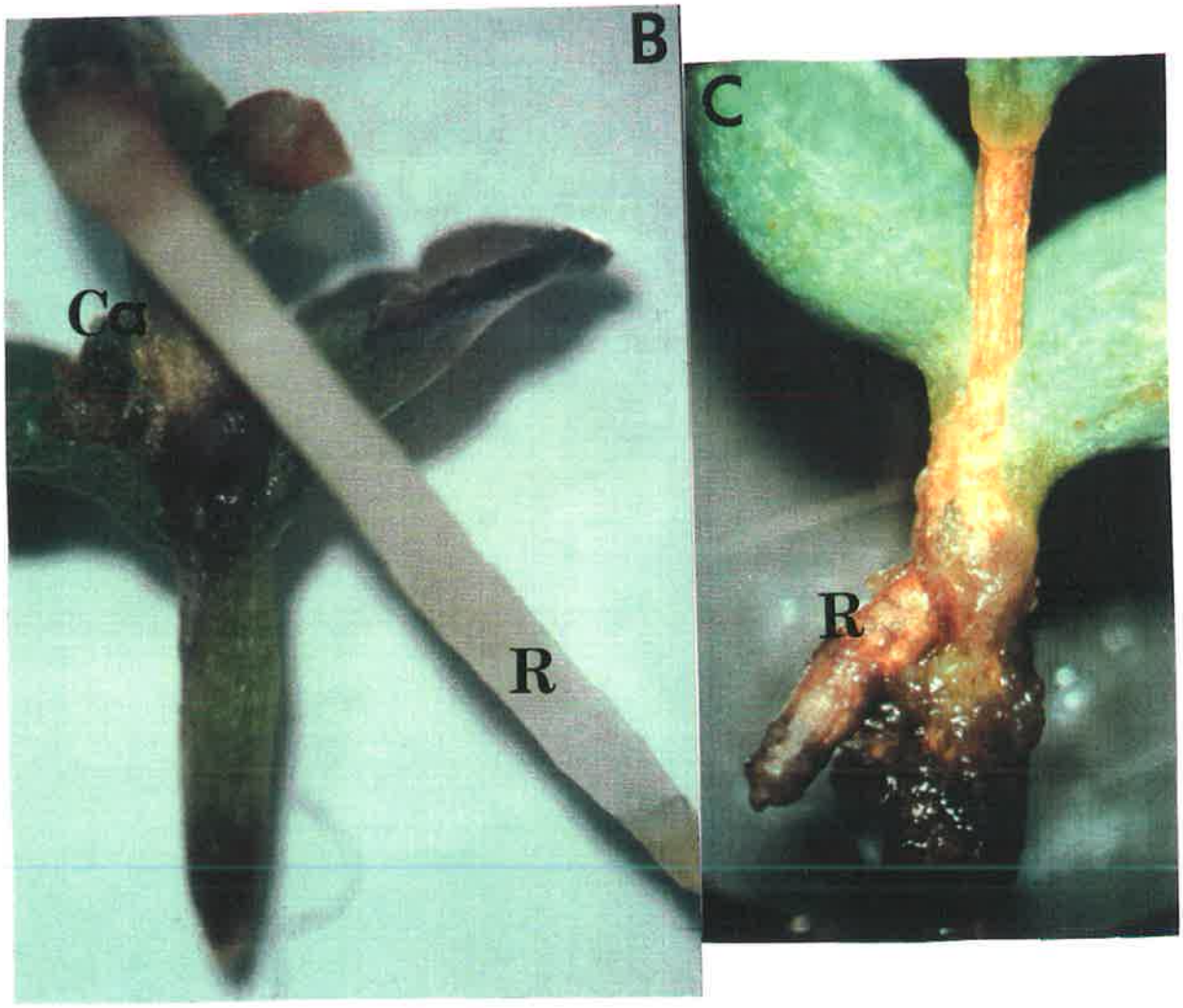
A: Apical root formation on an explant inoculated with strain K565 by the stem stab method.

B: Root formation on an explant inoculated with strain K568 by the stem stab method.

C: Root formation on an explant inoculated with strain K568 by the base dip method.

R= Roots, Ca= Callus


A
R


B
Ca
R
C
R

5.2.2 Target cells for *Agrobacterium* infection and regeneration of transformants

The method developed for regeneration of shoots from leaf discs, described in Chapter 3, was used for transformation experiments. At least one hundred leaves were inoculated with one of four virulent *A. rhizogenes* strains. The mean percentage of leaf discs that formed shoots, galls or roots was recorded after 8 weeks.

Table 5.2 shows the percentage of shoots, galls and roots which formed on leaf discs, 8 weeks after inoculation with *A. rhizogenes*. Strains K565 and K596 produced the highest percentage of leaf discs forming both shoots and galls. Similar to stem inoculation results, only a small percentage (1%) of all discs (including uninoculated controls) formed roots (strain K565). Furthermore, only traces of opines were detected in shoot tissue, whereas higher concentrations of opine were detected in gall tissue. It was not possible to obtain any shoot differentiation of gall tissue. On all media tested (MS basal medium with or without the addition of phytohormones), the gall tissue eventually browned and died.

Figure 5.3 shows examples of shoot regeneration from leaf discs of *V. grandis* inoculated with *A. rhizogenes*. These photos show that shoot and gall formation occurred at the inoculation site (leaf petiole area). Shoot formation was first observed on leaf discs 3 weeks after inoculation (Figure 5.3 A). Gall formation was most extensive on leaf discs inoculated with strain K565 or K596 and growth occurred on both the abaxial and adaxial sides of the leaf discs (Figure 5.3 B).

Table 5.2: Percentage of *V. grandis* (Clone 865) explants exhibiting shoot, gall or root formation, 8 weeks after inoculation and the percentage of these tissue which tested positive for opines.

Results were scored 8 weeks after inoculation with the *A. rhizogenes* strains. The standard error of the mean are given in the brackets.

Treatment	No. tested	% of explants producing			Opine result
		Shoots	Galls	Roots	% positive
Control	300	71(2.34)	10(6.2)	0	0
K596	300	31(13.1)	13(5.5)	0	S(45) G(100)
K565	300	28.3(17.1)	20(7.8)	0.7	S(38.5)
K597	50	3	8	0	0
K568	50	3	2	0	0

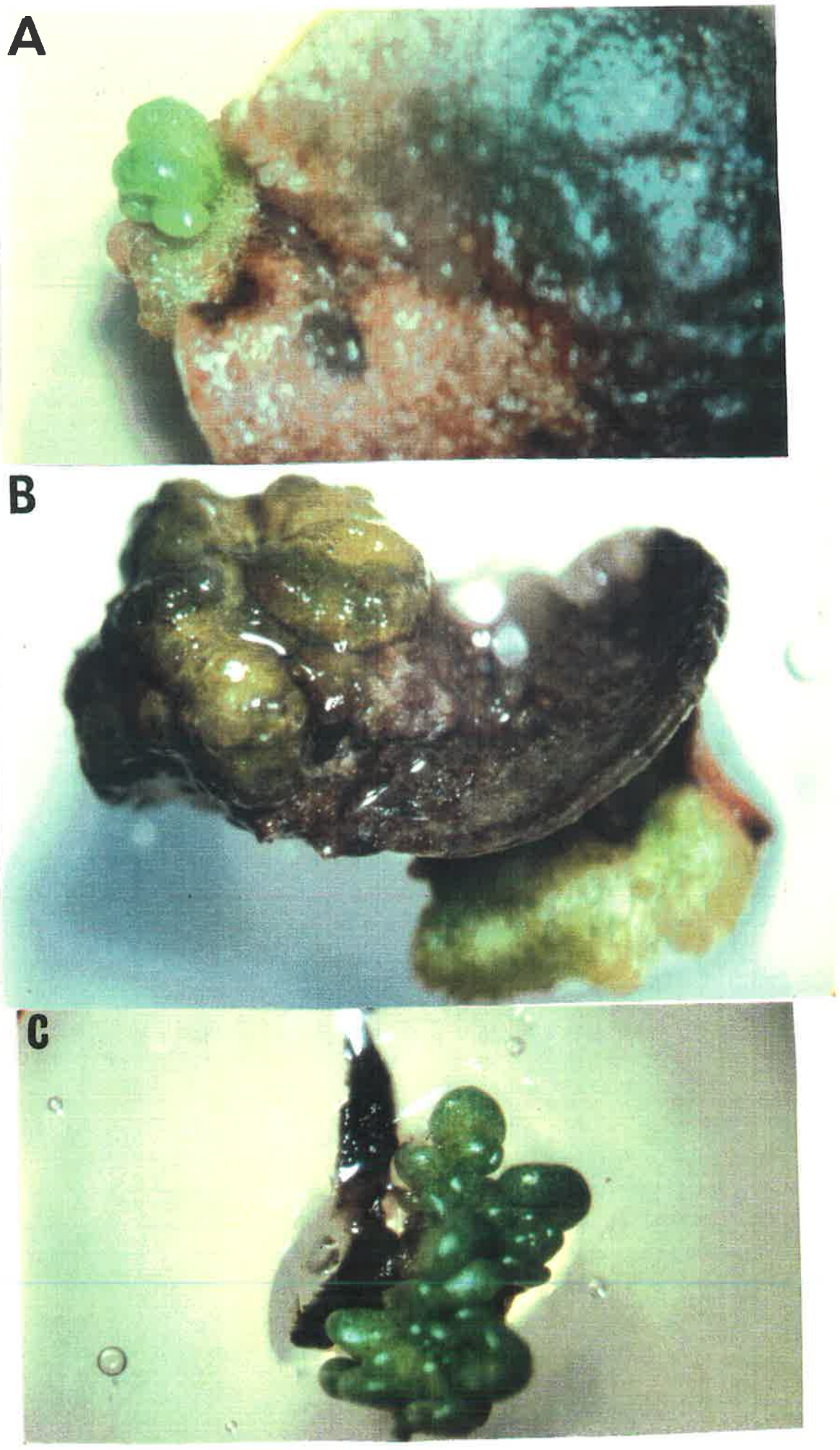
S=Shoots, G=Galls

Figure 5.3: Shoot regeneration from inoculated *V. grandis* leaf discs. (Magnification 1.6x).

A: Shoot formation from the leaf petiole region of a leaf disc 3 weeks after inoculation with *A. rhizogenes* strain K596.

B: Callus and gall formation from a leaf disc inoculated with *A. rhiozgenes* strain K596.

C: Shoot proliferation from a leaf disc 6 weeks after inoculated with *A. tumefaciens* strain AL4404 containing pBI121.


A
B
C

A

6 WKS
ex *in-vitro*

B

C

5.2.3 Phenotype of *V. grandis* plants regenerated from leaf disc inoculation experiments

Shoots and galls produced from *Agrobacterium* inoculation of leaf discs, were subcultured on shoot proliferation medium (MS basal supplemented with 4.5μM BA) to obtain shoots of a suitable size for root induction. Explants were then transferred to root induction medium. Shoots which produced roots were out-planted to the glasshouse. The survival rate of plants once transferred to glasshouse conditions and phenotype of the plants are discussed below.

Table 5.3 shows the multiplication rate over 2 subculture periods each of 4 weeks (number of shoots produced) and the percentage of these shoots which developed roots. Overall, multiplication rates for the inoculated treatments were similar to those of control plants. The percentage of shoots which developed roots for all treatments was lower than the control treatment, except for strain K568. Of interest, root growth of K568 explants was profuse with numerous lateral roots and aerial roots formed at the base of K596 explants (Figure 5.4). However, due to the aplagiotropic growth of these aerial roots it was not possible to out-plant any of these explants. These roots did not reposition themselves towards the soil and consequently these plants died.

