Tissue Culture of Ornamental Eucalypts *Eucalyptus erythronema, E. stricklandii* and their hybrids

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Abstract

This research project was carried out using the ornamental species *E. erythronema, E. stricklandii*, their natural interspecific hybrid *E.* 'Urrbrae Gem', and artificial hybrids of these two species, with the aim of identifying a clonal propagation method for hybrid ornamental eucalypts using the tissue culture techniques of micropropagation, organogenesis and somatic embryogenesis.

Seeds of *E. erythronema, E. stricklandii*, and *E.* 'Urrbrae Gem' were decontaminated and germinated in culture for experiments on callus growth and response to plant growth regulators. Murashige and Skoog (1962) medium supplemented with 1 μM BAP, 10 μM NAA, 20 g^{-1} sucrose, gelled with 7 g^{-1} Phytagel® proved the most suitable callus growth medium when compared to those of Gamborg (1968), Lloyd and McCown (woody plant medium) (1980), Almehdi and Parfitt (1996) and de Fossard(1976) (*Eucalyptus)*.

A range and combination of plant growth regulators was used to investigate organogenesis, somatic embryogenesis and axillary shoot proliferation. A broad based experiment was first conducted to assess the response of the hybrid and its parental species to wide concentrations of commonly used auxins and cytokinins known to induce organogenesis and somatic embryogenesis. Callus growth was consistent across all genotypes and tissue types with soft and spongy callus growth in the presence of 2,4-D and hard and friable callus in the presence of NAA, while callus growth was greater with auxin or auxin/cytokinin (BAP or kinetin) combinations compared to cytokinin alone. Healthy root development was observed from treatments with NAA, while roots that developed from

treatments with 2,4-D turned brown within 12 weeks. Bud development was observed with BAP alone or in combination with NAA with large bud clusters observed on *E. erythronema* and *E.* 'Urrbrae Gem' explants. Shoots were observed in treatments with NAA alone, or in combination with BAP or kinetin, but not in treatments with 2,4-D. No somatic embryos were observed with any treatment or taxon. Light microscopy of explants treated with 2,4-D alone showed calli consisting only of large parenchyma cells, while 2,4-D in combination with a cytokinin produced smaller parenchyma cells, with areas of vascular tissue and spherical growths. BAP, kinetin and NAA alone or in combination produced an increase in vascular tissue. Shoot and root initiation appeared to occur within the callus with connection to the vascular tissue.

Shoot organogenesis from *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' seedlings and *E. erythronema* x *E. stricklandii* hybrid 2.5 was further investigated using apex and leaf explants with 1 μM and lower levels of BAP. Apex and leaf explants of *E. erythronema* and *E.* 'Urrbrae Gem' developed clusters of buds from the apex base and leaf petiole while apex and leaf explants of *E. stricklandii* and hybrid 2.5 produced individual buds. Bud and shoot development was greater on apex than leaf explants with 0.5 and 1 μM BAP producing the greatest shoot numbers. Light microscopy showed that meristem development had occurred within the callus and bud structures, but there were few shoot primordia considering the amount of meristem development (meristemoids).

Somatic embryogenesis was further investigated in juvenile ad callus tissue using NAA, NAA/2,4-D combinations, and heat and smoked water treatments. Somatic embryogenesis

was not observed macroscopically with 2,4-D and NAA treatments. Globular somatic embryo structures were observed on apex explants of *E.* 'Urrbrae Gem' seedlings at 2 wk on MS medium supplemented with 16.11 μM NAA but there was no development beyond the globular stage. Areas of defined callus development, which did not contain typical embryogenic cells, were observed on all treatments and genotypes. Root development was observed from all auxin treatments but was less on explants treated with 5.37 μM NAA/ 4.5 μM 2,4-D compared to NAA alone while roots and shoots developed simultaneously on apex explants after culture for 1 wk on MS medium supplemented with 80.55 μM NAA. Heat and smoked water were detrimental to *E.* 'Urrbrae Gem' callus and no somatic embryogenesis was observed.

Explants from field trees of *E. erythronema, E. stricklandii*, *E.* 'Urrbrae Gem', *E. erythronema* x *E. stricklandii* hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, 21U and potted plants of *E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5 growing under glasshouse conditions, were harvested and initiated into culture. Of the selected plants only explants from glasshouse grown material could be initiated into culture and multiplied. *E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5 were multiplied by axillary shoot proliferation over 3 yr on Quoirin and Lepoivre medium (1977) with 2.2 μM BAP, 0.5 μM NAA and 0.5 μM GA_3, 20 g^{-1} sucrose, gelled with 7 g^{-1} Phytagel®. However root growth proved difficult as shoots developed short thick roots that failed to elongate and the plants could not be hardened off. By using zeatin in the multiplication medium and activated charcoal after the IBA pulse elongated roots suitable for hardening off were developed. By comparison seedlings of *E.* 'Urrbrae Gem' were successfully germinated in culture and

multiplied by axillary shoot proliferation using WPM, 2.2 μM BAP, 0.5 μM NAA and 0.5 μM GA_3, 20 g^{-1} sucrose, gelled with 7 g^{-1} Phytagel®. They were rooted on ½WPM, 20 μM IBA, 10 g^{-1} sucrose gelled with 7 g^{-1} Phytagel® for 7 d, followed by subculture to PGR free medium. Following root initiation and growth plants were hardened off to the external environment.

In conclusion genetic differences were observed between genotypes, in regards to initiation into culture, and response to nutrient medium, and plant growth regulators, with differences observed in explant deterioration, medium exudates, callus growth and appearance, somatic embryogenesis, bud, shoot and root organogenesis, axillary shoot development, and rooting of shoots. For organogenesis, juvenile explants were more responsive in culture than mature explants and potted plants more responsive than field plants for micropropagation. Shoot and root organogenesis was achieved from juvenile explants while shoot explants of *E. erythronema* x *E. stricklandii* hybrids were successfully micropropagated by axillary shoot proliferation. Somatic embryogenesis was observed with *E.* 'Urrbrae Gem' seedlings but was blocked at the globular stage. This research has provided a greater understanding of the clonal propagation requirements via tissue culture techniques for *E. erythronema, E. stricklandii* and their hybrids.

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Dedication

To the late Noel Lothian, former Director of the Adelaide Botanic Gardens, for his continued inspiration.

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Z 6[4-hydroxy-3-methylbut-2-enylamino] purine
2Ip dimethylallyl amino purine
K 6- furfuryl amino purine **153**

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List of abbreviations

AP	Almehdi and Parfitt (1986) culture medium
B5	Gamborg *et al.* (1968) culture medium
BAP	6-Benzylamino purine
2,4-D	2,4-Dichlorophenoxyacetic acid
EUC	de Fossards (1976) eucalypt medium
IAA	Indole-3-acetic acid
IBA	Idolebutyric acid
2iP	(Dimethylally amino) purine
kinetin	6-Furfuryl amino purine
MS	Murashige and Skoog (1962) medium
NAA	α-Naphthaleneacetic acid
PGR	Plant growth regulator
PVP	Polyvinlypriolidone
QL	Quoirin and Lepoivre (1977) medium
TDZ	1-Phyl-3-(1,2,3-thiadiazol-5-yl) or (thidiazuron)
TK	Tabachnik and Kester (1977) culture medium
WPM	Woody plant medium (Lloyd and McCown, 1980)
Zeatin	6[4-hydroxy-3-methylbut-2-enylamino] purine

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Chapter 1

General introduction, literature review and aims

1.1 General Introduction

The genus *Eucalyptus* comprises a diverse range of trees from small shrubby mallee plants to tall spreading trees, found in a variety of environments throughout Australia. Classification is challenging as eucalypts show high species variation and readily outcross. Eucalypts and their hybrids are grown for pulp, timber, fuel, honey, oils, amenity horticulture, cut flowers and foliage (Nicolle, 1997). Superior trees of ornamental and floriculture value can be found from individual species and natural and artificial hybrids. A breeding programme aimed at the development of interspecific hybrid eucalypts for ornamental horticulture is underway at the University of Adelaide, with many selections under evaluation (Neaylon *et al.,* 2001). The ornamental species *Eucalyptus erythronema* and *E. stricklandii* have been chosen as parents in the programme, as natural hybridisation between the two has given rise to the highly ornamental *E.* 'Urrbrae Gem', which was planted in the Waite Arboretum in 1936 (Delaporte *et al.*, 2001a,b). Several other hybrids between *Eucalyptus erythronema* and *E. stricklandii* are currently under evaluation in the programme in addition to *E.* 'Urrbrae Gem' which has yet to be clonally propagated. These superior hybrids require clonal propagation to maintain genetic integrity and their desired characteristics. Clonal propagation can be achieved through cuttings, air layering, budding, grafting and tissue culture.

The tissue culture techniques, micropropagation and somatic embryogenesis utilise the plant's ability to regenerate new plants from isolated plant tissues (explants) such as meristems, anthers, cotyledons, hypocotyls, leaves, and nodes. Micropropagation is the regeneration of plant shoots and roots from meristematic

tissue or callus via organogenesis. Somatic embryogenesis is the development of seed like embryos from non-zygotic cells. The origins and development of organs and of somatic embryos can be investigated by microscopy to assist in technique development and refinement.

Current research into tissue culture of eucalypts is focused mainly on forestry species and because these often come from taxonomically different subgenera to ornamental species, these methods may not be suitable. Difficulties may be encountered when developing tissue culture techniques for the clonal propagation of ornamental hybrid eucalypts. An understanding of the *in vitro* environment, mineral nutrient levels, plant growth regulator type and concentration, hardening of plants into the external environment, mother plant maturity and growing conditions is required for individual genotypes. In most cases, the selected hybrid is an individual mature tree growing in a field location. This creates difficulties with explant age, availability, and decontamination for initiation into culture. Juvenile explant material is more amenable to tissue culture techniques. To rejuvenate mature trees the methods of coppicing, regular grafting of the mature scion onto juvenile rootstock and taking explants from cutting grown plants can be used. The growing of grafted and cutting plants under controlled conditions in the glasshouse can also reduce the difficulties associated with sterilisation of field grown material.

Previous clonal propagation experiments involving the superior individual *E.* 'Urrbrae Gem', and selected hybrid specimens from a cross between *E. erythronema* var. *erythronema* x *E. stricklandii* encountered the following difficulties (Sedgley, personal communication):

- All specimens are field grown, making sterilisation for tissue culture difficult.

- There is limited, suitable experimental material available from an individual field grown specimen.
- Assessment for commercial value comes at maturity, but mature explants are difficult to propagate and may require rejuvenation.
- Hybrids could not be coppiced for rejuvenation for risk of losing the trees.
- *E.* 'Urrbrae Gem' has proven difficult to propagate by grafting and cuttings.
- *E.* 'Urrbrae Gem' is over 60 yr old and is in poor health.
- The *in vitro* culture requirements for *E. erythronema* x *E. stricklandii* hybrids are unknown.

The approach adopted in this study was to develop tissue culture techniques for the parental species *E. erythronema* and *E. stricklandii* and for open pollinated seedlings of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem'. The parental species are the most similar material genetically to the hybrids, so the techniques developed may be applicable. The use of parental species and seedlings also overcomes the problem of limited material. Both species are ornamental in their own right, so clonal propagation is a worthwhile goal. Seedlings offer the advantages of providing an adequate supply of juvenile explants, which are generally more responsive in culture than mature tissue. The techniques developed on juvenile explants can then be tested on the limited material available of mature hybrid explants. The juvenile plant material chosen was open pollinated seedlings the original hybrid *E.* 'Urrbrae Gem' and the parental species *E. erythronema* var. *erythronema,* and *E. stricklandii.*

To establish a tissue culture technique for these eucalypts several factors were investigated. These relate to explant selection, initiation, shoot, root and somatic embryo development and involved observations on time of year and type of explant collected, mother plant growing conditions, sterilisation, gelling agent, mineral nutrients, culture environmental conditions, type and concentration of plant growth regulators, and different genoptypes and tissue types required for callus production, bud induction, shoot growth, rooting and somatic embryo development. To further understand organogenesis and embryogenesis, microscopy was used to show the path of regeneration by either meristem or embryo development.

1. 2 The *Eucalyptus* Genus

1.2.1 Introduction

The genus *Eucalyptus* consists of over 800 named species plus hybrids (Brooker and Kleinig, 1999) most of which probably developed during Australia's isolation following the break-up of Gondwana over 50 million years ago. Their morphology and habitats are as diverse as their ecological range (White, 1998) and this, together with a diversity of form, has enabled eucalypts to become a valuable economic crop in forestry and horticulture.

1.2.2 Classification

The genus *Eucalyptus* is part of the *Myrtaceae* family, and is found throughout most of Australia and parts of South East Asia (Penfold and Wills 1961). The first known collection of a *Eucalyptus* specimen was in 1770 from eastern Australia by Joseph Banks and Daniel Solander (Brooker and Kleinig, 1999). New *Eucalyptus* species are still being discovered and the system of classification, which incorporates

a combination of genetics, ecology, morphology, and anatomy for identification, has undergone much debate and revision.

At present there are two different thoughts on eucalypt classification. One proposes three genera, *Angophora, Corymbia,* and *Eucalyptus* (Ladiges and Udovicic, 2000). However Brooker, (2000) includes these genera under *Eucalyptus*, and divides the genus into thirteen subgenera. The ornamental species selected for this project are classified in the subgenerus *Sympyomyrtus.*

1.2.3 Hybridisation

Hybridisation occurs where an individual is produced from taxonomically distinct parents, resulting in a phenotype that displays characteristics of both parents. This process occurs naturally between *Eucalyptus* species and can be utilised to breed trees of commercial value for forestry, ornamental horticulture, and floriculture (Griffin *et al.,* 1988). Specialist nurseries are developing eucalypt varieties and hybrids, which meet market demands. Crosses between *E. ficifolia* and *E. pytchocarpa* have produced *E.* 'Summer Beauty' and *E.* 'Summer Red' (Payne, 2000a). Other new releases include *E. maculata* 'Imagine' and *E. robusta* 'The Green and Gold', which both have leaves that change colour as they develop (Payne, 2000b). These plants are propagated by the clonal method of grafting to maintain their characteristics.

Natural hybrid eucalypts can have distinctive characteristics of commercial importance, such as flower colour, leaf colour, oil content, and timber quality. For example, in 1936 the gardener of the Waite Arboretum, Mr E.A. Couzens, discovered a chance seedling, and named it *E.* 'Urrbrae Gem'. The parents were recently confirmed, by DNA fingerprinting, as *Eucalyptus erythronema* var. *erythronema,* and

E. stricklandii (Delaporte, *et al.,* 2001a). *E* 'Urrbrae Gem' is a small tree with white smooth bark, spreading branches, glossy green leaves, and showy bright reddish blossoms 7-8 cm across (Gardner, 1987, unpublished).

1.2.4 Ecology and morphology

Eucalyptus plants have a broad range of morphology and are distributed throughout dry temperate, subtropical, and tropical climates within Australia (Brooker and Kleinig, 1999). There is great diversity within the species from low shrubby mallee trees, to tall erect forest trees (Nicolle, 1997). Some species display heteroblastic development where the leaves develop different morphology through the stages of seeding, juvenile, intermediate, and adult. Flowers, fruits, and buds vary in size, shape, and colour (Brooker and Kleinig, 1999).

1.3. Clonal propagation

1.3.1 Introduction

Eucalypt plants often show high phenotypic variability when grown from seed, thus making seed propagation commercially unacceptable for ornamentals. Clonal propagation is therefore the preferred method of reproducing individual plants that have desirable characteristics. Examples of interspecific hybrids that require clonal propagation include crosses between *E. ficifolia* and *E. pytchocarpa* such as *E.* 'Summer Beauty' (Payne, 2000b), the rare naturally occurring hybrid *E. graniticola* (Bunn and Dixon, 1997), and *E.* 'Urrbrae Gem' (Delaporte *et al.,* 2001b).

Methods of clonal propagation include stem, root, and leaf cuttings, air layering, grafting, and tissue culture. Plant regeneration through tissue culture can be

achieved either by micropropagation, organogenesis, or somatic embryogenesis. These tissue culture methods are the main focus of this review.

1.3.2 Micropropagation and organogenesis

1.3.2.1 Introduction

Micropropagation and organogenesis are used in ornamental horticulture for rapid clonal multiplication of individuals, and for species that are difficult to propagate (de Fossard, 1976). Micropropagation involves the multiplication of shoots from existing meristems, such as axillary shoot proliferation, followed by root development, and hardening off to the natural environment. Micropropagation usually provides genetically stable plant material (Razdan, 1993).

Organogenesis is the formation of adventitious shoots and roots from non-meristematic cells. Dedifferentiation of these cells gives rise to meristem and organ formation (direct organogenesis) or to callus growth, followed by meristem formation and organ development (indirect organogenesis). Micropropagation and organogenesis can be influenced by many factors including types and age of plant tissue (explant), type and concentration of plant growth regulators, culture medium, gelling agents, light and temperature (McComb and Bennett, 1986; Taji and Williams, 1996). Also, explant response in culture varies between and within species (Bonga and Pond, 1991; Vicitez *et al.,* 2003; Druart, 2003; Watt *et al.,* 2003). For each species, there is a need to develop suitable culture conditions considering each variable.

1.3.2.2 Plant tissue

Successful shoot multiplication and rooting is often related to the type of plant tissue collected, (termed the explant), its age, and the season of collection (Le Roux and van Staden, 1991a). This can be significant when selecting tissue for successful clonal propagation.

Le Roux and van Staden (1991a) reported that successful cultures of eucalypts were obtained from explants of seedlings, stump coppice shoots, scion shoots, and epicormic shoots, with the important requirement being selection of young, healthy, vigorous, stock plants. However, Gupta and Mascarenhas (1987) used axillary nodes from mature, fast growing trees of *E. camaldulensis, E. globulus, E. tereticornis,* and *E. torelliana* to micropropagate over 1,000 plants, and these were successfully transferred into the field, while Chang *et al.* (1992) successfully micropropagated *E. radiata* from adult nodal explants. Meristems from nodes are genetically stable, and therefore a good choice for micropropagation. However, meristems from the crowns of mature trees can show plagiotropic growth, which is undesirable for propagation of forestry species (Le Roux and van Staden, 1991a).

The ability to propagate by clonal methods appears to diminish with increasing maturity of tissue. Cellular changes influence the ability of mature tissue to regenerate (von Aderkas and Bonga, 2000). When both juvenile and mature stems and leaves are taken from the same plant, the juvenile tissue responds better *in vitro* (Burger, 1987; Vicitez *et al.,* 2003) and can also give better rates of multiplication (McComb and Bennett, 1986; Niccol *et al.,* 1994). This may be due to changes within the cell as it matures and through the production of rooting inhibitors (Bonga, 1982; Paton *et al.,* 1970). The removal of rooting inhibitors in stems and leaves was

achieved in *E. grandis* by a two hour soak in sterile water (Creswell and Nitsch, 1975).