Figure 5.5 shows examples of abnormal shoot characteristics of plants regenerated from leaf discs inoculated with *A. rhizogenes*. Regenerated control plants were all similar, whereas some explants from strain K596 displayed shoot growth with numerous lateral branches. Figure 5.5 (A) compares a control plant and a K596 transformant 6 weeks after transfer to the glasshouse. The control plant shows the characteristic apical dominant growth habit of *V. grandis* plants, whereas the K596 plant has numerous lateral shoots and is stunted.

Table 5.4 shows the percentage survival of *V. grandis* (Clone 865) explants at 20 weeks and one year after transfer to the glasshouse. The survival rate at 20 weeks for control plants was poor (15%). This is consistent with outplanting experiments described with

Clone 865 in Chapter 4. This represents a lower survival rate compared to treated plants K565 (40%), K597 (30%) and K568 (60%). With the exception of strain K568 (40% survival) and one K597 plant, all transformed and control plants had died within one year. After 20 weeks of growth, phenotypic alterations in leaf morphology (such as cupped leaves) were observed in K597 regenerated plants (Figure 5.5 B and C). Control plants did not show this leaf characteristic.

Table 5.3: Shoot proliferation of *V. grandis* Clone 865 and the percentage of shoots which formed roots, from leaf discs inoculated with *A. rhizogenes*.

Units = No. of shoots.

Treatment	Units at start	No. of units produced		
		1st sub	2nd sub	Rooting %
Control	67	80	300	100, n=20
K596	45	38	117	71, n=17
K565	27	95	181	59, n=78
K597	4	8	40	90, n=33
K568	1	2	10	100, n=10

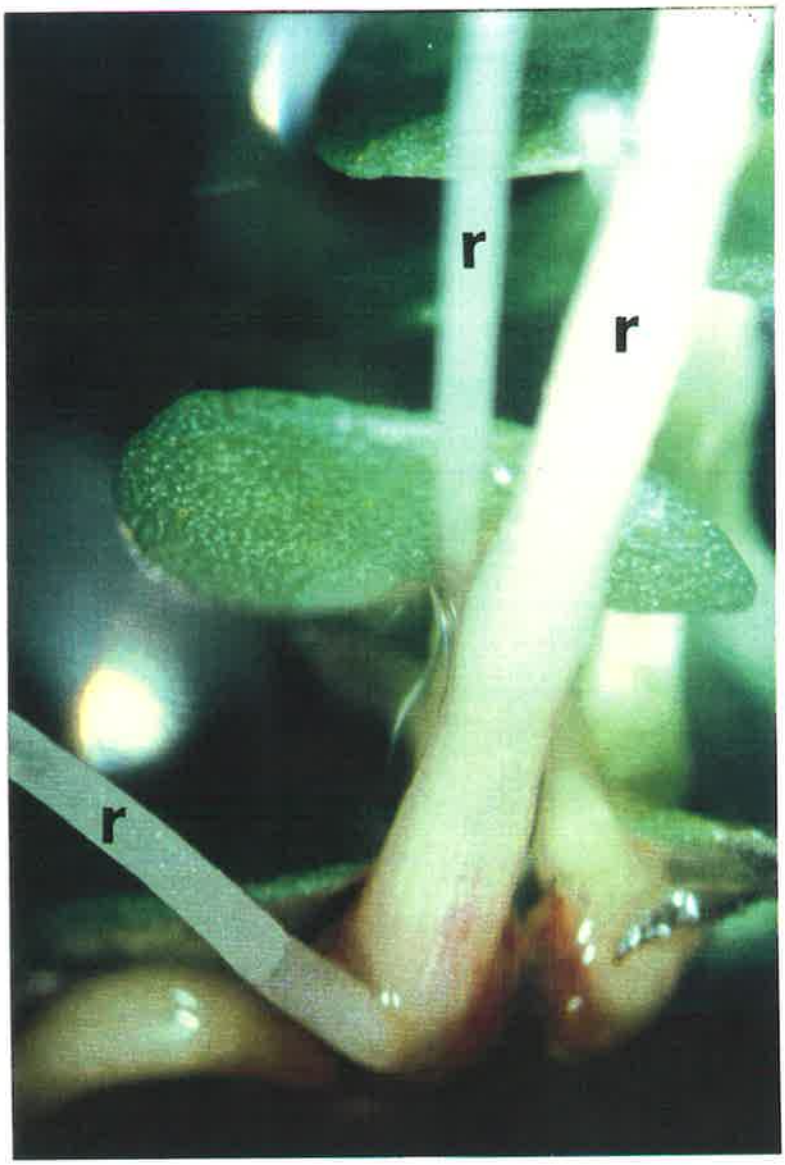


Figure 5.4: A shoot regenerated from a leaf disc inoculated with *A. rhizogenes* strain K596, showing aerial root formation after transfer to root induction medium. (r) indicates roots.

Figure 5.5: Phenotypic characteristics of plants regenerated from leaf discs inoculated with wild type *A. rhizogenes* strains.

A: A comparison of growth of a control plant and a regenerated K596 transformant after 6 weeks growth in the glasshouse.

B: Cupped leaf characteristics of K597 plants regenerated from leaf discs.

C: Examples of K597 plants and a control plant 10 weeks after transfer to glasshouse conditions.

Table 5.4: Survival of *V. grandis* Clone 865 plants after 20 weeks and 1 year in pots.

Treatment	No. planted	% survival	
		20 weeks	1 year
Control	20	15	0
K565	78	40	0
K596	17	6	0
K597	30	30	3.3
K568	10	60	40

5.2.4 The toxicity of antibiotics to *V. grandis*

An experiment was designed to test the toxicity of four antibiotics available as selection markers for *Agrobacteria* vector systems on *V. grandis* growth. The antibiotics, kanamycin, phosphinotricin, methatexate and hygromycin were added to shoot proliferation and root induction media (see Section 2.1) at varying concentrations (see Section 2.5.3). The most suitable antibiotic and concentration for selection purposes was determined after 6 weeks.

The antibiotics phosphinotricin (0.01mg/l), methatexate (0.5mg/l) and hygromycin (25mg/l) were extremely toxic to *V. grandis* tissue. At the lowest concentrations stated in brackets, *V. grandis* shoots died within 2 weeks. In contrast, kanamycin only inhibited *V. grandis* growth without causing death of tissue. Twenty five (mg/l) of kanamycin completely inhibited root growth of *V. grandis* explants *in vitro* (Figure 5.6 A). An example of root development on a putative *V. grandis* transformant on root induction medium with 25mg/l kanamycin is shown in (Figure 5.6 B). Leaf discs exposed to kanamycin at 25 mg/l produced a small percentage of escapes (5%). However these shoots died upon transfer to fresh medium containing kanamycin (25mg/l). Shoot induction of leaf discs was totally inhibited with 50mg/l kanamycin. Shoot growth was only suppressed with high levels of kanamycin 200mg/l. As a result of these experiments, kanamycin resistance was used as a selection marker in subsequent experiments.

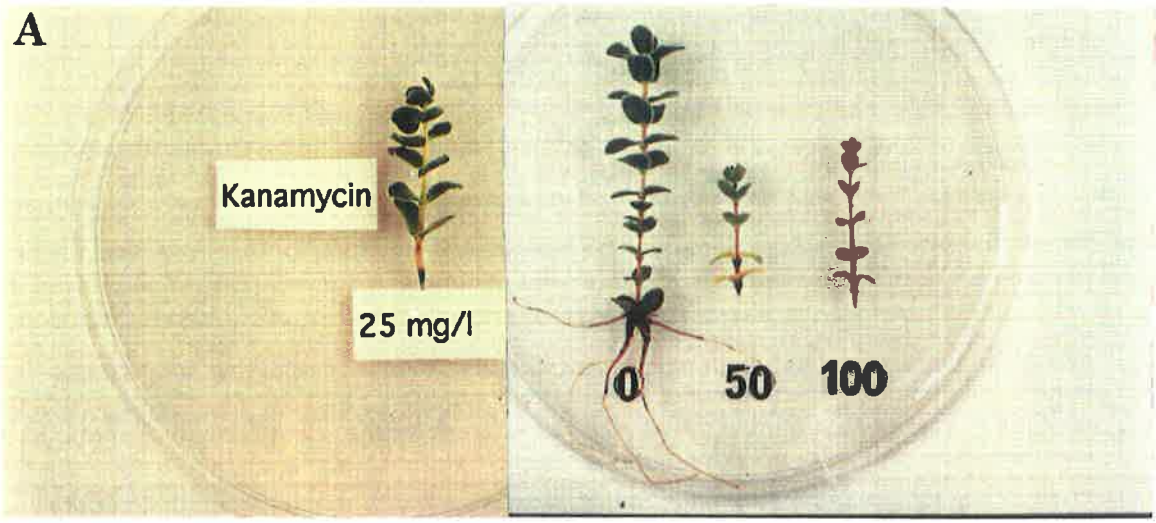
5.2.5 Selection of transformed *V. grandis* plants

The binary vector pBI121 was introduced into four virulent *A. rhizogenes* strains and leaf discs of *V. grandis* Clone 866 were inoculated with one of the *Agrobacterium.* Fifty discs were inoculated per treatment. One week after inoculation, the leaf discs were transferred to shoot proliferation medium containing 25mg/l kanamycin. The number and percentage of leaf discs forming shoots or callus tissue was recorded after 6 and 10 weeks. Each clone (ie individual leaf disc which formed a shoot) was kept as a separate line. The lines were then multiplied for a number of subcultures to obtain a sufficient number and height of shoots for root induction. The number and percentage of shoots which formed roots on root induction medium with 25mg/l kanamycin was recorded after 6 weeks. These plants were transferred back onto multiplication medium containing 200mg/l kanamycin to induce shoot proliferation for further analysis (see below).

Figure 5.6: Growth of *V. grandis* explants on kanamycin selection medium.

A: The effect of kanamycin (0, 25, 50 and 100mg/l) on root production of *V. grandis* explants on root induction medium (1/2 MS supplemented with 10μM IBA and kanamycin).

B: An example of a plant (inoculated with *A. tumefaciens* strain AL4404 (pBI121) which regenerated and rooted on 1/2 MS medium supplemented with 10μM IBA and 25 mg/l kanamycin.


A
Kanamycin
25 mg/l
0
50
100

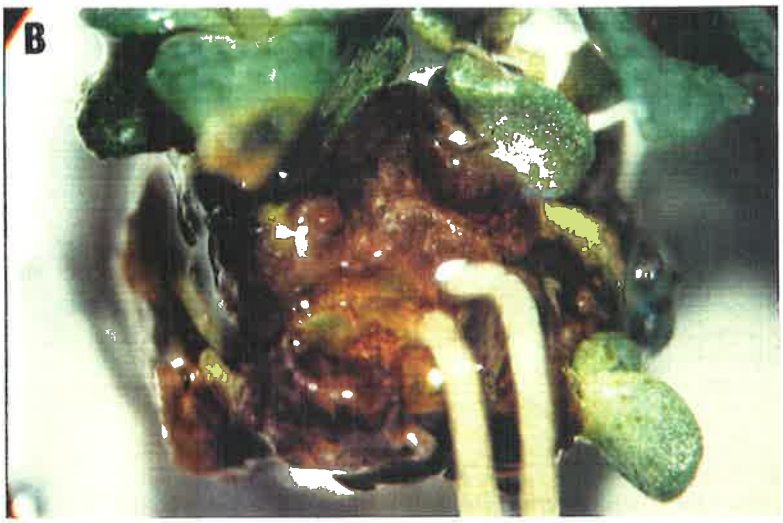

B

Table 5.5 shows the percentage of shoots and callus produced on inoculated leaf discs after selection on shoot proliferation medium containing kanamycin (25mg/l). Control leaf discs did not produce shoots on the selection medium. Six percent of control leaves produced callus. However the callus died following transfer to fresh selection medium. The percentage of leaf discs which produced shoots for the four strains varied from 4% (K597) to 12% (K568) and 16% for both strains K565 and K596. Attempts to regenerate callus tissue on a number of media failed (MS basal alone or supplemented with 4.5 or 9μM BA or 4.5μM BA with 1.5μM IAA). Regenerated shoots appeared normal.