Rejuvenation of mature tissue is a poorly understood process whereby mature tissue regains juvenile properties and the ability to produce roots (Bonga, 1982). Rejuvenation can be induced by coppicing (stimulation of juvenile tissue growth), taking cuttings from rooted cuttings, particularly rooted coppice cuttings, and from repeated grafting of mature scions onto juvenile rootstock (Le Roux and van Staden, 1991a) and *in vitro* micrografting (Revilla *et al.,* 1996). Rejuvenation of mature tissue can also be achieved through repeated *in vitro* subculturing (Durand-Creswell *et al.,* 1982). For example, McComb and Bennett (1986) achieved rooting of 20 yr old *E. citriodora* after the fourth subculture. Subculturing therefore appears to be an important process in the rejuvenation of mature tissue.

As well as changes occurring with maturity, the physiological state of the parent plant also appears to alter with the seasons. Explants collected in spring when growth is active appear to have a higher regeneration rate. Durand-Creswell *et al.* (1982) found a seasonal trend in the number of rooted cuttings of *E. grandis*. They found that active growth can be stimulated by environmental change and that exceptionally hot or cold conditions prevented rooting. *E. grandis* trees that were treated with gibberellin produced new growth that led to prolific rooting of cuttings. Sharma and Ramumurthy (2000) found for *E. tereticornis* that regeneration was greater in spring and autumn compared to summer, and McComb and Bennett (1986) found the optimum time to collect explants of eucalypts was from November to March.

Suitable plant material for successful *in vitro* culture can therefore be selected from juvenile tissue, following plant rejuvenation (coppicing) or rejuvenation of mature tissues (serial grafting, cuttings, or *in vitro* subculture).

1.3.2.3 Methods of decontamination

Plant material must first be surface cleaned to remove bacteria and fungi which can grow rapidly in culture, overgrowing the explant. Methods of decontamination vary slightly, but generally consist of soaking plant tissue in chemicals such as sodium hypochlorite, calcium hypochlorite, ethanol, or hydrochloric acid for a period of time (McComb and Bennett, 1986; Le Roux and van Staden, 1991a).

Decontamination success varies greatly between eucalypt species and the source of plant tissue. Field cuttings of mature tissue can be difficult to decontaminate due to heavy microbial populations. Also, high concentrations of chemicals can severely damage plant tissue. Floral buds and seeds can tolerate higher concentrations as the operculum and seed coat protect the internal tissue and are later removed. Young vigorously growing shoots tend to be more successful, as are cuttings from seedlings grown under controlled conditions in a greenhouse. Compared to field grown mature tissue, young and glasshouse material requires relatively mild decontamination chemicals due to low microbial populations, and therefore exhibits greater survival rates (McComb and Bennett, 1986; Le Roux and van Staden, 1991a).

1.3.2.4 Culture medium

The culture medium provides the nutrients necessary for plant growth and development that would normally come from photosynthesis and nutrient uptake from

the soil. These include various combinations of macro and microelements, carbohydrates, vitamins, and plant growth regulators (Taji and Williams, 1996), with different genotypes often requiring specific combinations (Razdan, 1993; Tang *et al.,* 2002). For eucalypts the most widely used medium is Murashige and Skoog, (MS) (Murashige and Skoog, 1962), which can be used either full strength, (Le Roux and van Staden, 1991b), half strength (Bunn and Dixon, 1997; Niccol *et al.,*, 1994), quarter strength, or modified (Gupta *et al.,* 1983; Gomes and Canhoto, *et al.,* 2003). Other media used for eucalypts include de Fossard's eucalypt medium (EUC) (de Fossard, 1974), Woody Plant Medium (WPM) (Lloyd and McCown, 1980), B5 (Gambourg *et al.,* 1968), and Knops (Tabachnik and Kester, 1977). Manipulating media components can affect organogenesis, such as the addition of riboflavin or removal of boron, which is reported to improve rooting in *E. globulus* (Trindade and Pais, 1997).

Transfer of the explant to different media is often required for shoot multiplication, elongation, and rooting (Warrag *et al.,* 1989a; Le Roux and van Staden, 1991b; Tonon *et al.,* 2001; Druart, 2003). This process was beneficial for *Eucalyptus globulus* (Bennett *et al.,* 1994), *E. viminalis* (Wiecheteck *et al.,* 1989), *E. dunnii* (Cortezzi Graca and Mendes, 1989), and *E. gunnii* (Franclet and Boulay, 1982) as it improved shoot multiplication, elongation and rooting. In contrast *E. microcorys* and *E. graniticola* appeared to progress satisfactorily through all stages on one basal medium (Burger, 1987; Niccol *et al.,* 1994; Bunn and Dixon, 1997).

Gelling agents are used to solidify the medium and can influence water availability and the movement of mineral nutrients to the explant (Williams, 1993). Agar, gelrite and Phytagel® are the most commonly used. Agar contains more impurities compared to Phytagel® and this may influence plant growth, while gelrite

has a better buffering capacity which can influence medium pH over time (Williams, 1993).

1.3.2.5 Culture environment

Incubation temperatures and daylength can be manipulated to optimise growing conditions, or to provide long term storage of plant tissue. The quality, intensity and wavelength of light are reported to influence organogenesis through stimulating specific gene synthesis (Stefano and Rosario, 2003). High light favours shoot growth; however, cultures kept in the dark for the first week are reported to show a benefit to shoot growth by reducing tissue browning and leaking of polyphenolics into the medium (Creswell and Nitsch, 1975; Durand-Creswell *et al.*, 1982; Franclet and Boulay, 1982). A dark period was found to improve shoot elongation in *E. dunnii* (Fantini and Cortezzi Graca, 1989) and *E. tereticornis* (Sharma and Ramamurthy, 2000). Low light intensities were shown to reduce prolific callus growth that can overgrow the explants of *E. graniticola* (Bunn and Dixon, 1997), while a dark period or darkening the medium improved root growth in *E. globulus* (Bennett *et al.*, 1994) and *E. grandis* x *E. urophylla* (Cid *et al.*, 1999).

Changes in temperature can benefit both explant development in culture, and explant storage, but the effect of temperature varies between eucalypt species (Taji and Williams, 1996). Improved shoot growth was achieved from mature nodes of *E. camaldulensis* and *E. torelliana* following a cold treatment of 15°C for 72 h in liquid medium, compared to no growth when mature nodes were placed directly into the growth room at 24°C (Gupta *et al.*, 1983). The optimum temperature for growth in eucalypt culture is generally 23–27°C, while storage temperatures of 10°C in *E. grandis* kept plant tissue viable for up to 10 months (Watt *et al.*, 2000).

In the closed system of *in vitro* culture, the build up of gases, particularly ethylene, may play a crucial role in reducing plant health (Williams, 1999). Improvements in regeneration, shoot growth, photosynthesis and survival of plantlets to the external environment have been achieved using enriched CO_2 or forced ventilation in *E. camaldulensis* (Zobayed *et al.,* 2002), *E. tereticornis* (Sha Valli Khan *et al.,* 2002), *Coffea arabusta* (Nguyen *et al.,* 2001), and carnation (Majada *et al.,* 2000). Improvements in plant health have also been achieved for woody plants using photoautotrophic, or sugar free, systems (Kozai and Nguyen, 2003), and in apricot by the use of ethylene inhibitors, such as silver nitrate (Burgos and Alburquerque, 2003).

1.3.2.6 Plant growth regulators

Plant growth regulators (PGR) such as auxins, cytokinins, and gibberellins are used to manipulate cell processes and encourage specific organ development. Auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and indole butyric acid (IBA), promote root induction, cell enlargement, and callus growth. Cytokinins, such as benzylamino purine (BAP), kinetin and zeatin stimulate the growth of existing meristems by promoting cell division, shoot multiplication, and axillary bud-growth, while gibberellins promote cell enlargement and stem elongation (Kyte and Kley, 1996).

The processes involved in initiation, multiplication and elongation, each appear to require different ratios of auxins to cytokinins for different genera (Franclet and Boulay, 1982; Gupta and Mascarenhas, 1987; Wiecheteck *et al.,* 1989; Warrag *et al.,* 1989a; Cid *et al.,* 1999; Quoirin, 2003; Rodriguez and Vendrame, 2003; Vicitez *et al.,* 2003). However, a simplified regime using BAP proved successful for *E. microcorys* (Niccol *et al.,* 1994), and with kinetin for *E. graniticola* (Bunn and

Dixion, 1997). Zeatin alone provided good shoot proliferation for *E. ficifolia* and *E. citriodora*, (Ostrowska *et al.,* 1998) and *E. grandis* (Lainé and David, 1994). Therefore once a species is established in culture, a more simplified micropropagation technique could be developed.

Difficulties with root induction also occur with eucalypts. This could be due to the age and physiology of the explant or a carry-over effect of PGR. Rooting was inhibited in *E. gunnii* when gibberellin was used for shoot elongation (Franclet and Boulay, 1982). Alternating shoots onto media with BAP then kinetin significantly improved shoot development and subsequent root production in *E. globulus* (Bennett *et al.,* 1994, 2003) and *E. gunnii* (Curir *et al.,* 1990). The addition of kinetin to the medium appears to produce an increase in endogenous flavonoids, which can be used as an energy source. BAP, however, appears to promote peroxidase activity which is detrimental to cell growth. This suggests that rooting ability is correlated with the type of cytokinin, and the level of flavonoids (Curir *et al.,* 1990). Auxins promote the induction of root meristems but continued exposure to auxin can have a negative effect on the explant and subsequently inhibit root growth. By reducing the exposure time to auxins it is possible to improve root growth. A pulse of IBA followed by a period on hormone free medium improved rooting with oak (Király *et al.,* 2001), *E. grandis x E. urophylla* (Cid *et al.,* 1999), *E. globulus* (Pelosi *et al.,* 1995), *E. regans* (Blomstedt *et al.,* 1991), and *E. nitens* (Willyams *et al.,*.1992). Therefore, eucalypt species appear specific to the type and concentration of PGR used and the time exposed (Tables 1.1 and 1.2).

Table 1.1 Shoot organogenesis of forestry eucalypt species closely related to *E. erythronema* and *E. stricklandii* and the ornamental species *E. sideroxylon*.

Species	Explant Source	Medium	Auxin	Cytokinin	Reference
grandis	S	MS	NAA	BAP/K	Watt *et al.*, 2000
tereticornis	MN	MSm	NAA	BAP	Sharma and Ramamurthy, 2000
grandis x *urophylla*	A	SP	NAA	BAP/Z/TDZ	Cid *et al*, 1999
graniticola	A	MS	K		Bunn and Dixon, 1997
grandis x *urophylla*	JN	MS	NAA	BAP	Yang *et al.*,1995
globulus	C	MSm	NAA	BAP	Bennett *et al.*, 1994
grandis x *E.* sp	JN	½MS	NAA	BAP	LeRoux and van Staden, 1991b
tereticornis	C	MSm	NAA	BAP	Das and Mitra, 1990
grandis	MN	MS	NAA	BAP	Warrag,*et al.*, 1989
tereticornis	MN	MSm	BAP/K		Gupta and Mascarenhas, 1987
sideroxylon	MN	½MS	NAA	BAP	Burger, 1987
camaldulensis	MN	MSm	BAP/K		Gupta *et al.*, 1983
grandis	MN	CN			Creswell and Nitsch, 1975
nitens	S	MSm	NAA	BAP	Gomes and Canhoto, 2003

Key

Explant source: A; aseptic seedling: C; coppice node: JN; juvenile node: MN; mature node: S; shoot

Medium: CN; Creswell and Nitsch (1975) medium
MS; Murashige and Skoog medium (1962): ½MS; half strength Murashige and Skoog: MSm; Murashige and Skoog modified
SP; highly modified MS medium

Auxin: NAA; α-Naphthaleneacetic acid

Cytokinin: BAP; 6-Benzylamino purine
K; kinetin
TDZ; 1-phenyl-3-(1,2,3-thiadiazol-5-yl) (Thidiazuron)
Z; zeatin

Table 1.2 Root organogenesis of forestry eucalypt species closely related to *E. erythronema* and *E. stricklandii* and the ornamental species *E. sideroxylon*.

Species	Explant Source	Medium	Auxin and other addatives	Reference
grandis	S	MS	IBA,P	Watt *et al.,* 2000
tereticornis	MN	½MSm	IBA	Sharma and Ramamurthy, 200
grandis x *urophylla*	A	SP	IBA,P,AC	*Cid et al.,* 1999
graniticola	A	½MS	IBA/NAA	Bunn and Dixon, 1997
grandis x urophylla	JN	MSm	IBA/NAA	Yang *et al.,*1995
globulus	C	MSm	IBA/D	Bennett *et al.,* 1994
grandis x E.sp	JN	½MSm	IBA	Le Roux and van Staden, 1991b
tereticornis	C	Knops	IBA	Das, Mitra, 1990
grandis	MN	¼MS	IBA	Warrag *et al.,* 1989a
tereticornis	MN	½MSm	IBA/NAA,P,AC	Gupta and Mascarenhas, 1987
sideroxylon	MN	½MS	IBA	Burger, 1987
camaldulensis	MN	MSm	IBA/NAA/IAA	Gupta *et al.,* 1983
grandis	MN,	CN	not rooted, studying phenols	Creswell, Nitsch, 1975

Key

Explant source	A; aseptic seedling: C; coppice node: JN; juvenile node: MN; mature node: S; shoot			
Medium	CN;	Creswell and Nitsch medium (1975)	Knops;	Tabachnik and Kester, 1977)
	MS;	Murashige and Skoog medium (1962)	½MS;	half strength Murashige and Skoog:
	¼MS;	quarter strength Murashige and Skoog	MSm;	Murashige and Skoog modified
	SP;	highly modified MS medium		
Auxin	IAA;	indole-3-acetic acid		
	IBA;	indole butyric acid		
	NAA;	α-Naphthaleneacetic acid		
Other	AC;	activated charcoal:		
	D;	initial dark period:		
	P;	PGR pulse		

1.3.2.7 Phenols

Phenols are produced by plants and appear to be associated with both pathogen protection and an interaction with growth hormones (Le Roux and van Staden, 1991a). When plant tissue is wounded, polyphenols and phenolic oxidases come in contact, and oxidation of polyphenols to quinones occurs. Quinones are highly toxic to plant tissue causing further death of surrounding cells (Taji and Williams, 1996). Phenols can leak into the surrounding medium and the polymerisation products appear as a brown exudate.

The problem of tissue browning and leakage can be reduced by removal of phenolic compounds through leaching, addition of antioxidants such as ascorbic acid, adsorption onto polyvinlyprriolidone (PVP) or activated charcoal, and manipulating environmental conditions (Taji and Williams, 1996). For example, soaking of *E. grandis* tissue in sterile water, followed by a period of darkness, reduced the concentration of phenolic compounds detected in the medium (Durand-Creswell, 1982). Reduction of the pH may also prevent enzyme activity and reduce oxidation (Taji and Williams, 1996).

1.3.2.8 Hyperhydricity

Plant morphology is affected by the physical and chemical nature of the *in vitro* environment. Explants can become swollen and brittle with a glassy appearance, a condition known as hyperhydricity. Hyperhydric shoots have large prominent stomata, reduced quality and quantity of epicuticular waxes and reduced chlorophyll efficiency (Ziv, 1991). Shoots show poor multiplication and rooting and cannot be hardened off to the external environment. This problem can be overcome for a wide range of woody plant species by a variety of methods. A reduction in hyperhydricity

can be achieved through manipulation of nutrient salts in the medium (Franclet and Boulay, 1982; Bouza *et al.,* 1992), reduced humidity (Bouza *et al.,* 1992; Williams, 1999), aeration of culture environment (Rossetto *et al.,* 1992; Johnson, 1997), changing type and concentration of gelling agents (Sutter, 1991; Bouza *et al.,* 1992; Johnson, 1997; de la Vina *et al.,* 2001; Druart, 2003) and type and concentration of plant growth regulators (Le Roux and van Staden, 1991a; Pereira *et al.,* 2000) and the use of commercial anti-hyperhydricity agents (Whitehouse *et al.,* 2002).

1.3.2.9 Fungal and bacterial contamination

Contamination of *in vitro* cultures by fungi and bacteria reduces explant health and vigour and results in lost cultures (Watt *et al.,* 1996; Taji, 1999; Cassells and O'Herlihy, 2003). Explant contamination with exogenous fungi or bacteria may become apparent soon after initiation into culture due to spores surviving the decontamination process or the proliferation of endogenous organisms may emerge months later. The need for sufficient decontamination procedures and good aseptic technique are the most important methods for reducing exogenous contamination problems (Taji, 1999). Endogenous organisms are more difficult to eliminate. Fungi may be controlled by the addition of fungicides to the medium (Shields *et al.,* 1984; Tynan *et al.,* 1993; Lizarraga *et al.,* 2001) while bacterial control can include the following methods; screening for contaminants at subculture (Cassells and O'Herlihy, 2003); acidification of the medium (Taji, 1999); additions to the medium such as antibiotics, egg white lysozyme (Marino *et al.,* 2003), and plant tissue culture preservative (formerly Plant Preservative Mixture) (Babaoglu and Yorgancilar, 2000). However, for each of these methods there are problems with effectiveness against a particular contaminant, or explant phytotoxicity to the agent used.

Bacteria and fungi can also be introduced via carriers such as mites. Mites are a very serious problem in the growth room environment, they are very difficult to eradicate and easily gain entry to the pot via the lid.

1.3.2.10 Transfer to the external environment

In vitro shoots can be transferred to the external environment before or after root initiation and growth. Prior to deflasking of shoots or plants the process of acclimatisation can commence with removal of culture pots from the growth room to either the general laboratory environment or the glasshouse. As the plant develops new leaves and roots, it becomes independent of the sterile, artificial environment (Louro *et al.,* 1999) and can be hardened off by transplanting into sterile soil, with a gradual reduction of humidity (Le Roux and van Staden, 1991b). The success of hardening off varies with eucalypt species. For example, *E. citriodora* is easier to transfer than *E. marginata* (McComb and Bennett, 1986).

Slow acclimatisation to the external environment appears necessary because of differences in the leaf structure of *in vitro* plants. *In vitro* eucalypt leaves have thin wax deposits and cuticle layers, reducing their resistance to low humidity compared to natural grown plants. The stomata can lose their ability to close and tend to be concentrated into clumps (Louro *et al.,* 1999). *E. grandis* showed a 40-80 day time period in which plantlets adjusted to greenhouse conditions and during this time, plantlets produced new leaves comparable to seedling leaves (Warrag *et al.,* 1989a).

Following hardening off, plants can be grown in the field and observed for their genotypic and phenotypic characteristics. The possibility of mutations occurring during *in vitro* culture must be assessed. During organogenesis the processes of dedifferentiation can result in chromosome changes resulting in physiological and

morphological changes in developing organ primordium (Schwarz and Beaty, 1996). This is termed somaclonal variation and can occur in high frequency in tissue culture propagation (Larkin and Scowcroft, 1981). Assessment of cloned plants in the field is essential to observe for genotypic and phenotypic variations in relation to the mother plant. For example, Bennett *et al.* (1986) found a difference in plant form between seed grown plants and micropropagated plants of *E. marginata.* Micropropagated plants produced no lignotuber or basal coppice growth and initially grew along the ground, but were taller than seedling plants after 2 years. Yang *et al.* (1995) found an advantage with micropropagated plants as they flowered one year earlier than seedlings. In contrast, micropropagated plants of *E. citriodora* showed identical concentrations of citronella oils with the parent tree (Gupta and Mascarenhas, 1987) and after three months in the field five year old micropropagated *E. grandis* plants showed no significant difference from seedlings of the parent plant (Warrag *et al.*, 1989b). Micropropagation, therefore, appears to be a viable method of clonal propagation for eucalypts.

1.3.3 Somatic embryogenesis

1.3.3.1 Introduction

Somatic embryogenesis is the development of seed-like embryos from individual non-zygotic cells (Berney, 2000). Embryogenesis can occur directly from somatic cells in zygotic embryos, microspores, reproductive tissue such as anther wall and ovary, young *in vitro* plantlets, or indirectly through callus production from juvenile tissue (hypocotyl, cotyledon, leaf, and stem), followed by the proliferation of embryos (Williams and Maheswaran, 1986). Embryos may then develop through the globular, heart, torpedo and cotyledonary stages, then mature and germinate. This

technique is applicable for large scale, rapid clonal propagation of selected individual plants.

The exact mechanisms that trigger somatic cells to form embryos are not known but probably include environmental conditions, the type and age of plant tissue, and the concentrations of plant growth regulators (Gray, 1996). The influence of these factors on somatic embryo development of eucalypts will be considered in the following sections.

1.3.3.2 Plant tissue

The age and type of plant tissue used appears important in the production of somatic embryos and the response of the explant varies greatly between and within species (Gray, 1999; von Aderkas and Bonga, 2000). Somatic embryogenesis of eucalypts has been achieved with plant tissue taken from mainly juvenile tissue and includes: 2-5 day old aseptic seedlings of *Eucalyptus citriodora* (Muralidharan and Mascarenhas, 1987; Muralidharan *et al.,* 1989), and *E. dunnii* (Termignoni *et al.,* 1996); three week old aseptic seedlings of *E. nitens* (Ruaud *et al.,* 1996); and for *E. globulus*, seedling leaf explants (Watt *et al.,* 1991) hypocotyls, cotyledons (Cranshaw and Smith, 1995; Nugent *et al.,* 2001; Pinto *et al.,* 2002), and mature zygotic embryos (Pinto *et al.,* 2002). Ruaud *et al.* (1997) found that hypocotyls of *E. nitens* produced the most embryos, and that this response varied between seedlings, indicating a genotype effect. The only report of somatic embryogenesis from mature tissue comes from Qin and Kirby (1992) using micropropagated epicormic shoots of *E. grandis x urophylla,* suggesting a possible rejuvenation of the tissue through subculturing. Maturation and germination of somatic embryos has been reported from *E. globulus*

(Pinto *et al.,* 2002), *E. grandis* (Watt *et al.,* 1991), and *E. citriodora* (Muralidharan *et al.,* 1989). These plants have been successfully transferred to the field.

1.3.3.3 Culture environment

The culture environment, including conditions of culture medium, light, and temperature all influence somatic embryogenesis and can be specific to, and vary with each species (Gray, 1996; Wilhelm, 2000). Of the six species of eucalypt and five media trialled by Qin and Kirby (1992) all produced callus, but embryogenesis occurred only when specific media were used for each species. Bandyopadhyay *et al.* (1999) found greater embryo production on MS medium compared to B5. However, other investigations with eucalypts have used B5 medium (Muralidharan and Mascarenhas, 1987; Muralidharan *et al.,* 1989; Termignoni *et al.,* 1996).

In other woody species such as *Picea stichensis,* a bubble bioreactor significantly improved embryogenesis compared to the shake flask (Ingram and Mavituna, 2000). This has not been tried with eucalypts.

Initial culture in the dark appears necessary to allow embryonic development and to prevent maturation processes triggered by light. Later, light is required for growth and germination of the embryo (Gray, 1996; Termignoni *et al.,* 1996). Temperature requirements for somatic embryogenesis appear slightly higher than those used for micropropagation. For example, initiation temperatures of 27-30°C have been applied to eucalypts (Muralidharan and Mascarenhas, 1987; Muralidharan *et al.,* 1989; Termignoni *et al.,* 1996) compared with 24°C for micropropagation.

1.3.3.4 Plant growth regulators

To stimulate somatic embryogenesis a specific ratio of plant growth regulators (PGR) such as auxins and cytokinins for a particular genotype appears necessary (Razdan, 1993; Wilhelm, 2000; Lambardi and Rugini, 2003; Mondal, 2003). Furthermore, the PGR ratio changes as the embryos develop. High auxin levels are used initially to suppress cell differentiation and increase embryogenesis through repeated cell division (Gray, 1996). Auxins are then removed in the proliferation phase to allow embryo development and maturation (Muralidharan *et al.,* 1989; Neuman *et al.,* 1993; Termignoni *et al.,* 1996).

Maturation of the embryo is an important step for subsequent plant development and can be promoted with the addition of plant growth regulators and other chemicals to the medium. Additions can include high sucrose levels, abscisic acid, polyethylene glycol, and glutamine (Gray 1996). For example, Qin and Kirby (1992) achieved maturation of somatic embryos in six species of eucalypt by changing the proportions of auxin, gibberellin, and abscisic acid. Qin and Kirby (1992) also found that the addition of spermidine to the medium improved embryo maturation with *E. grandis*.

1.3.3.5 Physical and chemical methods of somatic embryo induction

Somatic embryogenesis can be induced by applying physical and chemical treatments such as temperature and smoked water. Smoked water is known to improve seed germination in Australian native species (Wills and Read, 2000; Allen *et al.,* 2004). Senaratna *et al.* (1999) used smoked water to enhance somatic embryogenesis in hypocotyls of *Pelargonium hortorum*, although the relationship between smoked water and somatic embryos is unknown. Flematti *et al.* (2004) have

isolated an active compound, butenolide, from smoked water and suggest this chemical's action is similar to a plant growth regulator. Butenolide may therefore play a role in seed germination and/or somatic embryogenesis.

A correlation was observed in carrot (Apuya and Zimmerman, 1992), cork oak (Puigderrajols *et al.,* 2002), white spruce (Dong and Dunstan, 1996), and Douglas fir (Misra *et al.,* 1996), between the synthesis of heat shock proteins, in the absence of heat, and somatic embryogenesis. Heat, in the absence of PGR, is reported to induce somatic embryos in callus of the gladiolus cormel (Kumar *et al.,* 2002), *Brassica napus* microspores (Cordewener *et al.,* 2000), tobacco microspores (Touraeu *et al.,* 1996a; Touraeu *et al.,* 1996b), and sunflower seed callus (Perrotta *et al.,* 1992). Heat treatment may stimulate somatic embryo production in otherwise recalcitrant species, while the role of smoked water is unclear.

1.3.3.6 Embryo development

Somatic embryos may develop following callus induction and can be seen as either hard, white, compact organised sections (Termignoni, *et al.,* 1996), or white, friable areas on the callus (Bandyopadhyay *et al.,* 1999). However, in *E. nitens* a callus phase was not necessary, as somatic embryos grew along hypocotyl tissue from proliferating provascular cells (Ruaud *et al.,* 1997), and *E. grandis* produced embryo like structures directly from young stems and leaves (Quin and Kirby, 1992).

A comparison of zygotic and somatic embryo development shows there are some similarities (Bandyopadhyay and Hamill, 2000). While both develop similar structures, somatic embryos have a significantly greater incidence of abnormal development (Gray, 1996; Termignoni *et al.,* 1996) and are surrounded by a thin waxy cuticle instead of nutritive tissue and seed coat (Bandyopadhyay and Hamill,

2000). Structures in the zygote develop sequentially within the plant, whereas for the somatic embryo, all stages of development are occurring simultaneously on the explant (Gray, 1996) (Table 1.3). Sequential development by somatic embryos can be induced with modifications to the medium such as the addition of silver nitrate, a known ethylene inhibitor (Fuentes *et al.,* 2000).

Somatic embryos may be manipulated *in vitro* to improve embryo proliferation. Muralidharan *et al.* (1989), found that embryogenesis from explants of *E. citriodora* was encouraged by repeated subculturing to produce an embryonic mass with increased incidence of germination. This is one of four reports of successful eucalypt regeneration via somatic embryogenesis. Germination in other eucalypt species is limited, with embryo development often blocked at the globular, heart (Termignoni *et al.,* 1996) or torpedo stage (Bandyopadhyay and Hamill, 2000). Eucalypts have high levels of anthocyanins and these may play a role in embryo development. For example, *E. dunnii* embryos containing anthocyanin remained globular while those without anthocyanin developed to maturity followed by cotyledon growth (Termignoni *et al.,* 1996).

Table 1.3 A comparison of zygotic embryo and somatic embryo development. (from Gray, 1996)

Zygote	Somatic embryo
seed coat	no seed coat
nutritive seed tissue	no nutritive seed tissue, relies on growth medium
synchronised development on the explant of zygotes within the ovary globular heart torpedo cotyledonary	asynchrony, all stages occurring simultaneously
compressed shape	almost spherical
	significantly greater incidence of abnormalities extra and abnormal cotyledons poorly developed apical meristems do not mature properly cells disorganise, forms new embryonic cells secondary embryogenesis
non-clonal	clonal

1.3.3.7 Artificial seed production

Following embryogenesis the somatic embryo can be encapsulated with a gel like substance, which hardens to form an artificial seed coat, and either stored, transported, or planted (Razdan, 1993). Encapsulation would allow seed to be stored and handled in a similar way to normal seed, and it would be pathogen free and pass quarantine restrictions. The use of calcium alginate beads containing nutrients, hormones, and mycorrhiza, to encapsulate somatic embryos from a range of difficult to propagate Australian natives is being tested (Berney, 2000). Dupuis *et al.* (1997) have developed a cellulose acetate mini-plug as an artificial seed coat giving a 90% germination rate with carrot.

1.3.3.8 Light microscopy

Light microscopic investigation of developing somatic embryos can be used to study their origin and development, and to compare them with zygotic embryos (Watt *et al.,* 1991; Bandyopadhyay and Hamill, 2000). By examining morphological differentiation in this way, it should be possible to compare more accurately the factors that inhibit the development of somatic embryos and to avoid these in favour of contributing factors. Light microscopy is also required to determine that regeneration from callus is through somatic embryogenesis and not organogenesis.

1.4 Conclusion

Micropropagation of forestry eucalypts has been extensively studied, and the results indicate that there is great variation in plant regeneration response within and between species. The most successful regeneration of forestry species has been reported using juvenile tissue, with only limited reports of successful use of mature

tissue. Micropropagation of hybrid ornamental species has received little attention. Superior hybrid ornamental species can be selected only following maturity but mature tissue is more difficult to regenerate. A number of factors need to be considered, including sterilisation procedures, prevention of polyphenol oxidation, optimum nutrient requirements, type and concentration of plant growth regulators, and overcoming endogenous rooting inhibitors. These factors will form part of this research project to produce clones of *E. erythronema, E. stricklandii* and their hybrids, including *E.* 'Urrbrae Gem'.

Somatic embryogenesis has not been extensively studied in eucalypts and this project will also investigate the development of somatic embryos by *in vitro* cultures of the ornamental species *E. erythronema, E. stricklandii* and their hybrids, including *E.* 'Urrbrae Gem'. Microscopy will be used to follow organogenesis and somatic embryo development and aid in the comparison of different treatments and their effects on organogenesis and somatic embryogenesis.

1.5 Project aims

The main aim of this project was to investigate clonal propagation by tissue culture of hybrids between *Eucalyptus erythronema* and *E. stricklandii* including the selected superior specimen, *E.* 'Urrbrae Gem'. Work towards this aim involved research on the parental species *E. erythronema* var. *erythronema,* and *E. stricklandii* as well as open pollinated seedlings from *E.* 'Urrbrae Gem' to investigate methods of micropropagation and somatic embryogenesis. Optimal methods will be further developed as a basis for the propagation of other selected ornamental hybrid eucalypts from this cross and to enhance knowledge of the growth and development of shoots and somatic embryos using microscopy techniques. The development of a reliable and

rapid method of clonal propagation for ornamental hybrid eucalypts is essential for their commercial development.

Chapter 2

Plant Material

2.1 Introduction

The eucalypt plants selected for trial were:

- Mature tree of *Eucalyptus erythronema* var. *erythronema* Turcz and *E. erythronema* var *marginata* (Benth.) Domin
- Mature tree of *E. stricklandii* Maiden
- *E.* 'Urrbrae Gem', a natural inter-specific hybrid of *Eucalyptus erythronema* var. *erythronema* and *E. stricklandii*
- Hybrids of *Eucalyptus erythronema* var. *erythronema* x *E. stricklandii* from controlled hand pollination
- Open pollinated seedlings of *Eucalyptus erythronema* var. *erythronema*
- Open pollinated seedlings of *E. stricklandii*
- Open pollinated seedlings of *E.* 'Urrbrae Gem'

2.2 Mature trees of *Eucalyptus erythronema* var. *erythronema* Turcz and *E. erythronema* var. *marginata* (Benth.) Domin

E. erythronema (Plate 3.1) is classified in the series *Elongatae* Blakely, subsection *Glandulosae* Brooker, section *Bisectae* Maiden ex Brooker, subgenus *Sympyomyrtus* (Schauer) Brooker, genus *Eucalyptus* L'Hér (Myrtaceae) (Brooker, 2000). Natural distribution of *E. erythronema* var. *erythronema* is in Western Australia, from north of Watheroo to Kondinin, while *E. erythronema* var. *marginata* is found from Wubin to Wongan Hill. Both are found on flat areas, often in stony or gravelly soils, and in open

shrubland and woodland. An established tree of *E. erythronema* var. *marginata* (tree number 1867) planted in 1966, was growing in the Waite Arboretum, University of Adelaide.

2.3 Mature trees of *E. stricklandii* Maiden

E. stricklandii (Plate 3.1) is classified in the series *Stricklandianae* Brooker, with subsection, section, subgenus, and genus as for *E. erythronema* (Brooker, 2000). *E. stricklandii* is found from Coolgardie to Norsman in Western Australia on ridges, rocky hills and sometimes near creeks in open woodland (George, 1988). An established tree of *E. stricklandii* (tree number 1614) planted in 1970 was growing in the Waite Arboretum, University of Adelaide.

2.4 *Eucalyptus* 'Urrbrae Gem'

Eucalyptus 'Urrbrae Gem' (tree number 1867, plated 1937) (Plate 3.2) is a natural interspecific hybrid of *E. erythronema* var. *erythronema,* discovered by Mr. F. A. Couzens in 1936 and planted in the Waite Arboretum, University of Adelaide (Gardner, unpublished, 1987). *E.* 'Urrbrae Gem' is a small tree with spreading branches, smooth white bark, glossy green leaves, reddish blossoms with the umbel up to 8 cm across, and can flower for up to 10 months between August and May (Kelly, 1969; Gardner, unpublished, 1987). The identity of the male parent was confirmed as *E. stricklandii* by DNA fingerprinting (Delaporte *et al.*, 2001a) and morphology (Delaporte *et al.*, 2001b). Grafted scions of *Eucalyptus* 'Urrbrae Gem' in the glasshouse were on *E.* 'Urrbrae Gem' seedling rootstock.

2.5 Hybrids of *Eucalyptus erythronema* var. *erythronema* x *E. stricklandii*

Several artificial hybrids were produced by crossing *E. erythronema* var. *erythronema* x *E. stricklandii* (Delaporte *et al.*, 2001b) (Plate 3.3). Progeny are planted in the Laidlaw Plantation at the Waite Campus, University of Adelaide, and in pots in the glasshouse. These crosses are derived from the same parental species as *E.* 'Urrbrae Gem'. The genotypes vary in leaf size, shape, colour, habit and bark colour. The trees had not flowered when material was collected for the experiments from January 2000 to December 2002, but flowered subsequently in spring/summer 2003. Field grown hybrids used for experiments were 20A, 20C, 20E, 20G, 20H, 20I, 20K, 20P, 20R, 20T, 20V, 20W, 20Y, 21A, 21D, 21G, 21H, 21K, 21R and 21U. Glasshouse grown hybrids were 35.2 and 2.5.

2.6 Open pollinated seedlings of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem'.

Seeds of *E. erythronema* var. *erythronema* (Lot 1596EJI, October 1998 and lot 1395PV2, September 2002) were obtained from Royston Petrie Seeds Pty Ltd (Victoria, Australia), as insufficient seeds for experimentation were available from the Waite Arboretum tree. Seeds of *E. stricklandii* were collected from the Waite Arboretum (tree number 1614, planted 1970) in October 2002. Open pollinated seeds of *E.* 'Urrbrae Gem' were collected on January 1995. Prior to planting, seeds stored at room temperature were given a 3 day cold treatment to improve germination, and then isolated from the chaff.

Seeds of *E.* 'Urrbrae Gem' were germinated in January 1999 on moist vermiculite, transplanted to 4 inch pots and grown in the glasshouse. Soil mixture contained native mix, pinebark: sharp white sand (2:1). Each one L of this mix contained 1.45 g of nutrients comprising iron sulphate: dolomite: lime: gypsum: osmocote native controlled release fertiliser (N:P:K 17:1.6:8.7): pH 6, in the ratio of 8:10:5:5:30.

E. 'Urrbrae Gem', *E. erythronema* var. *erythronema* and *E. stricklandii* seeds were sterilised and germinated *in vitro*. Seeds were sterilised by soaking in 70% ethanol for 30 sec, then 3% NaOCl for 20 min, followed by three rinses in sterile Milli Q water. Sterilised seeds were placed in 250 mL polycarbonate pots with 40 mL per pot Murashige and Skoog (1962) (MS) medium with 30 g^{-1} sucrose, gelled with 7 g^{-1} Phytagel®, and placed in the dark at 24°C. After 7 days, light was given for 16 h per day at 75 µmoles m^{-2} sec^{-1} (Plate 3.4). Cultures from two seedlings of *E.* 'Urrbrae Gem' (line 1 and 3) were particularly responsive in culture.

Plate 2.1 Top) Flowers and buds *Eucalyptus erythronema* Turcz

Bottom) Flowers, buds and leaves *Eucalyptus stricklandii* Maiden

Plate 2.2 Top) *Eucalyptus* 'Urrbrae Gem', original tree planted in the Waite Arboretum, 1936.

Bottom) Flowers of *Eucalyptus* 'Urrbrae Gem'

Plate 2.3

A) A row of *E. erythronema* var. *erythronema* x *E. stricklandii* hybrids growing in the Laidlaw Plantation, Waite Campus, University of Adelaide 2001.