Table 5.5: *V. grandis* Clone 866 inoculated with 4 *A. rhizogenes* strains containing the pBI121 plasmid (binary vector).

The number and percentage of shoots and callus produced after 6 and 10 weeks. Inoculated leaf discs were transferred to selection medium (25mg/l kanamycin) after 1 week. Fifty replicate discs were used for each treatment.

Treatment	No. of discs forming shoots (% response)		No. of discs producing callus (% response)	
	6wks	10wks	6wks	10wks
Control	0(0)	0(0)	3(6)	0(0)
K565+pBI121	7(14)	8(16)	6(12)	12(24)
K596+pBI121	17(34)	8(16)	1(2)	0(0)
K568+pBI121	5(10)	6(12)	1(2)	0(0)
K597+pBI121	4(8)	2(4)	0(0)	0(0)

Table 5.6: Subculture of shoots regenerated from leaf discs inoculated with *A. rhizogenes* strains containing pBI121.

Subculture rate over 2 years for the inoculated clones. The number and percentage of shoots which produced roots on selection medium are shown.

Strain containing pBI121	No. of sub-cultures	Total No. of explants at start	Shoots transferred to rooting medium	Rooted plants obtained(%)	No. (%) of Plants surviving reselection
K565	22	1160	778	202(26)	34(16)
K596	22	1865	1140	208(18)	30(14)
K568	22	565	407	55(14)	7(13)
K597	22	177	50	11(22)	4(36)

Table 5.6 shows the results of the subculture of potentially transformed plants over a 2 year period. The percentage of kanamycin tolerant plants which rooted on selection medium ranged from 14-26%. No abnormal root phenotypes were observed in any of these plants. The overall percentage of kanamycin tolerant plants decreased for all strains except K597 (increase from 22 to 36%), when rooted plants were placed back onto shoot proliferation selection medium (200mg/l kanamycin).

5.2.6 Selectable marker genes in disarmed *A. tumefaciens* host strains

To test the effectiveness of a disarmed *A. tumefaciens* strain in *V. grandis* transformation experiments, two plasmid vectors were introduced into strain AL4404 to aid in the selection and screening of potential transformants. Leaf discs were inoculated with this strain carrying either the cointegrating vector plasmid pGV3850 or the binary vector pBI121. No selection pressure was placed on shoot growth. Plants were selected for their ability to form roots on root induction medium with or without kanamycin (25mg/l).

Table 5.7 shows the response of *V. grandis* (Clone 865) leaf discs to inoculation with the two vector constructs. The percentage of leaf discs forming shoots was highest for the control treatment (64%), followed by pBI121 (34%) and pGV3850 (24%). Root induction medium without kanamycin produced 83-90% rooting of shoots for all treatments. Control shoots did not produce roots on root induction medium containing kanamycin. Only a small percentage of regenerated shoots from the *Agrobacterium* treatments produced roots, 9 and 7% for pBI121 and pGV3850, respectively. ß-glucuronidase assays revealed low level expression in rooted pBI121 clones and nopaline was not detected in any of the rooted pGV3850 clones (data not shown). A nodal segment of each plant which rooted on selection medium was placed on multiplication medium to induce shoot growth for further analysis (see below). Plantlets were then out-planted to the glasshouse. However, the health of these plants was poor. Growth was woody and all plants died within 6 weeks.

Table 5.7: Response of *V. grandis* Clone 865 leaf discs to inoculation with disarmed *Agrobacterium tumefaciens* strain AL4404.

Results show the mean percentage of Clone 865 leaf discs forming shoots after 10weeks, and the percentage of these shoots which produced roots after 20 weeks. The root induction medium (1/2MS + 10µM IBA) contained 25mg/l kanamycin. Two hundred leaf discs were inoculated per treatment.

Treatment	Percentage Response		
	Shoots produced	Roots produced	
	No kanamycin	No kanamycin	with kanamycin
Control	64	90	0
pBI121	34	88	9
pGV3850:	24	83	7

5.2.7 Comparison of *A. tumefaciens* strains for *V. grandis* transformation

An experiment was performed to test the effectiveness of two *A. tumefaciens* strains for *V. grandis* transformation. A leaf disc inoculation experiment was designed using *V. grandis* Clone 866 and tobacco leaves as a control. The binary vector pBI121 was transferred to two disarmed *A. tumefaciens* strains; AL4404 and EHA101. Early transformation events were monitored by transient GUS expression assayed one week after inoculation. Leaf discs were then placed onto shoot proliferation medium with kanamycin selection at 25mg/l. The number and percentage of leaf discs forming shoots was recorded after 6 and 10 weeks. Histochemical GUS expression was assayed in the 10 week old shoots.

Table 5.8 shows the number and percentage of leaf discs forming shoots on selection medium after 6 and 10 weeks. A small percentage (5%) of *V. grandis* control shoots escaped selection but were poor in growth and died upon transfer to fresh kanamycin selection medium (week 10). No GUS expression was detected in control *V. grandis* or tobacco shoots. Some tobacco shoots (4-7%) also escaped selection during this period. Five percent of *V. grandis* and forty four percent of tobacco shoots regenerated from leaf discs inoculation with AL4404 (pBI121) were actively growing on the selection medium after 10 weeks. Strain EHA101 was less effective than AL4404 for transformation of both *V. grandis* and tobacco leaf discs. Figure 5.3 (C) shows an example of a *V. grandis* leaf disc with shoot regeneration on selection medium 6 weeks after inoculation with strain AL4404 containing the binary vector pBI121.

Transient GUS expression was only detected in plants inoculated with strain AL4404. This is shown in (Figure 5.7 A) and depicts transient GUS expression in *V. grandis* leaf discs one week after inoculation. Note that GUS expression is located on and around the inoculation site (leaf petiole area). GUS expression was also detected in *V. grandis* (strain AL4404) and tobacco (strains AL4404 and EHA101) shoots after 10 weeks growth.

Blue colouration can be seen throughout leaf and stem tissue especially around the vascular elements as is shown in (Figure 5.7 B). No GUS expression was detected in the control plants.

Table 5.8: Comparison of transformation efficiencies with disarmed *A. tumefaciens* strains AL4404 and EHA101 as hosts for the binary vector plasmid pBI121.

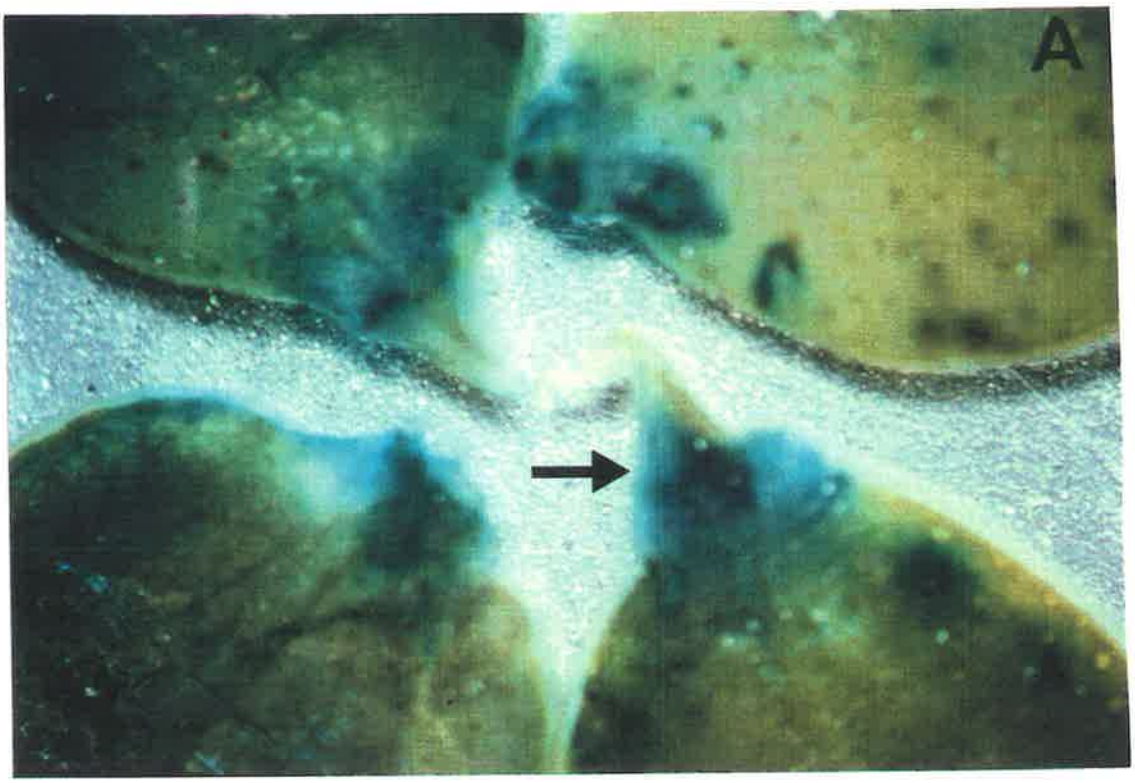
Results show the percentage of leaf discs producing shoots on selection medium (25mg/l kanamycin). Histochemical expression of GUS was determined 1 and 10 weeks after inoculation.

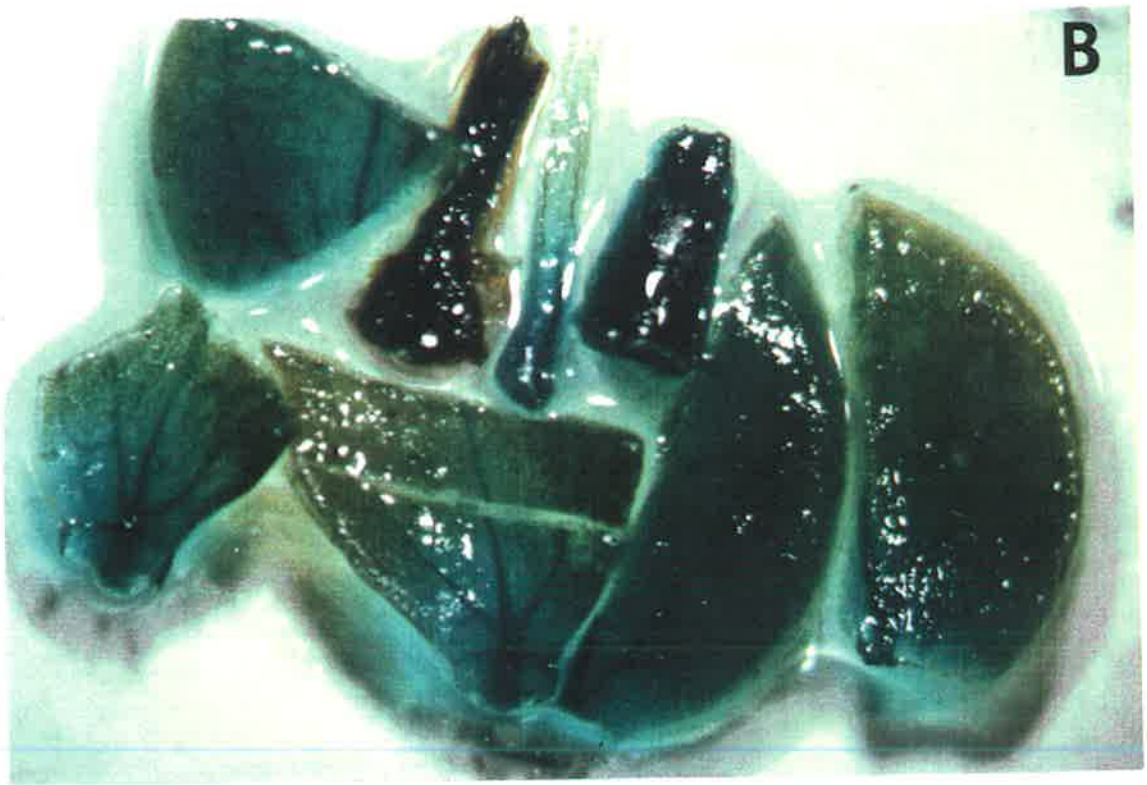
Treatment *A. tumefaciens* host	No. treated	% of discs forming shoots after		GUS expression detected after	
		6 weeks	10 weeks	1 week	10 weeks
Control					
866	150	5	0	none	none
Tobacco	70	7	4	none	none
AL4404					
866	200	8	5	positive	positive
Tobacco	50	44	44	positive	positive
EHA101					
866	94	8	0	none	none
Tobacco	30	13	13	none	positive

Figure 5.7: Histochemical GUS expression of *V. grandis* explants inoculated with the disarmed *A. tumefaciens* strain AL4404 harbouring the binary vector plasmid pBI121.

A: Transient GUS expression detected one week after leaf discs were inoculated. Note that GUS is expressed on and around the inoculation site. The arrow points to one inoculation site.

B: GUS expression in regenerated plants. GUS stained blue tissue is expressed in leaf and stem tissue.


A


B

5.2.8 Shoot proliferation and root induction of explants on kanamycin selection medium

In order to eliminate any possible *V. grandis* escapes, shoots were multiplied and selected for their ability to form roots in the presence of kanamycin. *V. grandis* plants, from transformation experiments with the strain AL4404 containing the vector pBI121 or pGV3850, were multiplied on shoot proliferation medium with 200mg/l kanamycin over a number of subcultures. Shoots were transferred to root induction medium containing 25mg/l kanamycin. At least 0.5 gms of fresh weight leaf tissue was required for extraction of DNA for analysing plants by PCR and Southern hybridisation (see below). Therefore shoots which had developed roots were transferred to shoot proliferation medium with kanamycin selection to induce shoot multiplication.

5.2.9 Transformation frequencies for Clones 865 and 866

Table 5.9 shows the combined data from the transformation experiments with Clones 865 and 866 inoculated with AL4404 harbouring the binary vector pBI121. Results obtained in Chapter 3 suggest that Clone 866 would be more applicable for transformation studies due to both its regenerative ability and ease of out-planting in comparison to Clone 865. The results shown in Table 5.9 confirm this hypothesis. Transformation experiments with Clone 866 using the binary vector pBI121 resulted in the highest percentage (32%) of plantlets which produced roots on kanamycin selection medium. Only 13% of Clone 865 plantlets produced roots. Twelve percent of explants of Clone 865 from the inoculation experiment with the vector PGV3850 produced roots. Analysis of these plants is discussed below.

Table 5.9: Subculture of *V. grandis* plants from transformation experiments with pBI121 and pGV3850.

Shoot proliferation of *V. grandis* Clone 865 or 866 over a number of subcultures and the number and percentage of these shoots which rooted on selection medium (kanamycin 25mg/l).

Host	Vector	*V. grandis* Clone	Total number of shoots (No. of subcultures)	Shoots transferred to rooting medium	Rooted plants obtained (%)
AL4404	**pBI121**	**865**	337 (10)	211	28 (13)
AL4404	**pBI121**	**866**	572 (13)	274	87 (32)
AL4404	**PGV3850**	**865**	631 (17)	383	46(12)

5.2.10 Histochemical analysis of GUS expression in kanamycin tolerant plants

To determine whether kanamycin resistant *V. grandis* plants also expressed GUS, histochemical GUS assays (Jefferson *et al.* 1987) were performed on *V. grandis* plants from transformation experiments. The assay was conducted on freehand sections of *V. grandis* stem and leaf tissue using the methods described Sections 2.6.2.

The results indicate that all vectors tested gave similar localisation of GUS expression. Figure 5.8 shows the histochemical localisation of GUS expression in free hand sections of kanamycin resistant *V. grandis* explants. Blue staining was not detected in any control tissue (Figure 5.8: A). Transverse sections through stem tissue revealed blue cells in every tissue layer except dead tissue of sclerenchyma and xylem fibres (B). Transverse sections of leaf tissue revealed blue areas throughout the leaf (B and C). Intense blue staining was also observed in leaf mesophyll cells (Fig D).

A Comparison of histochemical GUS expression in two *Agrobacterium* host vectors

Two vectors, pBI121 and pKiwi in the disarmed *A. tumefaciens* strain AL4404 , were used in leaf disc inoculation experiments with *V. grandis* and tobacco plants. The pKiwi vector was chosen because GUS activity is not expressed in bacteria. The efficiency of the initial gene transfer process was investigated by determining GUS expression one week after inoculation. Leaf discs where then transferred to shoot proliferation medium containing antibiotic selection (see Section 2.5.2). After 10 weeks, regenerated shoots were assayed for GUS expression.

Comparisons of GUS-expression between the vectors, revealed that all *V. grandis* and tobacco explants transformed with pBI121 showed blue staining on and around the inoculation site. Only 50% of *V. grandis* and 60% of tobacco leaf discs transformed with the pKiwi vector showed blue staining (Table 5.10). Transformation frequencies, as determined by the percentage of leaf disc that regenerated shoots, were similar for both vectors, 20% and 100% for *V. grandis* and tobacco plants, respectively (Table 5.10).

Figure 5.8: Histochemical localisation of GUS expression in free hand sections of kanamycin resistant *V. grandis* explants regenerated from leaf discs inoculated with A. tumefaciens strain AL4404 harbouring the vector pBI121 (A, B, C and D) or pKiwi (E). Bars indicate 100μM.

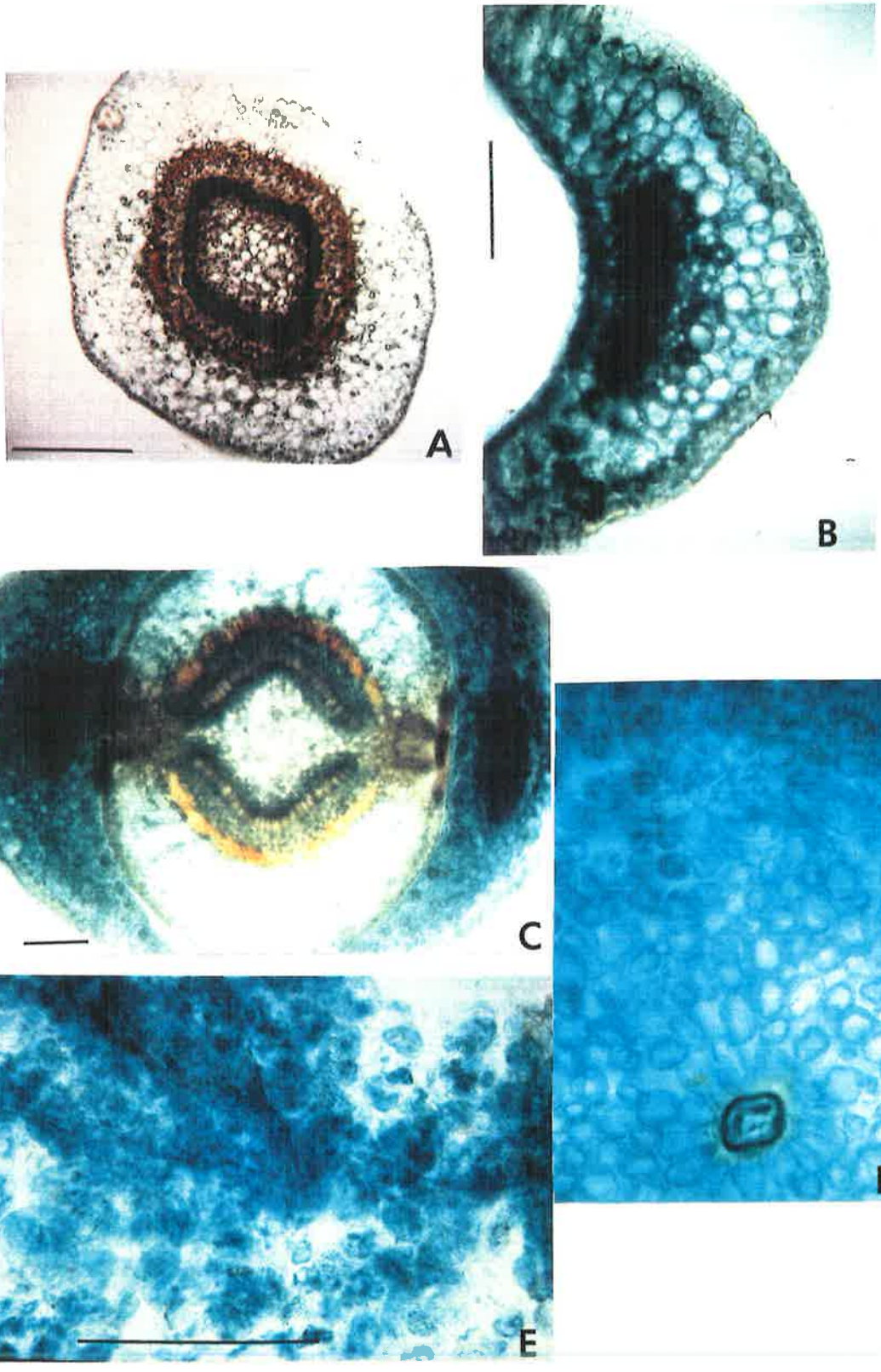
A: Transverse section of control *V. grandis* stem tissue. No blue staining is evident in any cells.

B: Transverse section of *V. grandis* leaf tissue. Blue staining is evident in all tissue.

C: Transverse section of a stem with two lateral leaves. Blue staining can be seen in all cells except for sclerenchyma and xylem fibres.

D: Higher magnification of mesophyll cells in a blue staining section of a leaf. GUS enzyme activity is concentrated in the chloroplasts.

E: A section through callus tissue of a pKiwi transformant (see Section 5.3.5 (A)).


A
B
C
D
E

GUS expression was detected in all of the regenerated shoots tested except for controls. Callus cells sectioned from the base of *V. grandis* (pKiwi) explant (Figure 5.8 E) shows intense blue colouration.

Table 5.10: GUS expression in *V. grandis* and Tobacco leaf discs

The percentage of plants which showed GUS-expression in the (A) leaf discs, (one week after inoculation) and in (B) shoots regenerated from leaf discs, (ten weeks after inoculation). One hundred *V. grandis* and twenty tobacco leaf discs were inoculated for each treatment. Only a small number of leaf discs (10 *V. grandis* and 5 tobacco) were tested for transient GUS expression one week after inoculation. A small portion of regenerated shoot tissue was removed and assayed for GUS expression after 10 weeks.

Treatment	No. tested	% positive for GUS expression (A) leaf discs	(B) shoots	% of regenerating leaf discs
Control				
866	10	0	0	0
Tobacco	5	0	0	0
pBI121				
866	10	100	100	20
Tobacco	5	20	100	100
pKiwi				
866	10	50	100	20
Tobacco	5	60	100	100

B Detection of endophytic bacteria within *V. grandis* transformants

The GUS expression seen in putative transgenic shoots may have resulted from endophytic bacteria. This possibility was examined by a histochemical GUS assay (see Section 2.6.2) performed on various kanamycin resistant explants which had previously been shown to be positive for GUS expression. At the time of the assay, leaf and stem material was used for indexing plants for bacterial growth (described in detail in Section 2.6.4). Treatments tested were *Agrobacterium* strain AL4404 carrying the vectors pBI121 or pKiwi; pBI121 plasmid only; nine putative *V. grandis* transformants from inoculation experiments with AL4404 (pBI121), and one *V. grandis* K596 (pBI121) transformant. All plantlets had produced roots on kanamycin selection medium and were previously positive for GUS-expression. Untransformed *V. grandis* tissue was used as a control.