B) Flowers of *E. erythronema* var. *erythronema* x *E. stricklandii* hybrid 20P growing in the above row in December 2003 (courtesy of Dr. K. Delaporte)

A

B

Plate 2.4 Three week old seedling of *E.* 'Urrbrae Gem' germinated *in vitro.*

Chapter 3

Explant initiation and optimisation of callus growth

3.1 Explant initiation

3.1.1 Introduction

The first two steps of tissue culture involve explant selection and initiation. In general, nodal explants from stems are selected for direct organogenesis via shoot proliferation while leaf, hypocotyls and cotyledons are selected for indirect organogenesis via callus production and bud development or somatic embryogenesis. Initiation involves the establishment of aseptic plant material into culture. Because there were no published reports for the ornamental species under investigation, culture techniques reported for forestry species (Sharma and Ramamurthy, 2000; Watt *et al.*, 2000; Le Roux and van Staden, 1991a; Gupta and Mascharenhas, 1987; McComb and Bennett, 1986) were used as a guide, with small empirical experiments conducted for each step. Factors studied on both stem and leaf explants were time of year for explant collection and location of source material, control of contamination using fungicides and antibiotics, methods of sterilisation, control of explant browning, the role of light and dark, gelling agents, and the effect of temperature, mineral nutrients, microelements and other additives. The aim was to provide a protocol suitable for establishing explants into culture for future experimentation. The protocol was derived by small empirical experiments using nodal and leaf explants of *Eucalyptus erythronema, E. stricklandii, E.* 'Urrbrae Gem', and glasshouse material from grafted plants of *E.* 'Urrbrae Gem' and *E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5

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3.1.2 Summary

From the small empirical experiments, material was successfully initiated into culture as follows.

- Leaf and nodal material was collected from flush growth of field-grown trees in late spring and autumn that had been sprayed weekly for three weeks with 0.5 g^{-1} Benlate® (DuPont, 500 g/kg benomyl) and 20 ml^{-1} Pyrethrum (Ausgro echnologies Pty Ltd, 4 g^{-1} pyrethrins, 16 g^{-1} piperonyl butoxide), or from plants growing in the glasshouse at any time of the year. The decontamination of explants was greater with pre-harvest sprays compared to dipping in fungicides or addition of fungicides and antibiotics into the medium.
- Shoots were washed for 1-2 h on an orbital shaker at 60 rpm in RO water, with 2 drops of Tween 20 per L.
- Material was decontaminated for 15 min in 1% NaOCl with gentle agitation, followed by 3 rinses in sterile Mill Q (MQ) water.
- Material was placed for 72 h in sterile water at 15°C in continuous light to allow for leaching of phenols.
- Stems were cut into 10 mm length sections with one node and petiole.
- The edges and tips of leaves were removed, and the lamina dissected across the mid rib in 2-3 mm wide sections.
- Media were autoclaved for 20 min at 121°C.
- Explants were placed individually into 30ml polycarbonate tubes with 10 mL of medium consisting of 20 g^{-1} sucrose, 1 mM ascorbic acid, gelled with 10 g^{-1} Phytagel® (high levels of Phytagel are required to set medium with low or no nutrient salts).

- Explants were placed in the growth room at 24°C with 16 h day at 11 μmoles m^{-2} sec^{-1}.
- Explants were subcultured onto the above medium every 48 h for 7 d to remove explants from media browning presumed to be polyphenols and/or quinones from the oxidation of polyphenols, which are highly toxic to plant tissue.
- Explants were subcultured to the above medium plus □ MS salts with full strength MS vitamins for 7 d.
- Explants were subcultured onto the above medium plus ¼ MS for 7 d, then ½ MS, 20 g^{-1} sucrose, gelled with 7 g^{-1} Phytagel®, supplemented with 1 μM BAP and 10 μM NAA for callus growth on leaf explants and 4.4 μM BAP and 1 μM NAA to encourage shoot growth on nodes.
- Callus and shoots were harvested and used for experiments as adequate material became available.

3.1.3 Discussion

Explants from *Eucalyptus erythronema, E. stricklandii, E.* 'Urrbrae Gem' (field and glasshouse), and *E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5 showed some degree of phytotoxic reaction to the various fungicides, decontamination agents, gelling agents and media nutrients that were employed. Phytotoxic effects were diminished by the following: using Phytagel® instead of agar (Sigma) as the gelling agent, use of the an antioxidant ascorbic acid within the medium, cold treatment, leaching of phenols in sterile water, light, frequent subculturing (to remove explants from phenols leached into the medium) and reduced nutrient salt concentration of the medium. Initiation of field explants into culture was difficult, even with healthy, rapidly growing, spring and autumn

shoots. Successful decontamination of field explants was only achieved during the growing season in November-December and March-April when rapid flush growth is produced and when this growth was sprayed with an insecticide and fungicide. During the cooler, wetter months of June to September the field explants could not be decontaminated. Following initiation on a basic medium comprising only sucrose, Phytagel®, and ascorbic acid, the introduction of nutrient salts to the explant required low levels of macro and micro MS elements together with full strength MS vitamins or rapid explant death occurred. After this low introduction to nutrients surviving explants could then be successfully introduced to media with higher nutrient salt concentration containing plant growth regulators.

Explants collected from plants growing under glasshouse conditions were generally more sensitive to sterilants, and fungicides, but showed less contamination in culture. This material also has the advantage that it can be collected all year round.

3.2 Optimisation of Callus Growth

3.2.1 Introduction

During the preparation of explants from plant tissues, some cells are inevitably injured. These injured cells produce ethylene, inducing inner cell layers to undergo dedifferentiation (Ziev, 2000). Plant growth regulators can be chosen to stimulate these dedifferentiated cells into either unorganised (callus) or organised cell division (meristematic centres) (Ziev, 2000). Callus, in turn, can be induced to form meristematic centres that can further develop into shoots, roots or somatic embryos, depending on the physical and chemical stimuli (Schwarz and Beaty, 1996). The components of these stimuli vary greatly from species to species (Caponetti, 1996) and include physical aspects such as light, temperature, and the style of container, as well as chemical aspects

such as plant growth regulators, medium, and gelling agent (Doran, 1996). The composition of the medium is considered to be one important aspect for callus growth (Taji and Williams, 1996) with different eucalypt species showing different medium requirements for initiation, growth of callus, and plant regeneration (Johnson, 1997; Watt *et al.*, 1991; Muralidharan and Mascarenhas, 1989). Initiation of *E.* 'Urrbrae Gem' explants into culture required low mineral nutrient levels (□ MS with full strength MS vitamins, Section 3.1.2), however, the growth of callus on this medium was slow and regeneration did not occur.

The production of callus is an important step in plant regeneration by organogenesis and somatic embryogenesis, and it is also considered to be a useful research tool when determining the physical and chemical requirements of a species or genotype in culture (Caponetti, 1996). This section describes the experiments that were conducted to test the effect of five different media, at three concentrations, on the growth and regeneration of callus originating from leaf explants of *E.* 'Urrbrae Gem' and *E.* 'Urrbrae Gem' open pollinated seedlings.

3.2.2 Materials and methods

Mature canopy leaves of *E.* 'Urrbrae Gem' were collected and initiated into culture were as described in Section 3.1.2. Callus derived from leaf explants was maintained by subculturing every 3 - 4 wk on □ MS with full strength MS vitamins, 20 g^{-1} sucrose, 10 g^{-1} Phytagel®, 1 μM benzylaminopurine (BAP) (Sigma) and 10 μM naphthaleneacetic acid (NAA) (Sigma) pH 5.7, 16 h per day at 75 μmoles m^{-2} sec^{-1}.

Open pollinated seeds of *E.* 'Urrbrae Gem' were sterilised by soaking in 70% ethanol for 5 min, then 3% NaOCl for 20 min, followed by three rinses in sterile Milli Q water. Sterilised seeds were placed on 40 mL MS medium, containing 30 g/L sucrose, and

gelled with 7 g^{-1} Phytagel®, in 250 mL polycarbonate pots and incubated in the dark at 24°C. After 7 days light was given for 16 hr/day at 75 µmoles m^{-2} sec^{-1}.

Pieces of *E.* 'Urrbrae Gem' callus of similar size and appearance in texture and colour (hard, friable, yellow), selected at 6 months, and *E.* 'Urrbrae Gem' seedling leaf explants, harvested at 6 wk, were allocated to the following media (Table 3.1)

- MS, Murashige and Skoog (1962)
- B5, Gamborg *et al.* (1968)
- WPM, Lloyd and McCown (1980)
- EUC, de Fossard *et al.* (1978)
- AP, Almehdi and Parfitt (1986)

All five media were used at the following strengths

- full strength salts and full strength vitamins
- ½ salts and ½ vitamins
- □ salts and full strength vitamins

The media contained 20 g^{-1} sucrose, were adjusted to pH 5.7, and Phytagel® was used as the gelling agent at 7 g^{-1} for full and ½ strength salts, and 10 g^{-1} for □ strength salts. This difference in the amount of Phytagel® was needed to achieve the same gel consistency at the different salt concentrations. The plant growth regulators remained the same as for *E.* 'Urrbrae Gem' callus maintenance (as above). The media were poured into petri dishes (55 x 14 mm) with 10 - 15 mL of media per dish. Each petri dish was allocated either 100-300 mg of *E.* 'Urrbrae Gem' callus, or one leaf explant from *E.* 'Urrbrae Gem' seedlings. The petri dishes were sealed with several layers of parafilm, and incubated at 24°C with 16 h light at 11 µmoles m^{-2} sec^{-1} and 8 h dark.

Table 3.1 Macro and micro elements, vitamins and growth factors for full strength MS, B5, WPM, EUC, and AP media (μmoles/L, mg/L) (Refer previous page for references to media)

Component			**Medium**		
	MS	**B5**	**WPM**	**EUC**	**AP**
Macronutrients μmoles/L					
Nitrogen	842	376	207	421	405
Phosphorous	39	30	39	32	35
Potassium	783	965	493	390	965
Calcium	120	41	94	80	41
Magnesium	36	24	36	36	18
Sulphur	55	71	238	74	97
Micronutrients μmoles/L					
Chloride	210	72	46	141	72
Iron	5.6	5.6	5.6	5.6	5.6
Sodium	0.0475	22.2	0.0475	38.5	25.5
Boron	1.1	0.35	1.1	0.35	0.53
Manganese	5.5	3.3	7.26	1.3	6.6
Zinc	2.0	0.5	2.0	1.3	0.5
Copper	0.0063	0.0063	0.0063	0.0063	0.0126
Molybdenum	0.1	0.1	0.1	0.0096	0.024
Cobalt	0.00625	0.00625	0:0	0.03	0.0075
Iodine	0.61	0.57	0.0	0.32	0.57
Vitamins & growth factors mg\L					
Thiamine HCL	0.1	10	0.1	13.50	
Nicotinic acid	0.5	1	0.5	4.90	
Pyridoxine HCL	0.5	1	0.5	1.20	2.0
Glycine	2.0		2.0	3.75	
Biotin				0.24	
D-Ca pantothenate				1.19	
Riboflavin				3.76	
Ascorbic acid				1.98	
Choline chloride				1.39	
L-Cysteine				14.5	
Myo-inositol	100	100	100	100	25
pH	5.7	5.7	5,8	5.7	5.7

The treatments were arranged as a randomised complete block design with 10 replicates per treatment. Callus was weighed at the beginning of the treatments and again after 5 wk, and the change in weight was calculated as a percentage of the original weight. The data were analysed for differences at the P=0.05 level of significance using Genstat 5 Release 4 (Lawes Agricultural Trust). As the normal plot was slightly curved, the data were transformed to the log scale. Due to the difficulty in accurately weighing the seedling leaf explants, treatment effects were assessed using the scoring system given in Table 3.2 and the means calculated. Levels of browning (exudates) in the medium were also scored.

Table 3.2 Scoring method for the assessment of leaf browning, callus growth and medium browning.

Explant score

Score	Description
0	No blackening
1	Blackening of cut edges of explant
2	Above plus small areas of explant
3	Greater than half explant black
4	All of the explant black

Callus growth on leaf explant

Score	Description
0	No callus growth
1	Small callus on leaf margins, cut edges (3-5 mm)
2	Above plus over explant surface (6-10 mm)
3	Callus covers explant surface (11-15 mm)
4	Explant not visible, callus growing throughout container (15+ mm)

Browning in the medium

Score	Description
0	No brown staining around the explant
1	Slight browning around the explant
2	Light brown staining around the explant
3	Browning extending away from explant
4	Medium in container is brown
5	Dark brown staining of medium throughout the container

3.2.3. Results

E. 'Urrbrae Gem' callus

Results are shown in Figure 3.1. After 5 wk, callus growth on media containing full strength salts and vitamins was significantly greater on MS, B5, WPM and AP compared to EUC. Callus growth on media containing ½ salts and ½ vitamins was significantly greater for MS and B5 compared to WPM, EUC and AP. Callus growth on

media containing □ salts and full strength vitamins was significantly greater for B5 and WPM compared to AP and EUC and MS.

When each medium was compared, there was significantly lower callus growth on those containing □ salts and full strength vitamins compared to those containing either ½ salts and ½ vitamins or full strength salts and vitamins. Generally, callus growth tended to increase with increasing nutrient salt concentrations for all media except for EUC.

Callus colour and appearance on MS and B5 was mainly yellow with varying sized red areas of hard, friable, and glassy callus, compared to callus on WPM which was hard, nodular, yellow and opaque. Callus on AP tended to be soft in consistency, with grey and white areas, while callus on EUC turned brown. Callus on □ salts and full strength vitamins tended to turn brown by five weeks with brown exudates in the medium. No buds, shoots or roots developed from the callus.

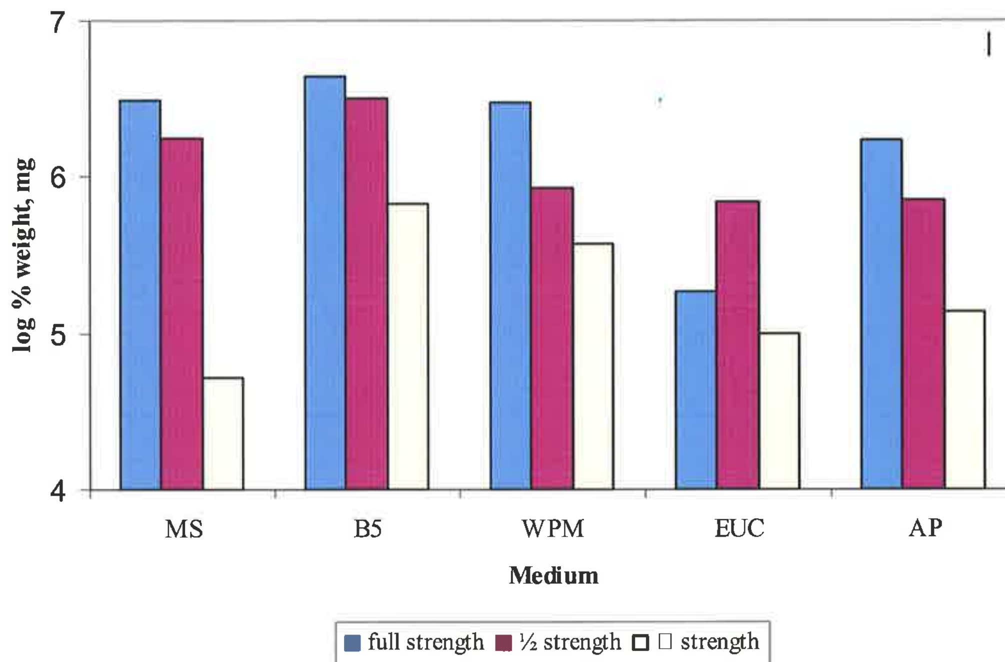


Figure 3.1: Effect of five nutrient media at three different concentrations, on callus growth, as scored per Table 3.2, of *E.* 'Urrbrae Gem', LSDs bar at the *P*=0.05 level (0.38).
Full strength = full strength salts and vitamins; ½ strength = ½ salts and ½ vitamins; □ strength = □ salts and full strength vitamins.

<u>Open pollinated seedlings of *E.* 'Urrbrae Gem'</u>

On MS media, explant blackening increased as the salt concentration increased, but callus growth also increased (Table 3.3). On B5 media, explant blackening was greater with ½ salts and ½ vitamins compared to full strength of both salts and vitamins, but callus growth was higher on full strength salts and vitamins and ½ salts and vitamins compared to □ salts and full strength vitamins. On WPM media, there was increasing explant blackening as the salt concentration of the medium decreased, with exudates observed for media containing □ salts and full strength vitamins. However, callus growth increased as the salt concentration of the medium increased. On EUC media, leaf blackening was most severe at □ salts and full strength vitamins and least at ½ salts and ½ vitamins, but explants on the latter developed more callus growth than either full strength salts and vitamins or □ salts and full strength vitamins. Overall, callus growth

was poor on all EUC media, with exudates in the medium at full strength. On AP media, explant blackening was consistent over all treatments (score 2.5-3.0) as well as callus growth (1.3-2.0) (Table 3.3).

Shoots developed from the petioles on three of the ten leaf explants cultured on EUC medium containing ½ salts and ½ vitamins. These shoots were subcultured on the same medium and regeneration occurred via shoot development from axillary nodes. Roots developed from the petiole of one leaf explant cultured on MS medium containing □ salts and full strength vitamins.

Table 3.3 Mean explant, callus and exudate score from leaf explants of open pollinated seedlings of *E.* 'Urrbrae Gem'. ½ = ½ salts and ½ vitamins; □ = □ salts and full strength vitamins; otherwise: full strength of both salts and vitamins

Medium	**mean explant score**	**mean callus score**	**exudates in medium**
MS	3.2 (de)	1.7 (ef)	0.0 (a)
½ MS	2.7 (bcde)	1.6 (def)	0.0 (a)
□ MS	1.7 (a)	0.1 (a)	0.1 (a)
B5	2.2 (ab)	1.9 (f)	0.0 (a)
½ B5	3.4 (e)	2.0 (f)	0.0 (a)
□ B5	3.3 (de)	1.5 (def)	0.0 (a)
WPM	2.4 (abc)	1.6 (def)	0.0 (a)
½ WPM	3.0 (cde)	1.6 (def)	0.0 (a)
□ WPM	3.4 (e)	1.1 (cd)	0.5 (c)
EUC	2.2 (ab)	0.5 (ab)	0.4 (bc)
½ EUC	1.8 (a)	0.7 (bc)	0.1 (a)
□ EUC	3.2 (de)	0.2 (ab)	0.2 (ab)
AP	2.6 (bcd)	1.3 (de)	0.0 (a)
½ AP	2.9 (bcde)	1.5 (def)	0.0 (a)
□ AP	2.7 (bcde)	1.3 (de)	0.0 (a)
LSD	±0.7	±0.5	±0.2

LSD *P*=0.05, means followed by the same letter are not significantly different

3.2.4 Discussion

The five media tested were used to examine the effects of different types and concentrations of macro-and micro-elements, vitamins, and growth factors on callus originating from leaf explants of *E.* 'Urrbrae Gem' and *E.* 'Urrbrae Gem' open pollinated seedlings. Macro-elements are used in structural components and enzyme cofactors (Taji and Williams, 1996) and are essential for plant life (Sutter, 1996). Micro-elements are used in enzyme activity and oxidation/reduction reactions while vitamins and myo-inositol are found to improve plant growth, while thiamine is essential for carbohydrate metabolism (Sutter, 1996). However, little is known of mineral nutrient uptake *in vitro* and of mineral element movement through the gel matrix, or the influence of different gelling agents and their relationship with pH (Williams, 1993).