No bacteria were cultured from any of the *V. grandis* tissue using the indexing technique. Further, the plasmid preparation of pBI121 did not contain any contaminating bacteria. Blue colouration was detected following GUS assay for the *Agrobacterium* vector AL4404 (pBI121). Blue colouration was not observed in AL4404 (pKiwi). Eight out of the nine AL4404 (pBI121) and the one K596 (pBI121) *V. grandis* transformants showed blue colouration in the histochemical GUS assays, whereas no blue colouration was evident in the control *V. grandis* tissue.

5.2.11 Molecular analysis of putative *V. grandis* transformants

A Isolation of DNA from *V. grandis*.

Molecular evidence of putative *V. grandis* transformants is necessary to confirm the integration of foreign DNA in the plant genome. PCR and Southern hybridisation techniques were employed for this purpose. These techniques require clean, undegraded DNA. Southern hybridisation and PCR procedures are described in detail in Sections 2.8 and 2.9, respectively. Leaf tissue from healthy actively growing shoot explants, *in vitro*

was used for extraction of DNA. The DNA extraction methods employed are described in detail in Section 2.7.

A number of methods were tested to obtain DNA of sufficient quantity and purity for PCR and for Southern analysis:

i) the phenol/chloroform method of Guidet *et al.* (1991);

ii) the CTAB method of Doyle and Doyle (1988);

iii) DNA extraction from Eucalypts (Bernatsky, pers. comm.);

Small amounts of DNA, of varying quality were extracted using either the phenol/chloroform method of Guidet *et al.* (1991) or the CTAB, method of Doyle and Doyle (1988). These methods produced low yields of DNA which would not cut with restriction endonucleases. Furthermore, the DNA solutions were quite viscous due to the presence of contaminating polysaccharides. It was assumed that the polysaccharides inhibited the activity of restriction endonucleases and interfered with determination of the concentration of DNA in the samples. Purification of DNA samples by the "Geneclean" method (Geneclean II Bio 101, No. 3106) or with CsCl gradients (Maniatis *et al.* 1982) were effective in removing the polysaccharides. However, only small quantities of DNA were recovered with either method. Both of these methods were time consuming and impractical for screening large numbers of samples. A satisfactory method adapted from Bernatsky (pers. comm.) for DNA extraction from Eucalypts (described in Section 2.7), gave good quantities (ca. 20µg/gm fresh weight of *V. grandis* leaves) of DNA. The DNA was further purified with an additional high salt precipitation (Fang *et al.* 1992). Examples of the quality of *V. grandis* leaf DNA preparations are shown in Figure 5.9 (A). It was possible to digest the DNA with a range of restriction endonucleases as depicted in Figure 5.9 (B).

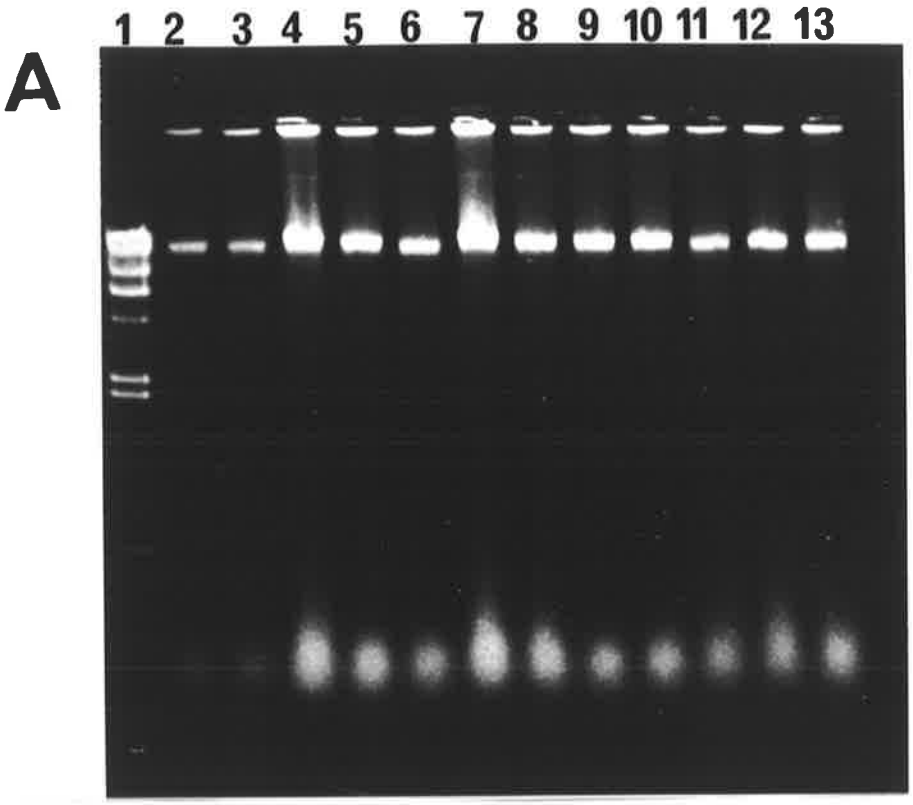
Figure 5.9: Quality of *V. grandis* leaf DNA preparations.

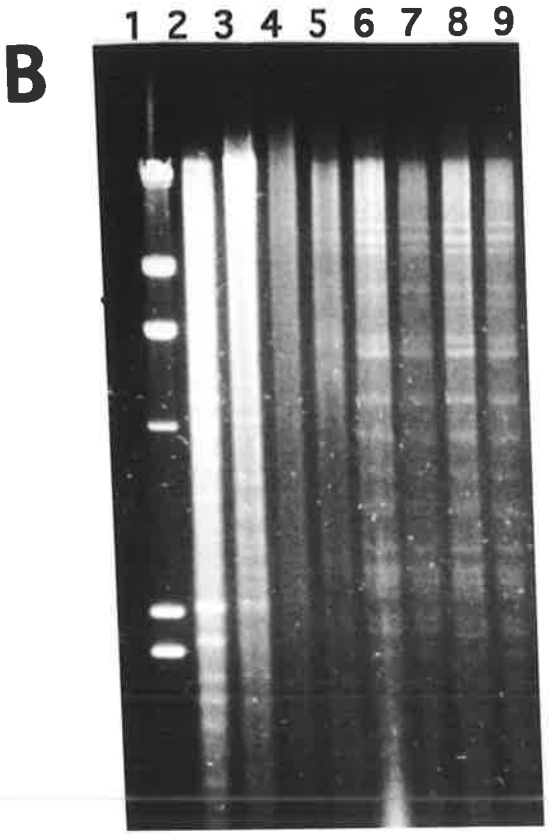
A: *V. grandis* leaf DNA samples electrophoresed on a 1% agarose gel, stained with ethidium bromide and visualised on a UV light box.

Lanes:
- 1 lamda Hind III marker
- 2-13 *V. grandis* leaf DNA

B: *V. grandis,* tobacco and barley leaf DNA digested with the restriction endonuclease BAM HI, electrophoresed on a 1% gel, stained with ethidium bromide and visualised on a UV light box. The quantity of DNA loaded per lane is given in the brackets.

Lanes:
- 1 lamda Hind III marker
- 2 barley (5µg)
- 3 tobacco transformed with AL4404 containing pBI121 (5µg)
- 4 tobacco control (2µg)
- 5 *Verticordia* control (2µg)
- 6-9 *Verticordia* transformed with AL4404 containing pBI121 (2µg).


A
1 2 3 4 5 6 7 8 9 10 11 12 13


B
1 2 3 4 5 6 7 8 9

B PCR and Southern blot analysis

DNA was isolated from shoots which had produced roots on kanamycin selection medium. The GUS gene was detected in kanamycin resistant *V. grandis* and tobacco plants by the PCR amplification method (see Section 2.9). Approximately 200ng of DNA was included in each PCR reaction and it was possible to screen large numbers of samples by this technique. The presence of the GUS gene in this tissue was confirmed by Southern hybridisation of the PCR product with a probe from within the GUS gene (see Section 2.8.2).

Figure 5.10 (A) represents PCR data of seventeen *V. grandis* transformants (lanes 2-18) and one tobacco transformant (lane 19). Untransformed *V. grandis* and tobacco DNA were included as negative controls (lanes 20 and 21, respectively). All plants from which DNA was extracted were tested for the presence of contaminating *Agrobacterium* by indexing plant material in a nutrient medium (see Section 2.6.3) but no bacteria grew from any of the cultures tested. A band corresponding to the expected GUS fragment of 514 bp was detected in all transformants, except lanes 9 and 10. However, a number of other bands of varying size were also detected.

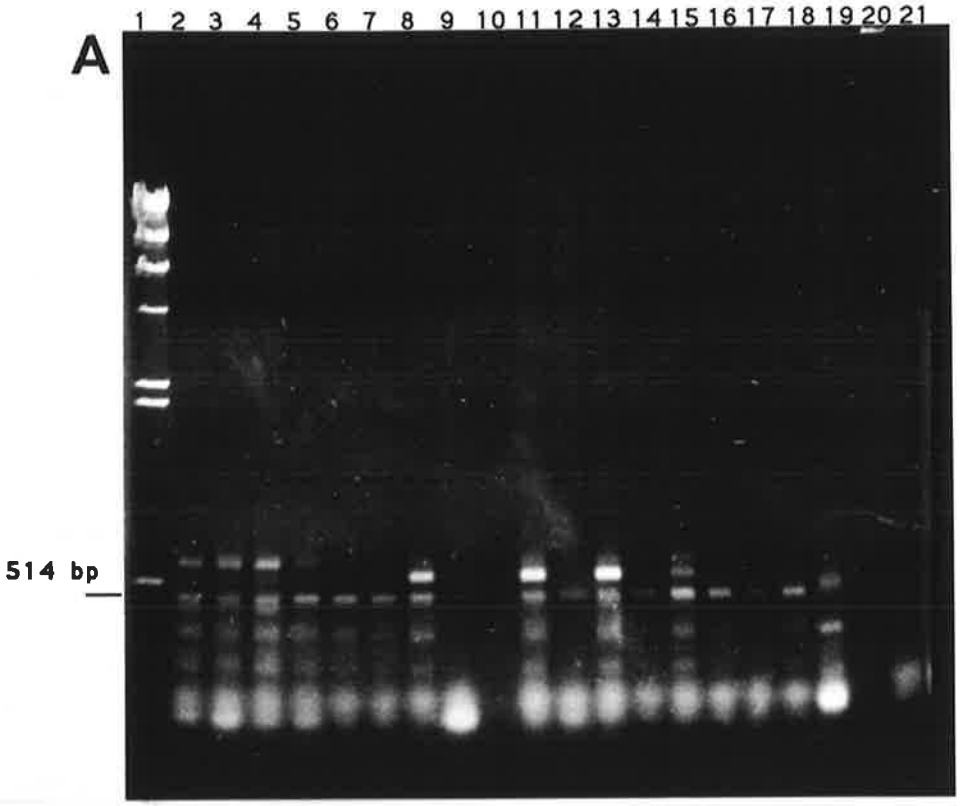
Figure 5.10 (B) shows the autoradiograph of the PCR gel depicted in (A) and probed with the GUS gene sequence. The presence of the 514 bp GUS fragment was confirmed by Southern hybridisation. The other bands observed in the PCR products did not hybridise to the GUS probe.

Figure 5.10: PCR analysis of kanamycin resistant putative transgenic plants.

A: PCR analysis of kanamycin resistant plants showing the presence of the expected 514bp DNA fragment, from the structural region of the GUS gene. A number of bands of varying size were also detected.