The pH of the medium may influence the availability of mineral nutrients to the explant. Although a buffering system is created when nitrogen is added as both ammonium and nitrate (Woodward and Bennett, 1997), the pH can alter during plant tissue growth (Williams, 1993) particularly as nitrogen is utilised. Explants on media containing only ammonium can show phytotoxicity, while media with only nitrate can show reduced plant growth (Sutter, 1996). The form of nitrogen in B5 and AP media is mostly as nitrate but WPM, EUC and MS contain both nitrate and ammonium, therefore, the pH of B5 and AP could be unstable over time due to lack of a buffering system. However, the type of nitrogen or its possible effects on the pH, did not appear to be a limiting factor as callus from both *E.* 'Urrbrae Gem' and its seedlings grew equally well on MS and B5.

WPM contains higher sulphur levels (238.5 μmoles/L) compared to the other media (55.4-97.3 μmoles/L). This did not appear to influence callus growth, but callus appearance was more yellow, compact and nodular on WPM compared to callus on other

media. The concentration of salts in the media had a greater influence on callus growth than the type of macro-elements, with the lowest levels of callus growth being seen on media containing □ salts and full strength vitamins. *E.* 'Urrbrae Gem' callus showed a significantly higher requirement for nutrient levels for growth compared to those found necessary for the initiation of explants into culture (Section 4.9). A search of the literature did not reveal any other examples of a difference between initiation of mature field explants and callus growth as regards the level of macro-and micro-nutrients required. It appears that once *E.* 'Urrbrae Gem' callus has been successfully initiated into culture the callus develops a requirement for higher nutrient levels. A comparison of energy (carbohydrate) use in these two stages would be interesting.

The type and concentration of vitamins and growth factors is the same for MS and WPM media while B5 has a higher concentration of thiamine, nicotinic acid and pyridoxine. The higher levels of vitamins, not the inclusion of glycine in B5 did not appear necessary as callus grew equally well on all three media. AP medium contains pyridoxine but has no thiamine or nicotinic acid and includes lower levels of myo-inositol compared to MS, B5 and WPM. Thiamine is known to be essential for the growth of tobacco callus (Taji and Williams, 1996) and is found in the coenzyme thiamine pyrophosphate, which is essential for metabolic reactions (Marschner, 1990), cytochromes, fatty acids, sterols and some hormones such as gibberellic acid and abscisic acid (Salisbury and Ross, 1978). Myo-inositol is important for membrane and cell wall development (Sutter, 1996). Callus on AP media was grey and soft and did not appear useful for regeneration. This appearance was possibly due to the lack of thiamine and myo-inositol as other media elements of AP are similar to B5. EUC medium contains a wider range and the highest concentration of vitamins and growth factors. This appeared to be phytotoxic as callus showed significantly lower growth on full strength of both salts

and vitamins compared to the medium with ½ salts and ½ vitamins, and callus growth could not be sustained on any of the EUC media.

Callus growth was significantly lower on all media containing □ salts and full strength vitamins compared to media with ½ salts and ½ vitamins and full strength salts and vitamins. Therefore, vitamins and growth factors did not appear to be the limiting factor for callus growth. However, the type and concentration of vitamins appeared to influence both the appearance and consistency of the callus that developed. Friable callus grew on MS and B5 media, whereas the callus on WPM was compact and nodular, and on AP it was soft. Callus on EUC tended to turn brown and die. Hard friable callus is suitable for regeneration via organogenesis, for callus growth via cell suspension culture and is associated with somatic embryo growth (Doran, 1996). Regeneration may also occur from a more compact nodular callus, whereas soft callus and callus that browns is unsuitable for regeneration.

The most important factor limiting the development of callus of *E.* 'Urrbrae Gem' and explants of its open pollinated seedlings appeared to be the concentration of macro- and/or micro-elements. Thiamine and myo-inositol appeared to have a greater effect on callus appearance rather than growth. From the results of these experiments, full strength MS medium was chosen for future callus experiments as this resulted in the production of callus that grew well and had a hard, friable appearance.

Chapter 4

Effect of smoked water and heat shock treatment on *E.* 'Urrbrae Gem' callus

4.1 Introduction

The regeneration of plant tissues under *in vitro* conditions can be induced by applying physical and chemical treatments such as temperature, osmotic stress and plant growth regulators. Although responses to these treatments have been observed through studies of DNA methylation, oxidative imbalance and specific protein production, the initiation of the regeneration processes of organogenesis and somatic embryogenesis are not well understood (von Aderkas and Bonga, 2000).

There are many reports on the success of smoked water and heat shock on improved seed germination and zygotic embryo development for a wide range of Australian native plant species (Allan *et al.,* 2004; Wills and Read, 2000; Gilmore *et al.*, 2000; Read *et al.*, 2000; Roche *et al.,* 1997; Dixon *et al.*, 1995). An active compound for seed germination within smoke from burning leaves has recently been identified as butenolide (Flematti *et al.,* 2004). Smoked water and heat shock have been further studied in relation to organogenesis and somatic embryogenesis. Smoked water was successfully used to enhance and accelerate somatic embryogenesis in *Pelargonium hortorum* hypocotyls (Senaratna *et al.,* 1999). Heat shock has been shown to enhance shoot and root proliferation from *Gladiolus hybridus* microshoots (Kumar *et al.,* 1999) and induce somatic embryos in gladiolus cormel callus (Kumar *et al.,* 2002), *Brassica napus* microspores (Cordewener *et al.,* 2000), tobacco microspores (Touraev *et al.,* 1996), and sunflower seed callus (Perrotta *et al.,* 1992). The synthesis of heat shock

proteins is known to occur in the absence of heat shock during somatic embryo development in cork oak (Puigderrajols *et al.*, 2002), white spruce (Dong and Dunstan 1996), Douglas fir (Misra *et al.*, 1996), and carrot (Apuya and Zimmerman, 1992). The treatment of explants with heat shock to induce heat shock protein synthesis is possibly the required stimulus for somatic embryo production in otherwise recalcitrant explants, while the role of smoked water could be similar to that of a plant growth regulator (Flematti *et al.,* 2004).

Many eucalypts have been induced to form callus but not all species go on to organogenesis, and few species produce somatic embryos (McComb *et al.,* 1996; LeRoux and van Staden, 1991). The experiments described in this chapter were conducted to test the ability of *E.* 'Urrbrae Gem' callus to regenerate following treatment with smoked water and heat shock.

4.2 Materials and methods

Collection of mature canopy leaves of *E.* 'Urrbrae Gem' leaf explants and their initiation into culture were as described in Section 3.1.2. Callus derived from leaf explants was maintained by subculturing at 3-4 weekly on ⅛ Murashige and Skoog (1962) (MS) with full strength vitamins, 20 g^{-1} sucrose, 10 g^{-1} Phytagel®, 1 μM benzylamino purine (BAP) (Sigma) and 10 μM nathphaleneacetic acid (NAA) (Sigma), 5.7 pH.

100-200 mg of hard, friable, yellow callus of *E.* 'Urrbrae Gem' was selected and allocated to the following smoked water and heat shock treatments:

- Regan 2000® Smokemaster (Society for Growing Australian Plants, South Australian Region, P.O Box 304, Unley SA 5061, purchased through Dealtry Native Plant Wholesalers, Lot 2 Trevilla Rd, One Tree Hill, SA 5224) was

added at 0 and 60 mL^{-1}, with 5 mL per polycarbonate tube. Smokemaster is a proprietary product and the active ingredients are not disclosed.

- Heat was applied at 22 (control, room temperature), 42, 45 or 50°C for 30 or 120 min. Sterile MQ water was added at 5 ml per tube to all heat treatments.

Calli were placed into sterile 30 mL polycarbonate tubes with and without smoked water and immersed in a water bath at the appropriate temperature. After treatment, calli were soaked in 10 mL sterile MQ water for 10 min to wash off the smoked water, before transfer to MS medium at 5.7 pH, with 30 g^{-1} sucrose, 5 g^{-1} Phytagel®, 5 μM BAP and 5 μM 2,4-D, in 55 x 14 mm petri-dishes, with 10 - 15 mL medium per dish, then sealed with parafilm and placed in the dark at 24°C for 4 wk. 2,4-D was chosen due to its known association with production of somatic embryos in plants such as *Pinus strobus* (Garin *et al.,* 2000), *Asparagus officinallis* (Mamiya and Sakamoto, 2001), *Olea europea* (Shibli *et al.,* 2001), and soyabean (Walker and Parrott, 2001), and with production of heat shock proteins in alfalfa (Davletova *et al.,* 2001). Callus growth and appearance was assessed immediately after treatment and at 4 and 8 wk (as per Table 3.2). After the 4 wk assessment, callus was subcultured to MS medium without plant growth regulators and placed under 16 hrs cool white light and 8 hrs dark with a light reading of 75 µmoles m^{-2} sec^{-1}.

Treatments were arranged as a randomised complete block design with 10 replicates per treatment. Data were analysed for significant differences using one-way ANOVA in PlotIT 3.2 (Scientific Programming Enterprises, Hasslet, MI, USA) with the least significant differences calculated at the P=0.05 level.

4.3 Results

Smoked water: Immediately after treatment, all callus treated with smoked water had changed colour from yellow to brown, with the liquid cloudy or brown, while callus without smoked water remained yellow with clear or slightly brown liquid (Table 4.1). No growth was observed from callus treated with smoked water (Figure 4.1) and brown exudate was observed in the medium at 4 wk for all treatments except 42°C for 120 min with no further browning following subculture (Table 4.2).

Heat shock: Immediately after heat treatment, callus incubated at either 45 or 50°C had softened and separated into small pieces, whereas callus incubated at either 22 or 42°C remained intact. Callus incubated at 45°C for 120 min, and 50°C for either 30 or 120 min turned brown (Table 4.1). Assessments at 4 and 8 wk showed callus incubated at 22 or 42°C had continued to grow, whereas callus incubated at either 45 or 50°C grew slowly or stopped. Callus incubated at 22°C showed a significant increase in growth over 4 wk compared to the other treatments. However, the growth of callus incubated at 42 and 45°C significantly increased between 4 and 8 wk (Figure 4.1). Some brown exudate was observed in the medium at 4 wk for all treatments except 42°C for 120 min and 22°C for 120 min, with no further browning following subculture (Table 4.2).

Table 4.1 Appearance of *E.* 'Urrbrae Gem' callus immediately after treatment with various combinations of smoked water and heat shock. Callus was yellow at the commencement of the experiment.

		Smoked water		**No smoked water**	
Heat (°C)	**Time (min)**	**Liquid**	**Callus**	**Liquid**	**Callus**
22	120	brown	brown	clear	yellow
42	30	brown	brown	clear	yellow
	120	cloudy	brown	clear	yellow
45	30	brown	brown	clear	yellow
	120	cloudy	brown	slight brown	brown
50	30	cloudy	brown	light brown	brown
	120	cloudy	brown	light brown	brown

Table 4.2 Appearance of *E.* 'Urrbrae Gem' callus at 4 and 8 wk after treatment with various combinations of heat shock and smoked water. Explants were subcultured after the four week assessment.

		Smoked water			No smoked water		
Heat	Time	Medium	Callus		Medium	Callus	
(°C)	(min)		Colour	growth		Colour	Growth
4 wk							
22	120	1	Brown	0.0	0	Yellow	4.0
42	30	1	Brown	0.0	1	Yellow	2.0
	120	0	Brown	0.0	1	Yellow	0.6
45	30	1	Brown	0.0	1	Yellow	0.6
	120	1	Brown	0.0	0	Yellow	0.2
50	30	1	Brown	0.0	1	Yellow	0.1
	120	1	Brown	0.0	1	Yellow	0.0
8 wk							
22	120	0	Brown	0.0	0	Yellow	5.0
42	30	0	Brown	0.0	0	Yellow	5.0
	120	0	Brown	0.0	0	Yellow	4.0
45	30	0	Brown	0.0	0	Brown	0.6
	120	0	Brown	0.0	0	Brown	0.3
50	30	0	Brown	0.0	0	Brown	0.1
	120	0	Brown	0.0	0	Brown	0.0

medium: mean level of exudate in the medium over 10 replicates, 0 = none; 1 = brown exudates in the medium immediately around the explant.
growth: mean increase of callus over 10 replicates. Score of 0 = no growth; 0-1 = 1-2 mm; 1-2 = 3-5 mm; 2-3 = 6-10 mm ; 3-4 = 11 –15 mm; 4-5 = 15+ mm, as per Table 3.2.

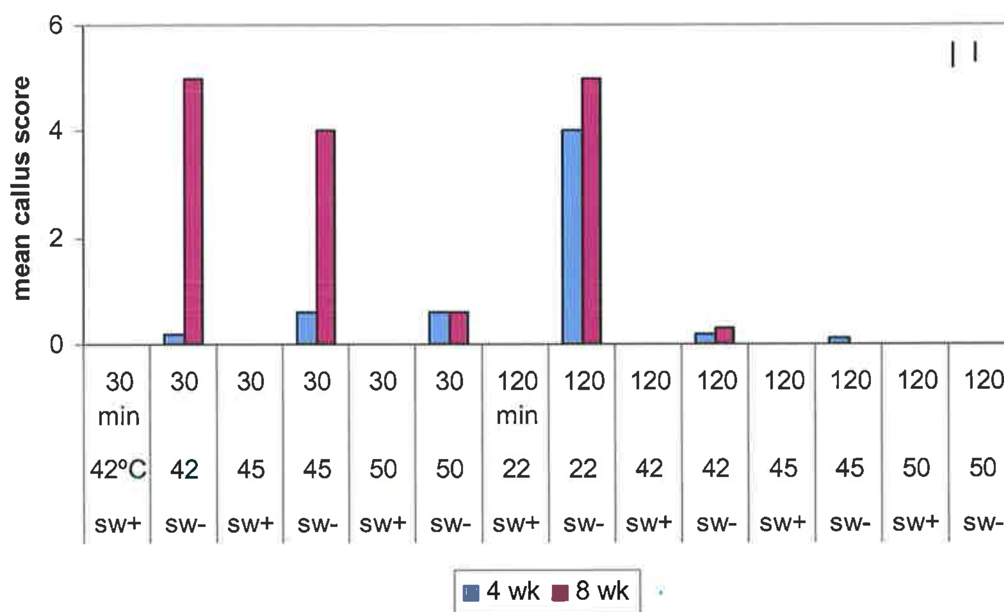


Figure 4.1 Effect of smoked water and heat over 30 or 120 min at 22, 42, 45 and 50°C on *E.* 'Urrbrae Gem' callus. LSDs bars at the *P*=0.05 level (4 wk, 0.34; 8 wk, 0.28). Mean callus score values represented in Table 4.2, callus growth. SW^+ = with smoked water; SW^- = without smoked water

4.4 Discussion

Kumar *et al.* (2002) reported that heat shock can induce somatic embryos on callus of *Gladiolus hybridus* without the use of plant growth regulators, and Senaratna *et al.* (1999) suggested that smoked water may affect the ratio of auxin to cytokinin or interact with other growth regulators. The callus used in these experiments was grown from canopy leaves of *E.* 'Urrbrae Gem' and had been in culture for 15 months. The presence of smoked water and the application of heat shock followed by exposure to 2,4-D, failed to induce either regeneration or somatic embryogenesis. There was rapid deterioration of callus after exposure to smoked water. It is possible that the lack of regeneration could be due to maturity of the original explant (von Aderkas and Bonga, 2000) or the loss of competence that can occur with callus cultures over time, as seen in gladiolus (Kumar *et al.,* 2002), maize (Emons and Kieft, 1995), and ryegrass (Albert *et al.,* 1995).

Heat shock proteins are reported to be directly associated with somatic embryogenesis (Puigderrajols *et al.*, 2002; Dong and Dunstan, 1996; Misra *et al.*, 1996; Apuya and Zimmerman, 1992), and the biosynthesis of polyamines such as spermidine, which is associated with somatic embryogenesis in gladiolus (Kumar *et al.,* 2002). Spermidine is also required for somatic embryo maturation of *Panax ginseng* (Kevers *et al.,* 2000). Somatic embryogenesis induced by heat shock in gladiolus was best at 50°C (Kumar *et al.,* 2002). However, *E.* 'Urrbrae Gem' callus exposed to 45°C and above for 30 and 120 min browned and died between 4 and 8 wk assessment following treatment.

Somatic embryos have yet to be induced from explants of *E.* 'Urrbrae Gem' or the parental species *E. erythronema* and *E. stricklandii*. Further work is required to develop a protocol for somatic embryogenesis in these species and their hybrids. This could be approached by looking at a range of factors such as plant growth regulator type and concentration, nutrient salt combinations, growth factors, environmental conditions, rejuvenation of plant tissue through techniques such as micrografting, and stress treatments such as starvation and chilling. Added to this, further work on the mode of action of butenolide (an active compound in smoked water for seed germination) and its role in plant growth regulation, may lead to increased understanding of the processes involved regards somatic embryogenesis and possible methods to overcome recalcitrance in species yet to develop somatic embryos.

Chapter 5

Effect of plant growth regulators on different genotypes and tissue types

5.1 Introduction

In this chapter, experiments were conducted to test the effects of auxins and cytokinins over a broad concentration range, on regeneration from juvenile tissue of the ornamental eucalypts *E. erythronema, E. stricklandii* and their interspecific hybrid, *E.* 'Urrbrae Gem'. There are no previous reports on organogenesis or somatic embryogenesis by tissue culture of these species, their hybrids, or of other ornamental eucalypts.

5.2 Materials and methods

Open-pollinated seeds of *E. erythronema* (Royston Petrie Seeds Pty Ltd, Vic. lot 1395PV2, September 2002), *E. stricklandii* (tree number 1614, planted in the Waite Arboretum, University of Adelaide) and *E.* 'Urrbrae Gem' (tree 1867 planted in the Waite Arboretum, University of Adelaide) were sterilised by soaking in 70% ethanol for 30 sec, then 3% NaOCl for 20 min, followed by three rinses in sterile Milli Q water. Sterilised seeds were allowed to imbibe in sterile water for 24 h, then 10 seeds each were placed 250 ml polycarbonate vials, each containing 40 ml of medium developed by Murashige and Skoog (MS) (1962), pH 5.7, with 30 g l^{-1} sucrose, gelled with 7 gl^{-1} Phytagel®, and kept in the dark at 24°C. After 7 d, light was given for 16 h per day at 75 µmoles m^{-2} sec^{-1}. Hypocotyls, cotyledons and the youngest expanding leaves were excised from 3 - 4 week old seedlings and placed on MS medium pH 5.7, containing 30 g l^{-1} sucrose, 7 g l^{-1} Phytagel®, that had been autoclaved at 121°C for 20 min and

poured into sterile 30 ml polycarbonate tubes (10 ml media per tube) with the various PGR treatments alone or in combination (Table 5.1).

Treatments were arranged in a randomised complete block design with one explant per tube, 10 replicates per treatment, and placed in a growth room at 24ºC in dim cool white light at 11 µmoles m^{-2} sec^{-1}. The effect of different treatments on callus growth was assessed by visual scoring (Table 5.2). Other data collected were the numbers of roots, buds, shoots (with one or more nodes visible) and somatic embryos at 6 and 12 weeks. Explants were sub-cultured onto MS medium with the same PGRs at 6 weeks for further calli, root, bud and shoot development. As no somatic embryos were observed by 12 weeks, callus was transferred to MS medium without PGR, and observed for somatic embryogenesis at 18 weeks. Data were analysed for significant differences using one-way ANOVA in PlotIT 3.2 (Scientific Programming Enterprises) with least significant differences (LSD) calculated at the 0.05 probability level (P□0.05).

At 12 weeks sections of callus 5 mm or smaller with and without roots, shoots, or buds, from hypocotyl, cotyledon and leaf explants were fixed, dehydrated, embedded, sectioned, and stained for observation under a light microscope by the method of O'Brien and McCully (1981) (Appendix 2).

Table 5.1 Combinations of PGR treatments applied to hypocotyl, cotyledon and leaf explants of *E. erythronema, E. stricklandii* and their interspecific hybrid *E.* 'Urrbrae Gem'

		Plant growth regulator (μM)				
		NAA			2,4-D	
		0	5	10	5	10
BAP	0	1	5	9	13	17
	1	2	6	10	14	18
	5	3	7	11	15	19
Kinetin	5	4	8	12	16	20

Numbers (1-20) represent treatment combinations used in the experiments and referred to in later Tables and in the text.

Table 5.2 Callus scoring method

Score	Description
0	no callus
1	callus < 1 mm long at cut ends
2	callus 1-2 mm at cut ends and/or along the explant
3	callus 2-4 mm, growing out from the explant into the medium
4	callus 4-10 mm overgrown the explant
5	callus 10-20 mm, or fills medium surface within the tube

5.3 Results

5.3.1 Callus growth from hypocotyl, cotyledon and leaf explants

Hypocotyl, cotyledon and leaf explants of E. erythronema, E. stricklandii and E. 'Urrbrae Gem' developed calli with all treatments, except for E. erythronema cotyledon explants with no PGR treatment (Table 5.3). Callus growth was larger at 12 weeks compared to 6 weeks. Callus growth on all explants treated with 2,4-D tended to be soft and spongy, while callus arising from treatment with BAP alone or in combination with NAA was hard and friable. Callus growth was generally greater with auxin or

auxin/cytokinin combinations (treatment 5-20, Table 5.1), compared to cytokinin alone (treatment 1-4, Table 5.1).

5.3.2 Root, shoot and bud development, and somatic embryogenesis on hypocotyl, cotyledon and leaf explants

Organogenesis was first observed at 6 weeks (data not shown), with an increase in root, bud and shoot numbers at 12 weeks, although numbers were low and the responses of explants within a given treatment were highly variable (Table 5.4). All three taxa produced roots, buds or shoots (Plates 5.1-3), with certain treatments (Table 5.4). Roots produced on MS medium with 2,4-D became brown within 12 weeks, while roots on NAA remained white. For hypocotyl, cotyledon and leaf explants, there was greater development of roots, shoots and buds with the auxin NAA, alone or in combination with a cytokinin (treatments 1-12, Table 5.1), compared to 2,4-D treatments (treatments 13-20, Table 5.1), as shown in Table 5.4. No somatic embryos were observed with any treatment, at any time.

Buds developed in large clusters on the petiole-end of leaf explants of *E. erythronema* and *E.* 'Urrbrae Gem' (Plates 5.1 and 5.3); while in *E. stricklandii,* buds developed individually along the midrib of the leaf explant. Greatest bud numbers per genotype were observed with leaf explants of *E. erythronema* on 5 μM BAP, 0 μM NAA, *E. stricklandii* on 5 μM BAP 5, μM NAA, and *E.* 'Urrbrae Gem' on 1 μM BAP, 0 μM NAA. Following transfer of explants to PGR-free medium, no further root, bud or shoot development occurred and all roots turned brown and died.

All explant types from all taxa produced both roots and buds, or roots and shoots, except for *E.* 'Urrbrae Gem' hypocotyls, with treatments 1, 3, 5, 6, 8, 9, 10, 12, 13 and 17 (Table 5.1; numbers highlighted in bold in Table 5.4). Treatments 2, 4, 7, 11,

14, 15, 18, 19 and 20 (Table 5.1 and 5.4) did not stimulate roots and buds, or roots and shoots in any genotype. No treatment was effective for all genotypes. *E. erythronema* responded to more treatments (12 of 20, Table 5.4) than *E. stricklandii* and *E.* 'Urrbrae Gem'.

Table 5.3 The effect of PGRs on callus growth from hypocotyl, cotyledon or leaf explants of *E. erythronema, E. stricklandii* or *E.* 'Urrbrae Gem' at 6 and 12 weeks

	E. erythronema						*E. stricklandii*						*E. 'Urrbrae Gem'*					
	Hypocotyl		Cotyledon		Leaf		Hypocotyl		Cotyledon		Leaf		Hypocotyl		Cotyledon		Leaf	
Ttmt	6	12	6	12	6	12	6	12	6	12	6	12	6	12	6	12	6	12
1	0.1^{a}	0.2^{a}	$-^{a}$	0.3^{a}	0.4^{a}	0.4^{a}	0.5^{a}	0.7^{a}	0.1^{a}	0.1^{a}	0.2^{a}	0.2a	0.2^{a}	0.2^{a}	0.2^{a}	0.3^{a}	0.4^{a}	0.5^{a}
2	1.0^{bcd}	1.6^{bcd}	0.9^{b}	1.2^{bc}	1.6^{bcd}	1.7^{bc}	1.7^{bc}	2.6^{bc}	1.5^{b}	2.3^{b}	1.2^{b}	1.8^{b}	2.1^{bcde}	3.1^{bcd}	2.5^{cd}	3.6^{cd}	1.5^{bc}	3.0^{c}
3	0.1^{cde}	2.7^{ef}	1.5^{c}	1.6^{cd}	1.4^{bc}	1.8^{bc}	2.3^{de}	4.1^{h}	2.0^{cd}	3.4^{gh}	1.1^{b}	2.6^{c}	2.2^{bcde}	3.3^{bcde}	2.0^{b}	3.8^{cdef}	1.9^{bcd}	3.1^{cd}
4	1.0^{bcd}	1.5^{bcd}	0.6^{b}	0.9^{b}	1.2^{b}	1.4^{b}	1.5^{bc}	2.7^{bcd}	1.8^{bc}	2.9^{cdef}	1.2^{b}	1.8^{b}	1.8^{bc}	2.5^{b}	2.1^{bc}	2.8^{b}	1.5^{b}	1.9^{b}
5	2.2^{fg}	3.0^{efg}	1.6^{cd}	1.9^{d}	1.8^{cde}	2.2^{c}	2.0^{cd}	3.7^{fgh}	2.1^{cd}	2.7^{bcd}	1.9^{cdef}	3.1^{cdef}	3.3^{gh}	3.3^{bcde}	2.9^{def}	4.2^{efgh}	2.2^{def}	3.0^{c}
6	1.7^{ef}	3.5^{fg}	2.5^{f}	3.4^{fgh}	2.5^{gh}	3.5^{efgh}	1.9^{bcd}	3.8^{gh}	2.8^{g}	4.2^{jk}	2.0^{cdef}	3.6^{fgh}	2.5^{def}	4.1^{ef}	3.3^{fgh}	4.9^{j}	2.8^{ghi}	4.9^{j}
7	1.4^{cde}	3.4^{fg}	2.7^{fgh}	3.8^{hi}	2.7^{h}	3.7^{gh}	2.3^{de}	4.1^{h}	2.7^{fg}	4.4^{k}	2.6^{gh}	4.3^{i}	2.5^{def}	4.0^{def}	3.0^{efg}	4.2^{efgh}	3.1^{ij}	4.5^{ij}
8	1.5^{de}	3.9^{g}	2.6^{fg}	3.8^{hij}	2.2^{efgh}	3.2^{defg}	2.0^{cd}	3.4^{defgh}	2.5^{ef}	3.9^{ij}	2.1^{def}	3.7^{gh}	2.2^{bcde}	3.8^{cdef}	3.0^{efg}	3.8^{cdef}	2.4^{defg}	4.0^{efghi}
9	2.9^{h}	2.9^{efg}	2.6^{fg}	3.2^{ef}	2.4^{fgh}	3.2^{defg}	2.8^{e}	3.9^{h}	2.2^{de}	3.5^{hi}	2.3^{fg}	3.2^{defg}	3.6^{h}	4.6^{f}	2.6^{de}	3.3^{bc}	2.5^{fgh}	3.7^{def}
10	1.4^{cde}	3.0^{efg}	2.6^{fg}	3.8^{hij}	2.3^{efgh}	3.6^{fgh}	2.0^{cd}	3.6^{efgh}	2.2^{de}	4.1^{jk}	1.8^{cd}	3.0^{cde}	2.1^{bcde}	3.7^{cdef}	3.1^{fg}	4.7^{ij}	2.9^{hij}	4.5^{hij}
11	1.4^{cde}	3.4^{fg}	2.9^{gh}	3.9^{ij}	2.3^{fgh}	3.8^{hi}	1.9^{bcd}	3.8^{gh}	2.2^{de}	4.5^{k}	2.1^{def}	4.0^{hi}	2.2^{bcde}	3.6^{cde}	3.4^{gh}	4.5^{ghij}	2.8^{ghi}	4.5^{ij}
12	1.5^{de}	3.4^{fg}	2.7^{fgh}	3.7^{ghi}	2.3^{efgh}	3.1^{def}	1.8^{bcd}	2.8^{cd}	2.0^{cd}	3.9^{ij}	1.7^{c}	3.2^{defg}	2.4^{cdef}	3.9^{cdef}	2.6^{de}	4.1^{defgh}	2.7^{ghi}	3.7^{efg}
13	2.0^{ef}	3.4^{fg}	2.8^{fgh}	3.1^{ef}	3.4^{i}	3.7^{h}	3.7^{f}	5.1^{i}	2.5^{h}	5.4^{l}	2.9^{hi}	5.1^{j}	2.7^{efg}	3.0^{bc}	3.3^{fgh}	4.6^{hij}	2.7^{ghi}	4.2^{fghi}
14	0.8^{bc}	3.0^{efg}	1.9^{de}	2.9^{ef}	2.7^{h}	3.4^{defgh}	1.4^{b}	2.9^{cde}	2.0^{cd}	3.2^{efgh}	2.2^{efg}	3.5^{efgh}	2.1^{bcde}	3.7^{cdef}	3.2^{fgh}	4.3^{fghi}	2.8^{ghi}	3.9^{efgh}
15	0.8^{bc}	3.2^{efg}	2.1^{c}	3.4^{fgh}	2.1^{defg}	3.6^{fgh}	1.6^{bc}	2.7^{bcd}	2.0^{cd}	2.9^{cdefg}	1.8^{cde}	2.9^{cde}	2.0^{bcd}	3.2^{bcde}	3.6^{h}	4.5^{ghij}	3.0^{ij}	4.3^{ghi}
16	1.0^{bcd}	2.9^{efg}	1.9^{de}	3.0^{ef}	2.1^{efg}	3.0^{de}	1.5^{bc}	3.0^{cdef}	2.0^{cd}	3.1^{defgh}	2.2^{defg}	3.3^{defg}	2.1^{bcde}	3.6^{cde}	3.2^{fgh}	4.1^{defgh}	2.8^{ghi}	4.0^{fghi}
17	0.8^{b}	1.2^{ab}	3.0^{h}	4.3^{j}	3.5^{i}	4.4^{j}	4.1^{f}	5.5^{i}	3.7^{h}	5.5^{l}	3.3^{i}	5.3^{j}	2.9^{fg}	3.3^{bcde}	3.4^{gh}	4.6^{hij}	3.3^{j}	4.3^{hij}
18	0.8^{b}	2.3^{cde}	1.9^{de}	3.2^{efg}	2.2^{efg}	3.1^{de}	1.6^{bc}	2.6^{bc}	2.0^{cd}	2.8^{bcde}	2.3^{fg}	3.3^{defg}	2.3^{bcdef}	3.7^{cdef}	2.9^{def}	4.3^{fghi}	2.4^{defg}	3.4^{cde}
19	0.4^{ab}	1.4^{bc}	2.0^{de}	3.1^{ef}	2.7^{h}	3.4^{efgh}	1.4^{b}	2.0^{b}	2.0^{cd}	2.5^{bc}	2.1^{cdef}	2.8^{cd}	1.7^{b}	3.1^{bcd}	3.2^{fgh}	4.0^{defg}	2.5^{efgh}	3.4^{cde}
20	0.8^{b}	2.5^{def}	1.7^{cde}	2.8^{e}	2.0^{def}	2.9^{d}	1.7^{bc}	3.1^{cdefg}	2.2^{de}	3.3^{fgh}	2.1^{cdef}	3.2^{defg}	2.2^{bcde}	3.7^{cdef}	2.9^{def}	3.7^{cde}	2.0^{cde}	3.4^{cde}
LSD *P*□0.05	0.6	1	0.4	0.4	0.6	0.5	0.5	0.8	0.3	0.4	0.5	0.7	0.7	0.9	0.04	0.04	0.5	0.6

Ttmt = refer to TABLE I for treatment details. Numbers represent the mean score, as described in materials and methods, of ten replicates per treatment. Different letters within each column indicate significant differences (*P*□0.05), starting with a as lowest

Table 5.4 The effect of PGR on organogenesis on hypocotyls, cotyledon and leaf explants of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' at 12 weeks

	E. erythronema									*E. stricklandii*									*E. 'Urrbrae Gem'*								
	Hypocotyl			Cotyledon			Leaf			Hypocotyl			Cotyledon			Leaf			Hypocotyl			Cotyledon			Leaf		
Ttmt	R	B	S	R	B	S	R	B	S	R	B	S	R	B	S	R	B	S	R	B	S	R	B	S	R	B	S
1	0.3^{a}	-	0.2^{a}	-	-	-	-	1.0^{ab}	0.4^{bc}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	3.2^{d}	-	-	3.2^{bc}	1.0^{d}	-	1.7^{ab}	0.9^{ab}	-	-	-	-	-	0.9^{bc}	-	0.3^{a}	-	-	0.3^{a}	-	-	13.8^{c}	0.9^{b}
3	0.1^{a}	1.4^{ab}	0.1^{a}	-	2.5^{cd}	-	-	9.7^{d}	0.3^{ab}	-	2.8^{b}	1.2^{b}	-	0.5^{ab}	0.4^{ab}	-	1.0^{a}	0.2^{ab}	-	-	-	-	2.5^{b}	-	-	1.0^{a}	-
4	-	-	1.0^{b}	-	0.5^{ab}	0.05^{a}	-	-	-	-	-	-	-	-	-	-	-	-	-	0.6^{ab}	-	-	-	-	-	0.4^{a}	-
5	4.5^{b}	-	0.5^{ab}	2.8^{b}	-	-	1.5^{c}	1.9^{ab}	0.1^{ab}	1.3^{b}	-	-	1.1^{b}	-	-	0.9^{bc}	-	-	4.4^{b}	-	-	0.8^{b}	-	-	1.9^{d}	0.4^{a}	-
6	0.1^{a}	2.2^{ab}	-	-	-	-	-	0.2^{a}	-	-	1.6^{ab}	-	-	1.6^{c}	0.3^{a}	-	1.5^{ab}	-	-	-	-	-	-	-	-	-	-
7	-	-	0.1^{a}	-	1.5^{abc}	-	-	0.5^{a}	-	-	0.7^{a}	1.3^{b}	-	0.6^{ab}	1.1^{c}	-	5.4^{c}	1.6^{c}	-	2.8^{c}	-	-	-	-	-	0.1^{a}	-
8	-	0.4^{a}	-	0.05^{a}	-	-	0.05^{a}	0.1^{a}	-	0.1^{a}	-	0.1^{a}	-	0.05^{a}	-	-	1.5^{a}	-	-	-	-	-	-	-	-	0.8^{a}	-
9	9.3^{c}	-	0.1^{a}	2.7^{b}	-	-	2.0^{c}	-	-	2.6^{d}	-	-	2.7^{d}	-	-	1.7^{d}	-	-	5.1^{b}	-	-	2.2^{c}	-	-	0.9^{bc}	0.2^{a}	0.1^{a}
10	-	3.8^{b}	0.2^{a}	0.1^{a}	0.05^{a}	-	0.1^{a}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.1^{a}	0.9^{ab}	-	-	1.5^{ab}	0.1^{a}
11	-	-	-	-	0.2^{ab}	0.05^{a}	-	0.2^{a}	-	-	0.1^{a}	-	-	1.2^{bc}	0.2^{a}	-	1.0^{a}	-	-	0.1^{a}	-	-	-	-	-	1.4^{a}	-
12	0.6^{a}	0.6^{a}	-	-	-	-	-	-	-	0.4^{ab}	1.7^{ab}	-	0.05^{a}	0.3^{ab}	-	0.1^{a}	0.4^{a}	-	0.1^{a}	-	-	-	-	-	-	0.5^{a}	-
13	-	-	-	0.3^{a}	1.7^{bcd}	-	0.20^{a}	0.3^{a}	-	1.3^{bc}	-	-	1.6^{bc}	-	-	0.5^{ab}	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
16	-	-	-	-	-	-	-	-	-	0.1^{a}	0.2^{a}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	-	-	-	0.05^{a}	0.3^{ab}	-	0.9^{b}	0.6^{a}	-	-	-	-	0.2^{a}	0.2^{a}	-	1.3^{cd}	-	-	-	-	-	-	-	-	0.4^{ab}	-	-
18	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	-	-	-	0.1^{a}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	-	-	-	-	-	-	-	-	-	-	0.3^{a}	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
LSD P□0.05	2.9	1.8	0.5	1.1	1.3	0.06	0.7	0.8	0.4	1.0	0.9	1.0	0.7	1.3	0.7	0.7	1.8	0.4	2.0	1.8	0	0.5	0.5	0	0.5	3.4	0.3

Ttmt = refer to TABLE I for treatment details. Treatment numbers in bold represents root, shoot or root, bud development with certain tissues. Numbers represent the mean value of root, bud and shoot numbers of ten replicates per treatment.
R = root number, B = bud number, S = shoot number, No somatic embryos were observed
Different letters within the columns indicate significant differences (*P*□0.05), starting with a as the lowest

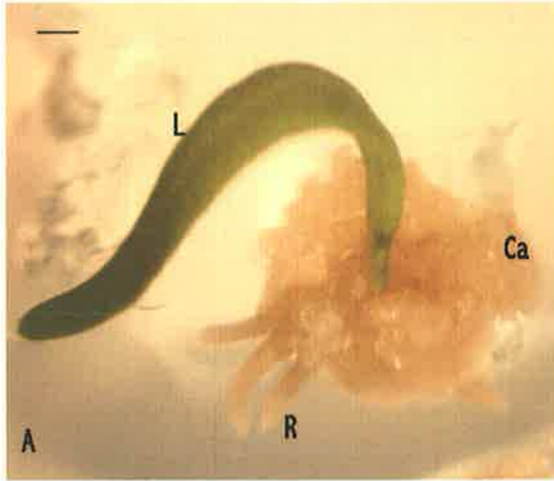
Plate 5.1 Roots, buds and shoots on leaf and roots and shoots on hypocotyl explants of *E. erythronema*