B: Hybridisation of the gel depicted in A, with a radiolabled sample of the 3kb GUS fragment isolated from pBI121, revealed only the presence of the expected 514bp fragment

Lanes:		
	1	lamda Hind III marker
	2-17	*V. grandis* transformants
	19	tobacco transformant
	20	*V. grandis* control
	21	tobacco control


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
514 bp
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
514 bp

To determine if the Southern hybridisation techniques employed during this study were adequate for detecting genes in *V. grandis* leaf DNA, two experiments were performed. Firstly, *V. grandis* and tobacco DNA were hybridised with a radiolabelled probe for the ribosomal, RNA genes. Figure 5.11 (A and B) shows autoradiograms of *V. grandis* and tobacco leaf DNA digested with (A) Hind III and (B) Eco R1+Pst 1 and hybridised to the radiolabelled ribosomal probe. As expected strong homology between the probe and plant DNA was detected. Secondly, it was important to show that other genes with low copy numbers could be detected in DNA from *V. grandis*. To test this an α-amylase cDNA clone from barley was used to probe *V. grandis* leaf DNA. This probe should detect low copies of α-amylase genes present in the *Verticordia* genome. Figure 5.11 (C) shows that *V. grandis* only weakly hybridised to the barley probe although a strong signal was found with barley DNA (lane 2).

Putative *V. grandis* transformants (shoots which produced roots on kanamycin medium, were positive for histochemical GUS expression and the GUS fragment was detected by PCR) were hybridised with GUS and NPT II radiolabelled probes (see Section 2.8). Examples of two autoradiograms, after hybridisation with these probes are shown in Figure 5.12 (A) and (B), respectively. Both autoradiograms contain DNA from 11 putative *V. grandis* transformants (lanes 1-12), control *V. grandis* (lane 13), tobacco control (lane 14), tobacco transformant (lane 15) and plasmid pBI121 (lane 16). The tobacco transformant showed strong homology with two fragments (2kb and 4kb) and the control plasmid pBI121 hybridised strongly with 2kb and 4.5kb fragments. One *V. grandis* clone, K568 (pBI121) showed 3 ECO R1 fragments (3, 5 and 15kb), indicated by an arrow. No hybridisation fragments were detected in either control tobacco or *V. grandis* DNA. Figure 5.12 (B) shows the same DNA samples after hybridisation with a radiolabelled NPT II probe. The *V. grandis* clone K568(pBI121), lane 3-as above, and an additional clone AL4404(pBI121) lane 8, produced a 3kb fragment (indicated with an arrow).

Figure 5.11: Southern hybridisation detecting ribosomal (A&B) and α -amylase (C) genes in *V. grandis*, tobacco and barley leaf DNA. Lamda DNA was the size marker.

A: *V. grandis* and tobacco leaf DNA was digested with the restriction endonuclease Eco R1 and Pst1

Lanes:		
	1	tobacco transformant
	2	tobacco control
	3	*V. grandis* control
	4-17	*V. grandis* transformants

B Autogradiogram of *V. grandis* and tobacco leaf DNA digested with Hind III and hybridised with a radiolabelled ribosomal probe.

Lanes:		
	1&5-10	*V. grandis* transformants
	2&4	*V. grandis* controls
	3	tobacco control
	11-12	tobacco transformants

C Autoradiogram of the gel depicted in Figure 5.9(B), after hybridisation with the α-amylase gene. The probe, α-amylase is a 800bp PstI fragment in the plasmid pUC 18. The quantity of DNA loaded per well is given in the brackets.

Lanes:		
	1	lamda Hind III marker
	2	barley (5μg)
	3	tobacco transformant (5μg)
	4	tobacco control (2μg)
	5	*V. grandis* control (2μg)
	6-9	*V. grandis* transformants (2μg).

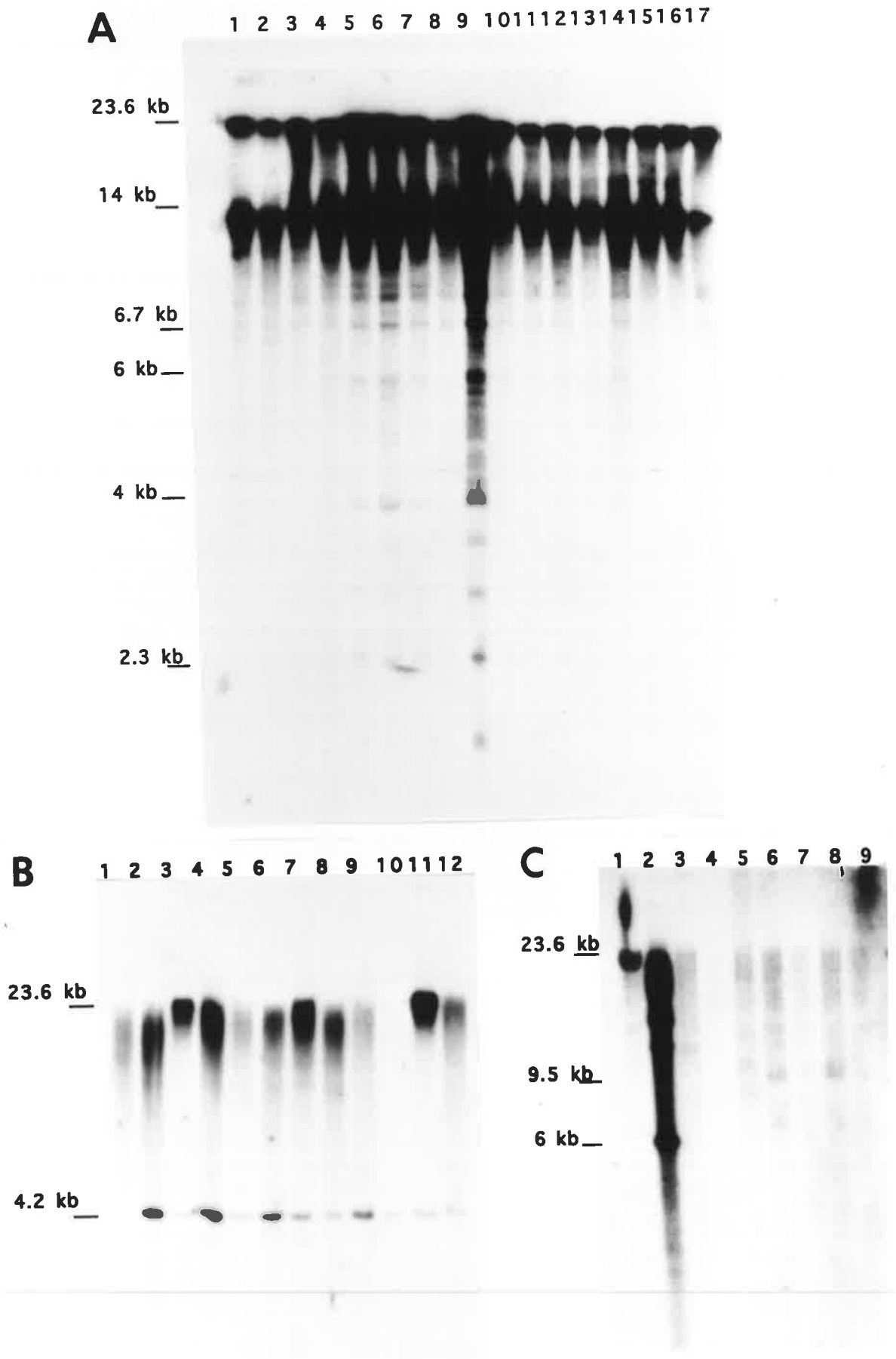
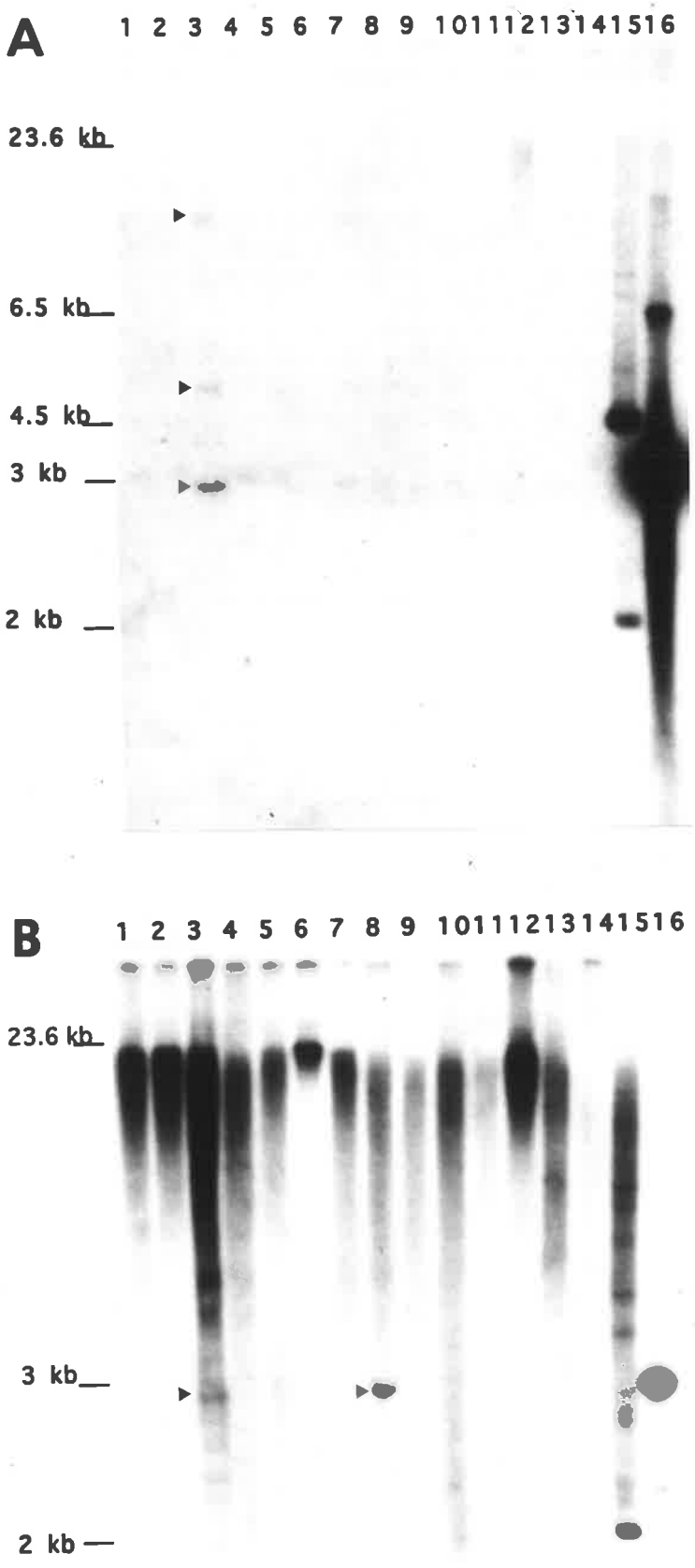

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
23.6 kb
14 kb
6.7 kb
6 kb
4 kb
2.3 kb
B
1 2 3 4 5 6 7 8 9 10 11 12
23.6 kb
4.2 kb
C
1 2 3 4 5 6 7 8 9
23.6 kb
9.5 kb
6 kb

Figure 5.12: Southern hybridisation detecting the GUS and NPT II fragments in tobacco and *V. grandis* leaf DNA (see text) transformed with various *Agrobacterium* vectors. The samples were digested with the restriction endonuclease Eco R1. Lamda Hind III was the size marker.

A: Samples were probed with a radiolabelled fragment of the GUS gene. Lane 3 shows three bands (3, 5 and 15kb GUS fragments).

B: Samples were probed with a radiolabelled fragment of the NPT II gene. *V. grandis* transformants K568(pBI121) and AL4404(pBI121), lanes 3 and 8, respectively, hybridised to a 3kb NPTII fragment.

Lanes:	1-12	*V. grandis* transformants
	13	*V. grandis* control
	14	tobacco control
	15	tobacco transformant
	16	plasmid (pBI121) control


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
23.6 kb
6.5 kb
4.5 kb
3 kb
2 kb
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
23.6 kb
3 kb
2 kb

High background hybridisation smears were seen along the tracks of autoradiograms after hybridisation with the NPT II probe (Figure 5.12, B). Nevertheless, the hybridisation bands present in the *V. grandis* clones (lanes 3 and 8), the tobacco clone (lane 15) and the plasmid control (lane 16) were not present in DNA from control *V. grandis* and tobacco DNA (lanes 13 and 14, respectively).