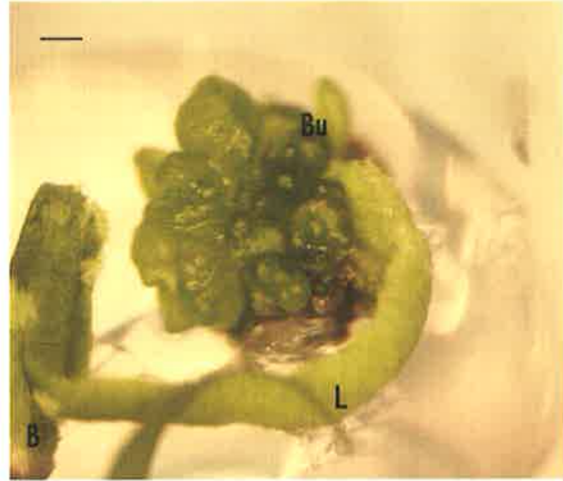
A) Root (R) and callus (Ca) from the petiole end of the leaf explant (L) on 5 μM NAA after 6 weeks showing callus lifting explant above medium and roots emerging from the callus. Bar = 1 mm

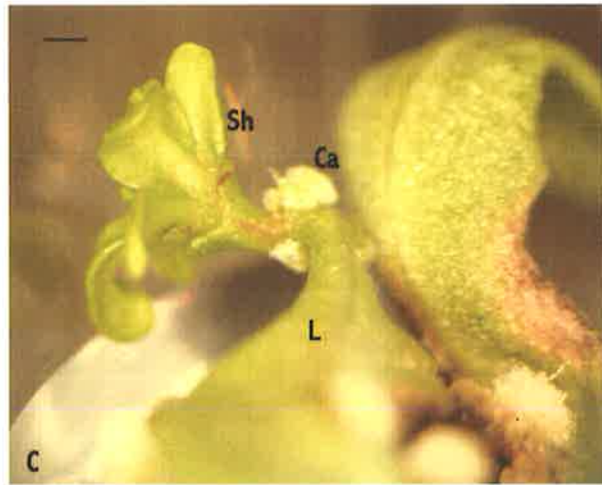
B) Bud (Bu) mass developing at petiole end of the leaf (L) after 6 wk on 1 μM BAP. Bar = 1 mm

C) Shoots (Sh) and callus (Ca) at the petiole end of the leaf (L) on 1 μM BAP at 6 wk. Bar = 1 mm

D) Shoot (Sh), root (R) and callus (Ca) from an individual hypocotyl explant on 5 μM NAA at 6 wk, showing callus only at the root growth end with roots emerging from callus close to the hypocotyl (H). Bar = 1 mm


L
Ca
R
A


Bu
L
B


Sh
Ca
L
C

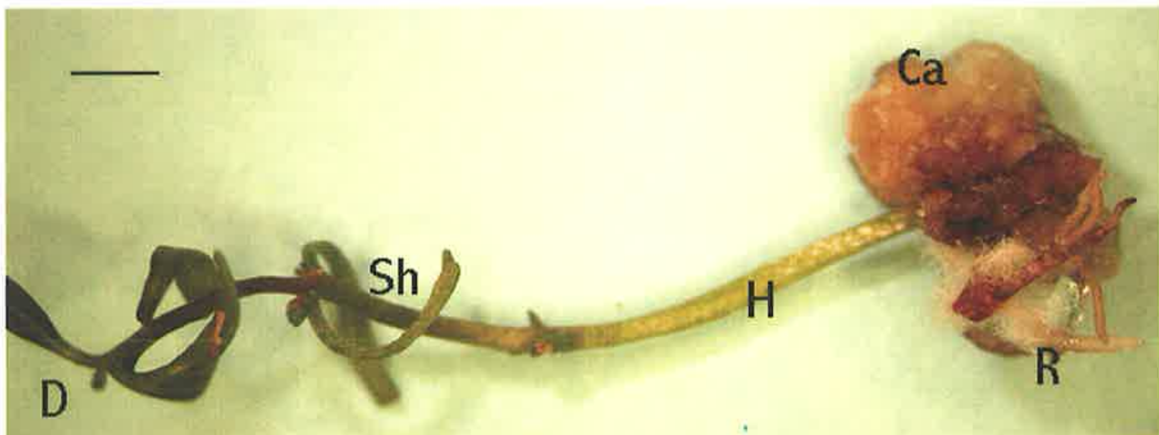

Ca
Sh
H
R
D

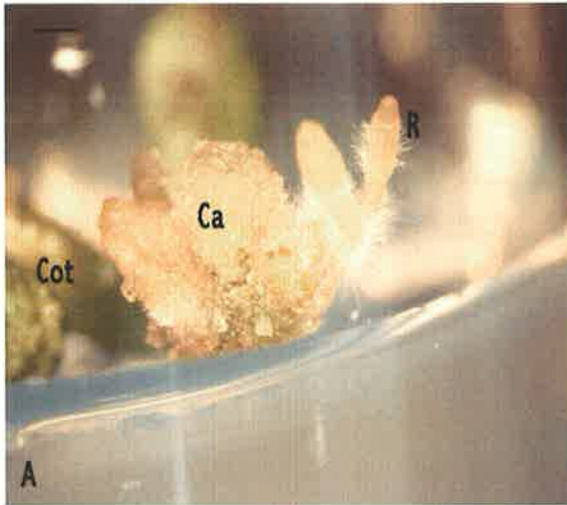
Plate 5.2 Callus, roots, buds and shoots on *E. stricklandii* hypocotyl, cotyledon and leaf explants.

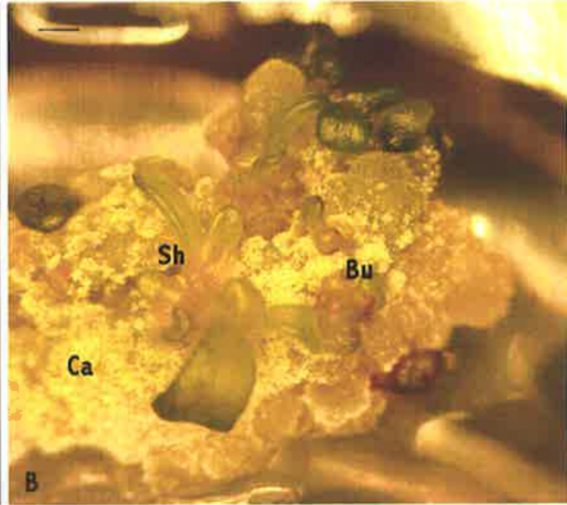
A) Root (R) and callus (Ca) on cotyledon (Cot) explant showing root emergence from callus near the explant on 10 μM NAA at 6 wk. Bar = 1mm

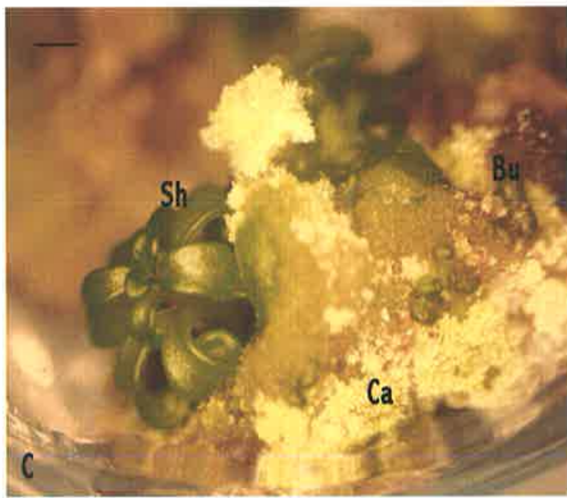
B) Callus, bud (Bu) and shoot (Sh) on hypocotyl explant on 5 μM BAP, 5 μM NAA at 6 wk. Bar = 1mm

C) Callus (Ca) bud (Bu) and shoot (Sh) on cotyledon explant on 1 μM BAP, 5 μM NAA at 12 wk. Bar = 1mm

D) Shoot (Sh) from the petiole end of a leaf explant on 5μM BAP, 5 μM NAA at 6 wk. Bar = 1mm


R
Ca
Cot
A


Sh
Bu
Ca
B


Sh
Bu
Ca
C

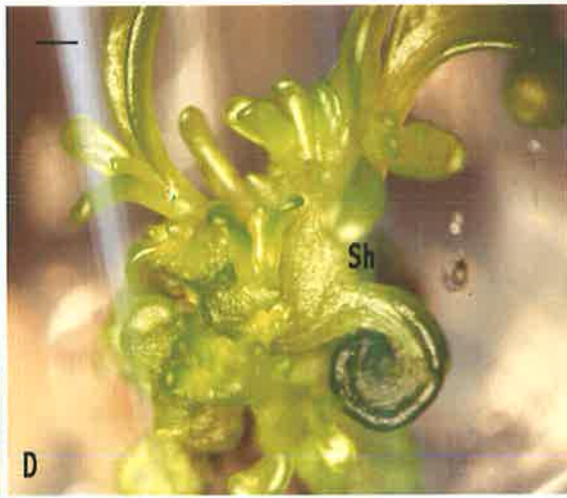
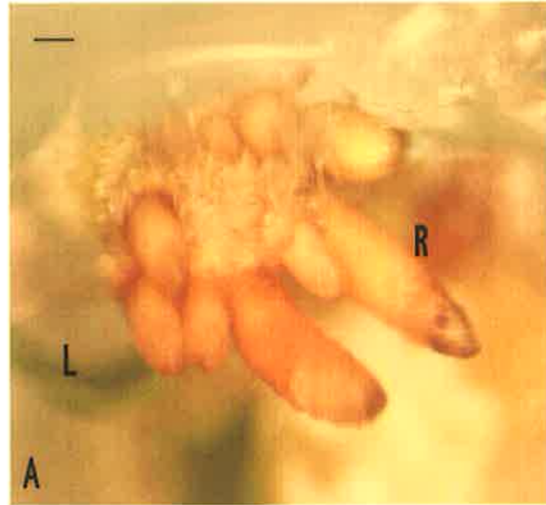

Sh
D

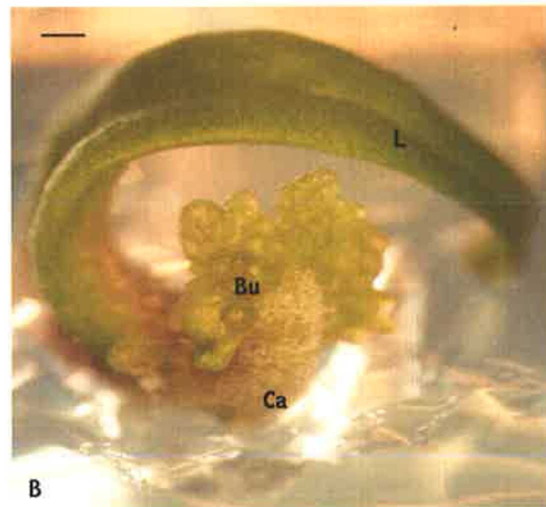
Plate 5.3 Callus, roots, buds and shoots on *E.* 'Urrbrae Gem' leaf explants

A) Roots (R) growing into the medium from the leaf (L) explant, which is just visible through the medium, on 5 μM NAA at 6 wk. Bar = 1mm

B) Callus (Ca) and bud (Bu) mass on 1 μM BAP at 6 wk. Bar = 1mm

C) Shoots (Sh) from the bud mass at 12 wk on 1 μM BAP, many buds failed to develop shoots and became brown. Bar = 1mm


R
L
A


L
Bu
Ca
B

Sh
C

5.3.3 Light microscopy

Microscopic observation was applied to explants of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' that developed callus, and/or shoots or roots.

Explants treated with 2,4-D alone produced large amounts of callus tissue consisting only of parenchyma cells (Plate 5.4). 2,4-D in combination with BAP or kinetin, and NAA alone or in combination with BAP or kinetin, produced less callus tissue, which also consisted of parenchyma cells, plus vascular tissue and defined areas of cell growth (Plate 5.4). Groups of densly cytoplasmic cells were observed within the callus (Plate 5.4), but no somatic embryos were observed.

Within the explants there was proliferation of vascular tissue and callus that distorted and ruptured the epidermis (Plate 5.5), followed by organogenesis of buds, shoots (Plate 5.6) and roots (Plate 5.7). Vascular tissue within the regenerated roots and shoots could be traced back to vascular tissue in the leaf or hypocotyl explant.

Plate 5.4 Callus growth from *E.* 'Urrbrae Gem' leaf explant at 12 wk;

A) Callus growth induced by 5 μM 2,4-D showing large parenchyma cells, and no vascular tissue. This callus was soft and spongy (x 20). Bar = 50 μm

B) Cells of callus explants induced by 5 μM NAA showing a group of densely cytoplasmic cells (meristemoid region) with prominent nuclei, no shoot or root primordia were observed (x 20). Bar = 50 μm

C) Cells of callus explant induced by 5 μM BAP 5 μM NAA showing the circular nature of the callus growth (arrow) with development of vascular tissue (x 20). Bar = 50 μm

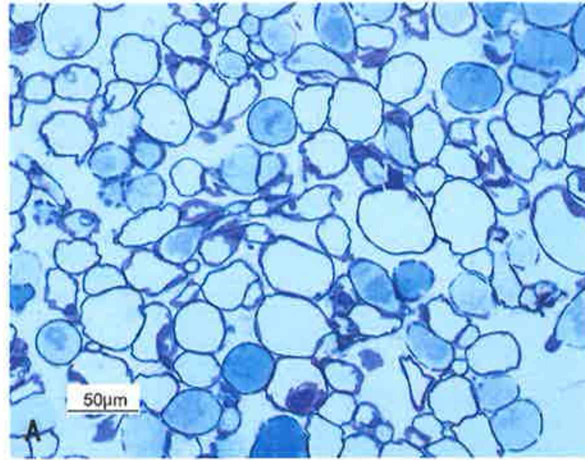
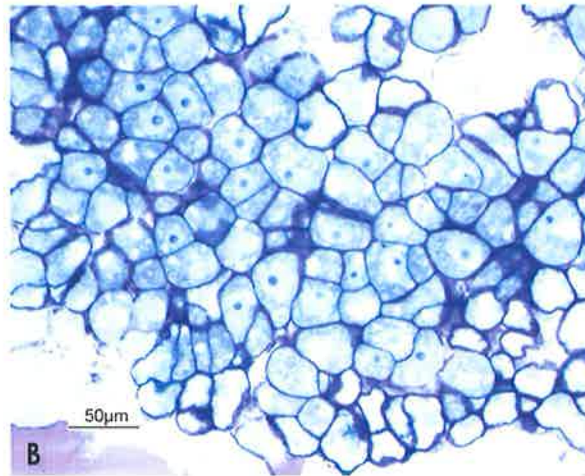
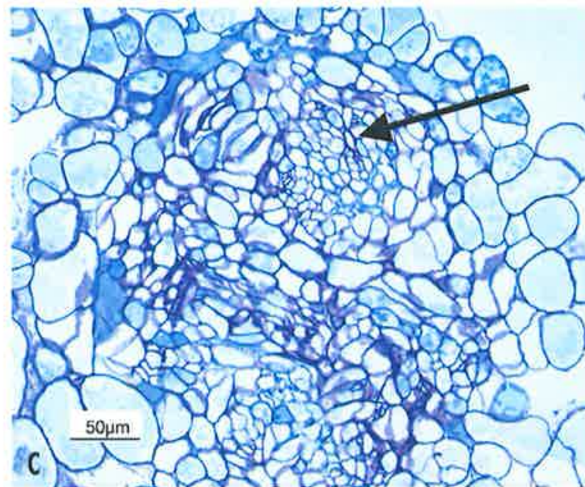
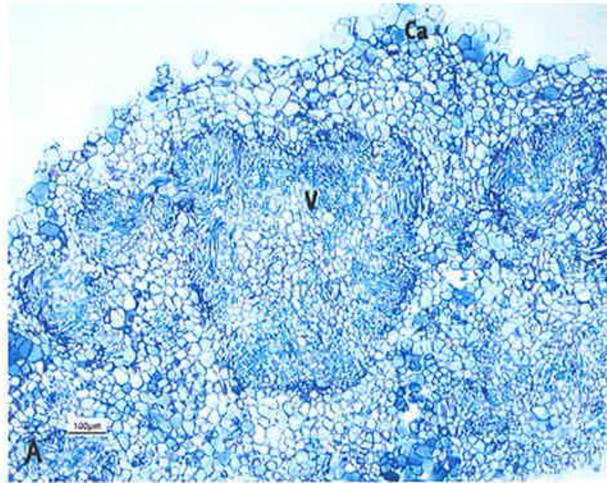

50µm
A

50µm
B

50µm
C

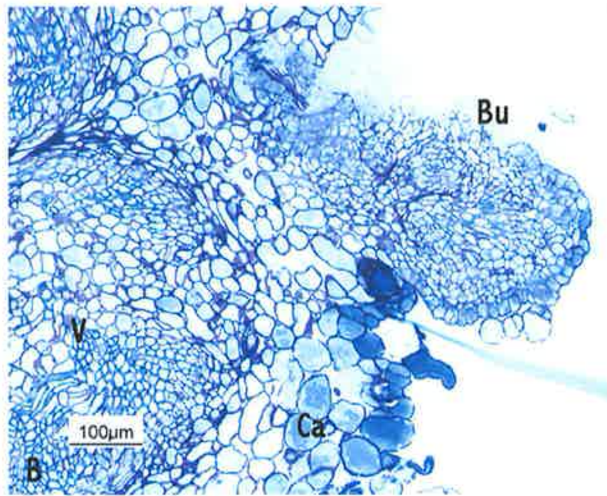
Plate 5.5 Callus, vascular tissue and buds on *E.* 'Urrbrae Gem' leaf explants after treatment with 5 μM BAP 5 μM NAA at 12 wk;

A) Callus (Ca) and vascular tissue (V) (x 5). Bar = 100 μm

B) Bud (Bu) with meristematic cells (M) (x 10). Bar = 100 μm

C) Bud (Bu) with meristematic cells (M) (x 20). Bar = 20 μm


Ca
V
A


Bu
V
100µm
Ca
B

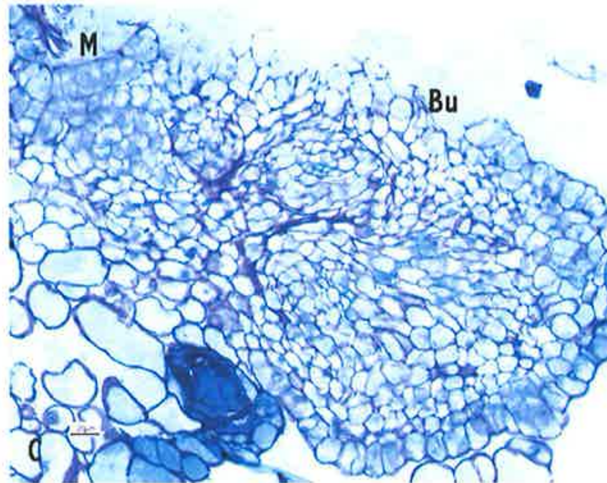

M
Bu
C

Plate 5.6 A well developed bud originating from the proximal part of an *E. stricklandii* leaf explant induced by 1 μM BAP at 12 weeks. Vascular tissue (Vt) can be traced back to vascular tissue within callus (x 10). Bar = 100 μm