5.3 DISCUSSION

The results presented in this chapter indicate that: 1) *V. grandis* is susceptible to infection with wild-type *A. rhizogenes* as determined by gall formation and opine analysis; 2) wild type *A. rhizogenes* does not induce hairy root formation on *V. grandis* stem explants; 3) abnormal root and shoot phenotypes result from *Agrobacterium* infection and are expressed at various stages of plant development (root induction stage and 5 months after potting); 4) *V. grandis* can be transformed with *Agrobacterium* vectors.

The stages involved in developing a transformation system using *Agrobacterium* were investigated in this study. Firstly, it was important to show that *Agrobacterium* will infect *V. grandis*. Evidence of infection comes from the production of roots of abnormal morphology and gall formations induced on *V. grandis* stem explants after inoculation with wilt-type *A. rhizogenes*. It is important to note that none of the control plants produced galls by either inoculation method. Therefore, gall formation cannot be attributed to a wounding response. Furthermore, gall formation could not have been due to the presence of hormones in the medium as all explants were inoculated and left to grow on MS basal medium (see Appendix A, Table 1) without the addition of phytohormones. Further evidence of infection was obtained by the detection of opines in root and gall tissue following inoculation with all strains (except the cucumopine strain K597). Inoculation of explants with this strain resulted in gall formation in only 6% of explants. These results indicate that strain K597 is only poorly pathogenic on *V. grandis*. Opines were not detected in control *V. grandis* tissue.

Gall formation was highest in stems inoculated with strains K565 and K596. These are both agropine strains and have previously been reported to express high virulence when compared to the other strains (Phelep *et al.* 1991). The T-DNA of agropine strains contain both a TL and TR region. The TR region contains *aux* genes, which are absent in the other strains (see Chapter 1). These genes are involved in the synthesis of phytohormones, namely the auxin biosynthesis function. The abundant gall formation induced by these strains may therefore be the result of a phytohormone imbalance.

A characteristic of Ti and Ri induced galls and hairy roots is their ability to grow on medium in the absence of phytohormones (Tepfer 1984 and Gelvin 1990). In contrast, roots and galls induced after inoculation of *V. grandis* with any of the *A. rhizogenes* strains, used in this study, did not display phytohormone independent growth. Gall tissue eventually browned and died on MS medium. Indeed, it was not possible to obtain any growth or shoot differentiation of gall or root tissue using a number of shoot induction media. This contrasts with poplar and wild cherry, in which tumors induced by nopaline strains grew hormone-independently and spontaneously developed shoots which had a normal phenotype (Brasileiro *et al.* 1991). Other reports indicate that, with the addition of phytohormones, regeneration may be possible (Han *et al.* 1993). More work will need to be carried out to achieve the objective of regeneration of *V. grandis* callus, gall and root tissue.

The production of galls and roots of abnormal morphology provided evidence that *Agrobacterium* does infect *V. grandis*. However, *V. grandis* stem explants inoculated with *A. rhizogenes* strains did not show any of the characteristic "hairy root" formation reported in a number of herbaceous (Tepfer 1984) and Birot *et al. 1987*) and woody plants (Rugini *et al.* 1991, Phelep *et al.* 1991 and Han *et al* 1993), nor did it increase the rooting percentage of *V. grandis* explants. Furthermore, root development was phenotypically different when compared to root growth on control explants. This observation has been made previously in both *Vitis* and *Rubus sp* where thickening of roots and lack of geotropism were observed after *A. rhizogenes* inoculation (Hemstad and Reisch 1985).

Species-specific root abnormalities induced by *A. rhizogenes* may be due to differences in responsiveness to a particular gene product (e.g. auxin due to *aux* gene expression) or to species-specific differences in expression of the T-DNA genes on the Ri plasmid (Amselem and Tepfer 1992). The addition of auxin to the medium on which black locust explants were co-cultivated with *A. rhizogenes* R1601, increased the percentage of explants that produced hairy roots (Han *et al.* 1993). This supports the theory that hairy root formation is the result of an increased sensitivity to auxin conferred by the T-DNA (Shen *et al.* 1988 and Spano *et al.* 1988). Further studies are necessary to test this theory with *V. grandis* explants.

The leaf disc regeneration method developed in Chapter 3 was utilised in subsequent transformation experiments using *A. rhizogenes*. Shoot proliferation from leaf discs was possible on MS basal medium containing 4.5µM BA. Control discs regenerated shoots at a much higher frequency (70%) than inoculated discs (highest percentage was 31% for strain K596)-see Table 5.2. This suggests that infection with *A. rhizogenes* stimulated the synthesis of phytohormones thereby influencing shoot development.

Shoots regenerating from leaf discs inoculated with wild-type *A. rhizogenes* strains displayed abnormal shoot and root phenotypes. However, the abnormal phenotypic features were not expressed during early shoot growth, but became evident in later stages of development, on root induction medium and 20 weeks after potting. For example, regenerated shoots of strain K596, after transfer to root induction medium, produced numerous lateral shoots in addition to roots. Furthermore, aerial roots formed at the base of a number of these shoots. Phenotypic alterations in leaf morphology were observed (cupped leaves) in strain K597 transformed plants 20 weeks after potting (Plate 5.5 B and C). The cupped leaf characteristics reported in this study are unlike other leaf abnormalities previously reported. Control plants did not show any of these leaf characteristics. These abnormalities may have originated from genomic disturbance due to the insertion of foreign DNA, rather than from the expression of T-DNA genes in the transformants, as suggested by Han *et al.* (1993). Similarly, delayed abnormal leaf

morphology phenotypes were reported in transgenic black locust plants, and included asymmetrical leaflets, variegated leaves and reduced spine length (Han *et al.* 1993).

Unlike the phenotypes discussed above, it was interesting to note that plants transformed with strain K568 grew faster than other transformants and control plants. The survival rate of K568 plants (40%), 2 years after potting, may be related to the excellent rooting of these plants *in vitro* (100% with profuse lateral roots). However, this result requires further investigation since *V. grandis* Clone 865 was used for these experiments, which judging by the controls, has poor out-planting success (see Chapter 4). The poor outplanting results obtained with plants transformed with the other strains may be due to culture conditions. During this period there were problems in the glasshouse with vinegar fly infestation. The larvae live on root tissue and root systems were damaged, as a consequence shoot growth deteriorated and plants eventually died.

Only faint traces of opines were detected in shoot tissue regenerated from inoculated leaf discs. Opine concentration in gall tissue was higher. These results suggest that shoots regenerated from leaf discs without selection pressure contain both transgenic and non-transgenic cells. Meristematic tissues are known to yield chimeric transformants (Mukhopadhyay *et al.* 1992). Since it was not possible to regenerate transformed gall and root tissue, a system which selects for transgenic cells and subsequent growth of these cells was necessary. Therefore, subsequent experiments with the vector pBI121 facilitated early selection and screening of transformants on kanamycin medium.

The vector pBI121 contains the selection marker NPT II which confers kanamycin resistance to transformed cells. Kanamycin at 25 mg/l completely inhibited root growth of *V. grandis* explants. Transformation experiments without initial antibiotic selection of leaf discs, produced only a small percentage (9%) of regenerated shoots that produced roots on the selection medium. This compares with 14-26% of explants producing roots when antibiotic selection was applied after the co-cultivation period. These results indicate that antibiotic selection should be applied to leaf discs immediately after co-cultivation with

Agrobacterium. For selection at the leaf disc stage, it was necessary to transfer cultures every 2-3 weeks due to loss of kanamycin activity.

It is interesting to note that *V. grandis* transformants resulting from inoculation with *A. rhizogenes* carrying the binary vector pBI121 did not show any of the abnormal shoot or root characteristics observed in earlier experiments using wild-type *Agrobacterium* strains without the binary plasmid. Similar results were reported with black locust plants, where the addition of kanamycin after the co-cultivation period inhibited hairy root formation (Han *et al.* 1993). Furthermore, morphologically normal poplar shoots isolated from nopaline tumors on selection medium did not contain oncogenic T-DNA, but were shown to contain and to express the NPT II and GUS genes of the binary vector pBI121 (Brasileiro *et al.* 1991). Since binary vectors contain two independent regions of T-DNA, the T-DNA from the Ti or Ri plasmid and the binary plasmid, it is possible that some transformed cells will contain only one of the T-DNAs (Klee *et al.* 1987). Since the selective agent, kanamycin, was used for selecting transformants in this study, only the cells carrying the binary plasmid may have survived. Nonetheless, the only way to avoid the problem of abnormal phenotypes and regeneration difficulties due to the transfer of wild-type T-DNA hormone genes is to disarm the Ti plasmid. Subsequent transformation experiments with *V. grandis* utilised the disarmed *A. tumefaciens* strain AL4404.

Transformation experiments using the disarmed *A. tumefaciens* strain AL4404 containing pBI121, indicated that the two *V. grandis* Clones 865 and 866 differed in their response to infection, as determined by the percentage of plants which produced roots on kanamycin selection medium. Thirty two percent of Clone 866 putative transformants produced roots on the selection medium compared with only 13% of Clone 865 putative transformants. The rooting ability of *V. grandis* Clone 866 explants *in vitro,* was superior to that of Clone 865 (see Chapter 3). These results suggest that:

1) transformation efficiency may be related to the regenerative ability of *V. grandis*,

2) susceptibility to AL4404 is genotype dependent.

Such genotype specificity has been described for many different host species (Bush and Pueppke 1991). These authors demonstrated that genotype-strain specificity in *Chrysanthemum* was due to "differential transfer, integration or expression of T-DNA".

Results of histochemical GUS expression in kanamycin resistant plants indicate expression of the introduced gene in all living cells. Blue colouration was not seen in woody tissue eg, sclerenchyma and xylem fibres. Cell type specific expression of the CaMV 35S-GUS gene has been reported in transgenic soybean plants (Yang and Christou 1990). However, no evidence of cell type specific expression or of apparent chimeric effects were apparent in *V. grandis* tissue which expressed GUS in histochemical assays.

A variety of plants species express endogenous GUS activity (Hu *et al.* 1990 and Hodal *et al.* 1992), however GUS expression was not detected in control *V. grandis* tissue. Transient GUS expression in *Chrysanthemum* explants transformed with an GUS-intron gene showed distinct blue spots, whereas large amounts of a blue precipitate were found in explants transformed with the *Agrobacterium* vector A281 (pBI121) (Wordragen *et al.* 1992). The GUS gene in this vector is expressed in *Agrobacterium* and this can produce false positives in GUS assays (Tör *et al.* 1992). In the experiments described here higher levels of GUS expression were detected in leaf discs inoculated with pBI121 (100%) than with pKiwi (50%)-see Table 5.10. The pKiwi vector gives negligible bacterial GUS expression due to the lack of bacterial ribomes-binding sites (Putterill and Gardner 1989). Therefore, the higher levels of GUS expression detected with pBI121 inoculated leaf discs, were possibly due to bacterial GUS activity.

Importantly, endogenous bacteria were not detected in any kanamycin resistant *V. grandis* tissue tested (Section 5.3.5 B). Therefore, the blue colouration reported in these plants appears indicative of GUS gene transfer. In addition, all regenerated plants were kanamycin resistant, indicating that the NPT II gene was stably expressed. However, histochemical GUS expression was only detected in some of the kanamycin resistant plants (7-86%) and varied with respect to the *Agrobacterium* strain used for transformation

(see Table 5.11). Similar results have been reported in *Chrysanthemum* (Wordragen *et al.* 1991) and potato (Ottaviani and Hanisch ten Cate 1991) transformants.

In addition to biochemical analysis, evidence of transformation should include molecular studies. A summary of the results obtained from transformation experiments conducted throughout this study are presented in Table 5.11, showing the combined data of all transformation experiments. Results present the number and percentage of kanamycin resistant *V. grandis* clones which tested positive for GUS histochemical assays, PCR amplification of a GUS fragment and Southern blot hybridisation with GUS and NPT II probes.

The PCR technique has proved useful to screen large numbers of plants and to detect single copy genes in large plant genomes (Wordragen *et al.* 1991). The GUS gene was detected in a percentage (7-30%) of kanamycin resistant *V. grandis* regenerates produced by a range of transformation vectors (Table 5.11). However, a number of other bands of varying size were also detected (Figure 5.10 A). These are assumed to be non-specific PCR products since Southern hybridisation with the GUS genes as a probe revealed only the expected 514bp fragment (Figure 5.10. B). The additional bands may have resulted from high DNA concentrations and self priming of the DNA. Amplification thus either stops or produces non-specific products after a certain number of cycles (McPherson *et al.* 1991).

Analysis of putative *V. grandis* transformants by Southern hybridisation revealed only a small percentage of the clones (0-29%) which showed homology to either the GUS or NPT II probes (Table 5.11). Furthermore, the GUS gene was only detected in 0-14% of the clones, whereas a higher percentage (0-29%) of clones contained fragments which hybridised to the NPT II probe.

Table 5.11: Summary of transformation results.

Results present the number and percentage of kanamycin resistant *V. grandis* plants which tested positive for GUS histochemical assays, PCR amplification of a GUS fragment and Southern blot hybridisation with GUS and NPT II probes.

Agrobacterium host	Vector	*V. grandis* Clone	No. of plants tested	Percentage of plants testing positive for			
				GUS	PCR	Southern probe GUS gene	Southern probe NPT 11 gene
A. tumefaciens							
AL4404	pBI121	865	28	7	7	0	11
AL4404	pBI121	866	87	21	21	2	2
AL4404	pGV3850	865	26	n/t	n/t	n/t	15
AL4404	pKiwi	866	1	100	n/t	n/t	n/t
EHA101	pBI121	866	8	0	n/t	n/t	n/t
A. rhizogenes							
K565	pBI121	866	34	18	27	0	6
K596	pBI121	866	30	57	30	10	18
K568	pBI121	866	7	86	0	14	29
K597	pBI121	866	4	25	25	0	0

n/t= not tested.

Problems in detecting either gene by Southern hybridisation may be related to difficulties in isolating good quality DNA from *V. grandis* leaves. Polysaccharide contamination is a common problem in DNA samples isolated from woody plants species (Murray and Thompson 1980). Contaminated 'DNA pellets' are extremely viscous (Fang *et al.* 1992) and difficult to concentrate (Manning 1991). The method chosen for DNA extraction of *V. grandis* leaf tissue produced good quantities (ca. 20µg/gm fresh weight) of DNA. Approximately 2µg of DNA was used for standard Southern blots. This compares with 5-20µg of DNA commonly used for Southern hybridisation studies to detect the presence of a foreign gene in the plant genome [Ottaviani and Hänisch ten Cate (1991), McCown *et al.* (1991), Brasilerio *et al.* (1991) and Fillatti *et al.* (1987)]. This together with the fact that *V. grandis* showed only weak hybridisation to the barley a-amylase probe, suggests that higher concentrations of *Verticordia* DNA are required for the detection of low copy gene sequences. More work will need to be carried out to achieve this aim.

This study has been important in that it has developed a potential genetic transformation system for *V. grandis*. This system employs *Agrobacterium* infection of *V. grandis* leaf discs and the method developed for plantlet regeneration from leaf discs for the recovery of transformed plants. Briefly, leaf discs were inoculated with an *Agrobacterium* strain containing a marker gene for antibiotic resistance, the NPT II gene and a reporter gene (GUS) for screening of transformants. Following co-culture leaf discs were transferred to shoot proliferation medium containing kanamycin. Shoots were then rooted in the presence of kanamycin.

This is the first report to date of genetic engineering of a plant from the Myrtaceae. The techniques developed in this study may have applications to other plants within this family.

CHAPTER 6 GENERAL CONCLUSIONS AND DISCUSSION

Results obtained during this study have demonstrated techniques for the micropropagation, out-planting and genetic transformation of *Verticordia grandis*. A commercial micropropagation protocol was developed for shoot proliferation, root induction and for the successful transfer of these plantlets to glasshouse conditions and for long term pot culture. The leaf disc regeneration method developed in this study formed the basis for the development of the genetic transformation system. This system utilises the *Agrobacterium* infection process for transfer of foreign DNA into host plants. The binary vector system containing a marker gene for antibiotic resistance (NPT II gene) and a reporter gene (GUS) were employed. These results represent the first report to date of genetic engineering of a plant from the Myrtaceae.

The transformation results were confirmed by PCR and Southern hybridisation. However, for the transformation system to be viable it is essential that expression of the introduced DNA be confirmed in the progeny. It is important to show that the foreign DNA is stably incorporated into the host's genome and subsequently inherited through meiosis as the result of a single dominant Mendelian trait.

The stages involved in the development of a transformation system using *Agrobacterium* were systematically investigated in this study. These stages include, 1) the susceptibility of plants to *Agrobacterium* infection and 2) the regeneration of plants from transformed cells. Experiments were carried out to test whether wild-type *A. rhizogenes* strains would increase the rooting ability of *V. grandis* explants, *in vitro*. These results indicated that infection with *A. rhizogenes* did not improve the rooting ability of *V. grandis*. However, these experiments did indicate that *V. grandis* is susceptible to infection with *A. rhizogenes* as determined by gall formation and opine analysis. For regeneration of transformed plants a leaf disc system was developed. This system employed *Agrobacterium* infection of apical meristematic cells located in the petiole of *V. grandis* leaves. These cells have a

high capacity for shoot regeneration. Therefore, after infection of these cells with the appropriate *Agrobacterium* vector it was possible to regenerate transformed plants.

The future applications of genetic engineering require the introduction of foreign genes of importance to floriculture, crop and forestry improvement programs. Three essential and interacting components are needed to achieve this aim:

1) a suitable regeneration system to produce transgenic plants (targeted cells need to have the ability to regenerate),

2) an efficient transformation system to deliver foreign DNA to individual cells (for *Agrobacterium* mediated transformation susceptibility trials are also necessary) and

3) the isolation and cloning of suitable genes which can impart favourable traits.

The transformation success of woody plants has been limited, partly because suitable regeneration systems have not been developed. This study has highlighted the importance of a reliable shoot regeneration system for the successful recovery of transformed plants. Considerations for genotypic variation in both regenerative potential and susceptibility to infection by *Agrobacterium* are also important. Genotypic variation in regenerative response of species has been widely reported (see Chapter 1: Section 1.2.7). Differences in the regeneration potential, in particular root induction, were found between the two *V. grandis* Clones (865 and 866) used in this study and these results were reflected in subsequent transformation frequencies. Therefore, the identification and screening of commercially useful genotypes for shoot proliferation and root induction are necessary for both successful commercial micropropagation and for genetic transformation studies.

Difficulties of transformation of woody species can also be attributed to the host range of *Agrobacterium*. The host range is dependent upon both the type of Ti or Ri plasmid of the *Agrobacterium* strain and on the type of wound response induced in the plant. Recent evidence (Godwin *et al.* 1992), indicates that co-cultivation conditions are important for infection of plant tissue with *Agrobacterium*. Additional factors to consider in assessing

the ability of *Agrobacterium* to transfer DNA into the host plant include 'genetic, physiological and physical components of the bacterium/plant interaction' (Godwin *et al.* 1992). Specific genotypic variation in susceptibility to *Agrobacterium* infection, which also includes *Agrobacterium* tissue specificity, have been well documented (see Chapter 1: Section 1.6.5). The type of cells, tissues and organs targeted for *Agrobacterium* infection can affect transformation frequencies in different host species. Therefore, successful transformation of plant species requires both *Agrobacterium* susceptibility trials and well defined culture conditions for the regeneration of transformants.

The choice of suitable vector systems for efficient screening and selection of transformants is also important in determining transformation protocols for a given plant species. The selection, growth and regeneration of transformed cells is critical for the recovery of transformed plants.

Finally, the application of genetic engineering to improvement programs is dependent upon the availability of suitable genes of potential importance to the industry which will impart favourable traits to ornamental, crop and forestry plant species. However, the availability of 'useful genes' is hampered by difficulties involved in isolating genes determining agronomic traits. Furthermore, at present only single genes can be easily applied with the gene transfer techniques currently available. Several genes of interest have been isolated and successfully applied to improvement programs. These include resistance to pests, diseases and herbicides and, for flower breeding, include manipulation of flower colour, shape and vase life.

In addition to the transfer of useful genes into host plants, the genetic manipulation of plants can provide valuable information on basic plant processes involved in gene regulation and plant development and provide important tools for a further understanding of plant molecular biology. The developments described here would allow the use of *Verticordia* as a model system for the genetic engineering and micropropogation of a woody species. However, the extension of these techniques to Eucalypts would have even greater economic impact.

APPENDIX A CULTURE MEDIA

Table 1 MS salts and vitamins (Murashige and Skoog 1962)

MACRONUTRIENTS	Final concentration(mg/l)
NH_4N0_3	1650
$KN0_3$	1900
$CaCL_2.2H_20$	440
$MgSO_4.7H_20$	370
KH_2PO_4	170
MICRONUTRIENTS	
$MnSO_4.H_20$	22.3
$ZnSO_4.7H_20$	8.6
H_3BO_3	6.2
KI	0.83
$NaMo0_4.2H_20$	0.25
$CoCl_2.6H_20$	0.025
$CuSO_4.5H_20$	0.025
Fe SOURCE	
$FeSO_4.7H_20$	27.8
Na_2.EDTA	37.3
VITAMINS	
Myo-inositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine	0.5
Thiamine-HCL	1.0

Table 2: Composition of bacterial growth media

A. YM medium

K_2HPO_4	0.5g
$MgSO_4$	0.2g
NaCl	0.2g
$FeCl_3$	0.01g
$CaCl_2$	0.2g
Bacto-yeast extract	1g
mannitol	10g
dist. water	to 1 litre

B. YEB medium

Bacto-Nutrient broth	13.3g
Bacto-yeast extract	1g
sucrose	5g
$MgSO_4$	0.24g
dist. water	to 1 litre

Table 3: Kendon liquid manure-nutrient analysis

Analysis	Total product (% W/V)
Total Nitrogen	5.4
Total Phosphorous	1.3
Total Potassium	4.5
Trace Elements	
Boron(B)	0.0036
Copper(Cu)	0.005
Manganese (Mn)	0.01
Molybdenum (Mo)	0.001
Zinc (Zn)	0.005
Maximum Biuret	0.08
Iron (Fe)	0.13

APPENDIX B Glasshouse Temperature and Light measurements

Table 1: Average temperature (°C) recordings (min, max, mean) for the months of January, April, August and November and the corresponding mean light intensity ($\mu E/s/m^2$).

TEMP°C	JAN	APRIL	AUG	NOV
MIN	19.1	17.9	14.4	19.1
MAX	30.7	26.4	25.6	27.8
MEAN	24.9	21.7	20.4	23.8
LIGHT INTENSITY	881	542	382	620

APPENDIX C: DNA extraction solutions

1) DNA isolation from cereals

DNA extraction buffer

1% sarkosyl
100mM Tris-HCL
100mM NaCl
10mM EDTA
pH 8.5

2) CTAB

extraction buffer	Wash buffer
2% (w/v) CTAB (Sigma)	76% (v/v) ethanol
1.4M NaCl	10mM ammonium acetate
0.2% (v/v) 2-mercaptoethanol	
20mM EDTA	
100mM Tris-HCl	
pH 8.0	

3) DNA isolation (Bernatsky)

DNA extraction buffer	Lysis buffer
64g sorbitol	200ml 1.0M Tris pH 8.0
12g Tris	200ml 0.25M EDTA
1.85g EDTA disodium salt	200ml H_20
2% PVP	20g CTAB
20mM sodium metabisulphite	400ml 5.0M NaCl
pH7.5	

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