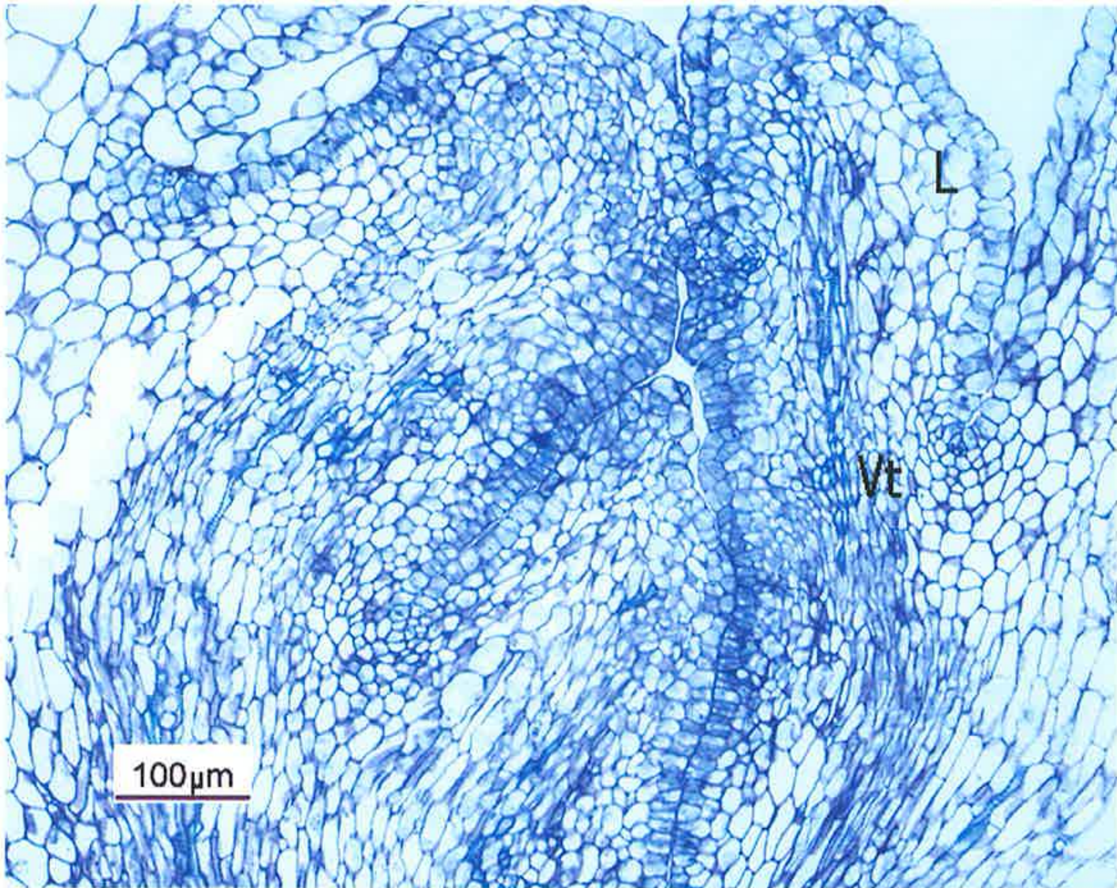
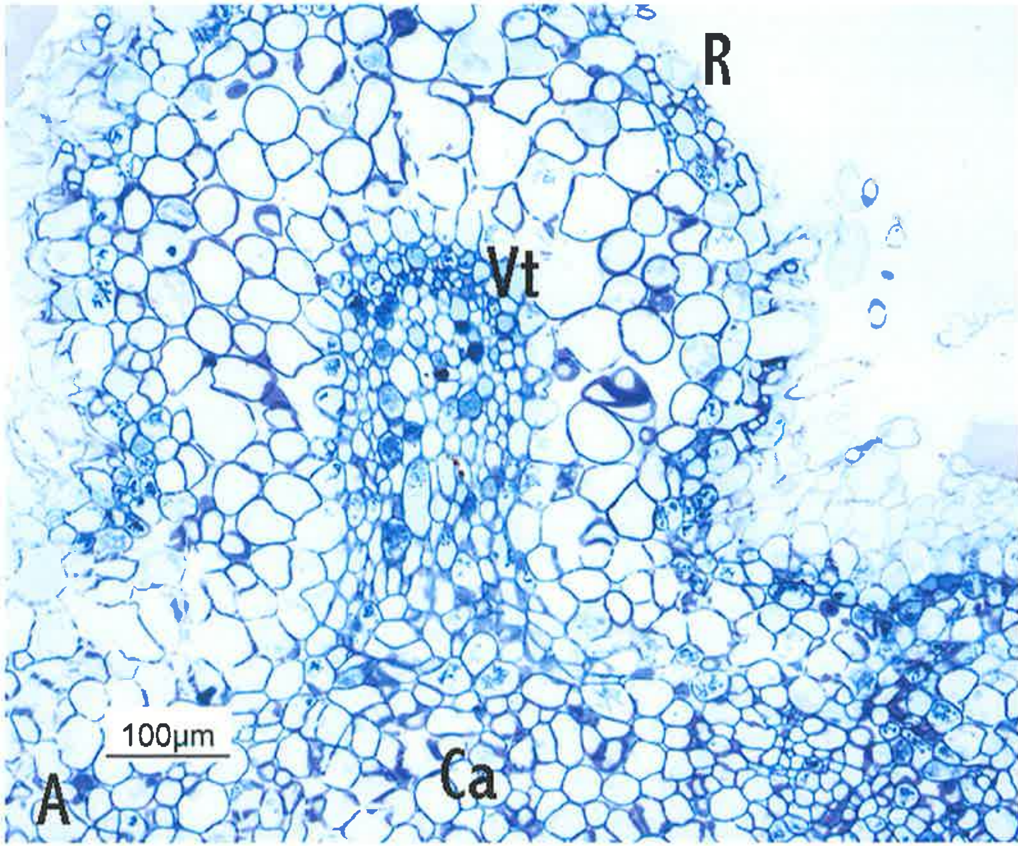
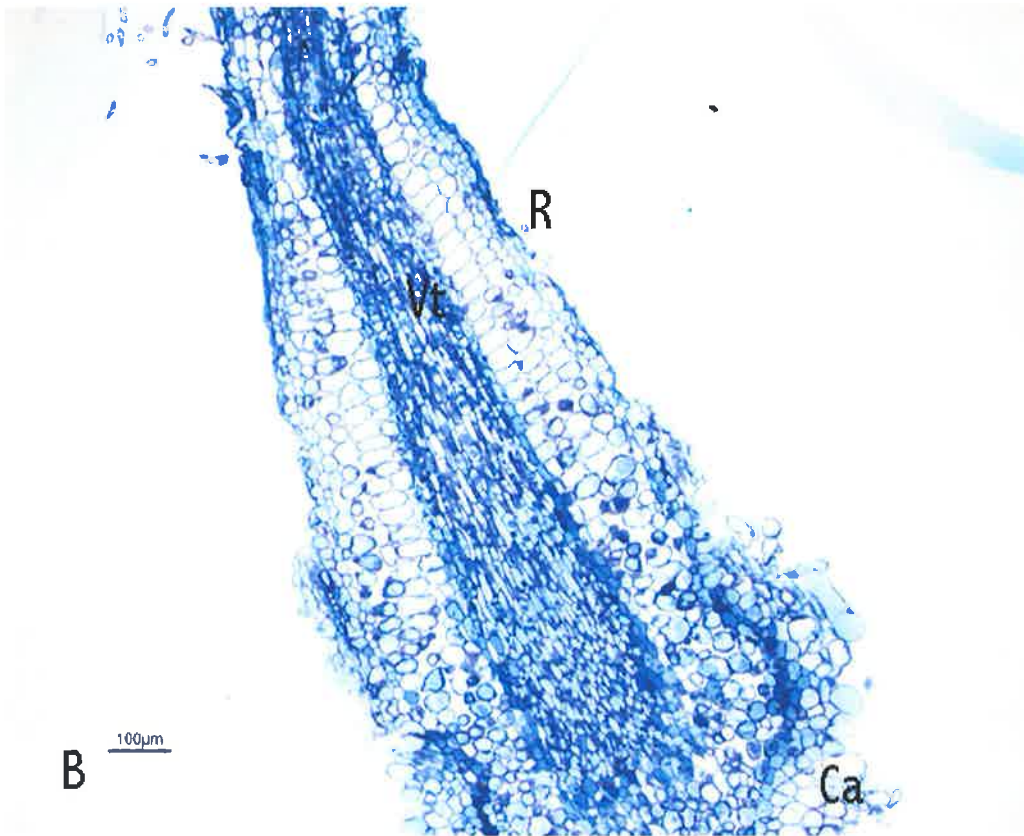

L
Vt
100µm

Plate 5.7 Roots on *E. erythronema* leaf explants at 12 wk;

A) Transverse section of a root (R) induced by 10 μM NAA. Vascular tissue (Vt) within the root can be traced back to callus (x 5). Bar = 100 μm

B) Longitudinal section of root growth induced by 5 μM NAA. Vascular tissue (Vt) within the root can be traced back to callus (x 10). Bar = 100 μm


R
Vt
100µm
A
Ca


R
Vt
100µm
B
Ca

5.4 Discussion

Callus development and the regeneration of roots, buds and shoots has been achieved from hypocotyl, cotyledon and leaf explants of *E. erythronema, E. stricklandii* and their interspecific hybrid *E.* 'Urrbrae Gem'. Some treatments induced both roots and buds, or roots and shoots, demonstrating the potential for whole plant regeneration. Somatic embryogenesis was not observed, either macroscopically or microscopically. This is the first report of successful organogenesis in an ornamental eucalypt.

The type, concentration and combination of PGRs are factors that can be used to manipulate *in vitro* plant tissue along the developmental pathways of organogenesis or somatic embryogenesis (Razdan, 1993). Successful regeneration is further influenced by the choice of explant and the genome of the plant (Watt *et al.,* 2003). The response of hypocotyls, cotyledons and leaf explants of open-pollinated seedlings of *E. erythronema, E. stricklandii,* and *E.* 'Urrbrae Gem' showed similarities in the pattern and appearance of callus growth and root development, but differences in shoot and bud development.

NAA and 2,4-D are commonly used to induce callus and plant regeneration through organogenesis and somatic embryogenesis in a wide range of woody and herbaceous plants (Zaerr and Mapes, 1982). Hard, friable callus is found suitable for organogenesis or somatic embryogenesis (Doran, 1996) and this was produced on NAA alone or in combination with BAP or kinetin. The soft spongy callus produced on 2,4-D was not suitable for somatic embryogenesis, although 2,4-D is used to induce somatic embryos in herbaceous (Mamiya and Sakamoto, 2001) and in some woody plants (Ewald, 1998; Wilhelm, 2000). The ability for organogenesis was supported by light microscopy with callus from NAA containing smaller cells, areas of dividing cells with

prominent nuclei, and vascular tissue, while callus from 2,4-D contained only large, loosely packed cells.

The ability to produce roots is important for successful propagation. Rooting conditions for eucalypt shoots varies between species and is achieved mainly with NAA, as for *E. camaldulensis* (Gupta *et al.,* 1983), indole-butyric acid (IBA), as for *E. tereticornis* (Sharma and Ramamurthy, 2000), or combinations of NAA and IBA as for *E. graniticola* (Bunn and Dixon, 1997). In this study all genotypes and tissue types developed roots with 5 and 10 μM NAA. These results can be applied in future experiments on the rooting of *in vitro* shoots.

Bud-development differed between tissue type, genotypes and PGR treatments. Bud growth was observed mainly on treatments with cytokinin or cytokinin/NAA combinations, while few buds were produced on treatments with 2,4-D. Microscopy showed buds to contain an epidermal layer, parenchyma, vascular tissue, and areas of densely packed cytoplasmic cells with prominent nuclei. *E. erythronema* and *E.* 'Urrbrae Gem' leaf explants produced large numbers of buds. This high-frequency bud induction has the potential to increase micropropagation rates and has been reported on seedling material of *E. camaldulensis* (Arezki *et al.,* 2000) using IBA and on *E. globulus* explants using a range of NAA or 2,4-D, in combination with thidiazuron (Nugent *et al.,* 2001) and BAP/IBA combinations (Trindade and Pais, 2003). However, the development of buds into shoots remained low, as was observed in these experiments. This problem has been overcome in other eucalypts by introducing a rotating culture system (Ito *et al.*,1996) with significant increases in shoot regeneration from buds on a number of *Eucalyptus* species, or by the introduction of other PGR such as TDZ as found with *E. globulus* (Nugent *et al.,* 2001).

Shoot development was low, varied between genotypes and tissue types, and was not observed on treatments with 2,4-D. There was high variability in shoot development, with a low number of explants within a treatment producing shoots while others developed none. Among reports on organogenesis of shoots from seedling material, hypocotyls appear more successful than cotyledons and leaves (McComb *et al.,* 1996). However, a comparison between callus cultures of hypocotyls, cotyledons and leaf explants from the same taxon, *E. grandis* x *urophylla*, showed cotyledon callus gave the highest level of shoot organogenesis (33-55%; Cid *et al.,* 1999). In comparison, the genotypes tested here produced greater numbers of shoots from hypocotyl and leaf explants compared to cotyledons.

There are few reports addressing the cellular as well as macroscopic development of roots, buds and shoots in eucalypts. Azmi *et al.,* (1997) demonstrated a connection of shoot regeneration with oil gland cells, while Hervé *et al.,* (2001) found regeneration from vascular tissue suggesting a link with cambial cells. In this study root, bud and shoot development showed linkages with the vascular tissue in the callus, and through to the explant vascular tissue. There was little vascular tissue differentiation with 2,4-D treatments, less root and bud growth, and no shoot growth. Buds tended to initiate on the epidermis and could develop into separate entities. Callus, roots, buds and shoots tended to develop at the cut areas of the explant in particular the petiole-end of the leaf as reported in other eucalypts (Hervé *et al.,* 2001; Cid *et al.,* 1999).

Work on the parental species and juvenile tissues of the hybrids can be used to establish suitable media, PGR and environmental requirements for initiating explants from clonal material of the mother plant. These results can be applied in future experiments on organogenesis and somatic embryogenesis of selected hybrid eucalypts.

This was achieved with the ornamental *E. ficifolia* (de Fossard *et al.,* 1978) where nodal material from the mother plant was limited so experiments were initially conducted on seedlings.

The plant material used here was from open-pollinated seedlings, with each seed being genetically different, so the variation between and within genotypes came from internal factors that could be genetically controlled. Although the levels were low, these experiments show that seedling explants of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' can be regenerated through organogenesis, but successful combinations for somatic embryogenesis have yet to be determined. Suggested causes for such recalcitrance of regeneration include oxidative stress from explant tissue damage (Cassells and Curry, 2001), and maturity of tissue (Von Aderkas and Bonga, 2000). Manipulation of explant preparation, nutrient media, PGRs, and environmental factors are suggested to overcome recalcitrance.

This study was initiated as a broad based experiment to assess the response of the hybrid and its parental species to wide concentrations of commonly used auxins and cytokinins known to induce organogenesis and somatic embryogenesis. From these results, future work can focus on successful PGR combinations and tissue types with a view to improve root, bud and shoot development, development of buds into shoots and the rooting of *in vitro* shoots, as well as other factors outlined above. These results can be used as a starting point for organogenesis and multiplication of other selected ornamental hybrid eucalypts. Also of importance will be an assessment of other PGR combinations and culture conditions for somatic embryogenesis.

Chapter 6

Stimulation by 6-benzylamino purine of *in vitro* shoot organogenesis for the ornamentals *Eucalyptus erythronema, E. stricklandii* and their interspecific hybrids

6.1. Introduction

Experiments described in Chapter Five using seedling explants of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' gave bud and shoot development on leaf explants and to a lesser extent hypocotyl and cotyledon explants using low levels of BAP alone. The aim of the experiment described in this chapter was to further improve bud and shoot organogenesis from *E. erythronema, E. stricklandii,* and their interspecific hybrids *E.* 'Urrbrae Gem' and hybrid 2.5. The approach was to treat apex and leaf explants with BAP at levels less than, or equal to, those known to induce shoots in these genotypes, and to observe regeneration at the cellular level by light microscopy.

6.2. Materials and methods

6.2.1. Plant material

Seed collection, decontamination and germination of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' were as per Section 5.2. *E. erythronema* var. *erythronema* x *E. stricklandii* hybrid 2.5, derived via controlled pollination from a breeding program to produce ornamental eucalypts (Delaporte *et al.,* 2001ab), was growing as an 18 mth old potted plant under controlled conditions in a glasshouse. *In vitro* shoots of hybrid 2.5 were maintained and multiplied by monthly subculture onto woody fruit tree medium (QL) (Quoirin and Lepoivre, 1977) comprising 2.2 μM

benzylamino purine (BAP) (Sigma), 0.5 μM naphthaleneacetic acid (NAA) (Sigma), and 0.5 μM gibberellic acid (Sigma), 20 g^{-1} sucrose, gelled with 5 g^{-1} Phytagel®, pH 5.7.

6.2.2. Callus, bud and shoot induction

Apex and leaf explants, excised from 2-3 wk old seedlings and *in vitro* shoots of hybrid 2.5 were placed on MS medium, containing 30 g^{-1} sucrose, and supplemented with 0.0, 0.1, 0.25, 0.5, or 1.0 μM BAP and gelled with 7 g^{-1} Phytagel®, autoclaved at 121°C for 20 min and poured into sterile 30 ml polycarbonate tubes, with 10 ml medium per tube. The treatments were arranged in a randomised complete block design with one explant per tube, 10 replicates per treatment and incubated at 24°C under dim light at 11 µmoles m^{-2} sec^{-1}. Data collected included callus growth, and the number of buds and shoots at 6 and 12 wk. The effect of different treatments on callus growth was assessed by visual scoring as per Table 5.2. Explants were sub-cultured onto MS medium with the same PGRs at 6 weeks for further callus, bud and shoot development. Data were analysed for significant differences using ANOVA in PlotIT 3.2 (Scientific Programming Enterprises, Haslett, MI, USA) with least significant differences (LSD) calculated at the 0.05 probability level (*P*□0.05).

6.2.3. Light microscopy

At 12 weeks specimens of callus 5 mm or smaller with and without shoots or buds, from apex and leaf explants were fixed in 3% glutaraldehyde, dehydrated via an ethanol series, and embedded in glycol methacrylate. Sections 3 μm in thickness were stained with periodic acid-schiffs reagent and toluidine blue O for observation under a light microscope by the method of O'Brien and McCully (1981) (Appendix 2).

6.2.4. Multiplication

Regenerated shoots from leaf and apex explants were harvested at 12 wk and multiplied on QL medium, with 2.2 μM BAP, 0.5 μM NAA, 20 g^{-1} sucrose, gelled with 4 g^{-1} Phytagel®.

6.3. Results

6.3.1. Callus, bud and shoot induction

Callus growth on apex and leaf explants of *E. erythronema* and *E. stricklandii* increased with BAP (Figure 6.1) and was greater at 12 wk compared to 6 wk (data not shown). Callus was beginning to brown at 12 wk, particularly callus from hybrid 2.5 explants. Callus growth on *E.* 'Urrbrae Gem' and hybrid 2.5 significantly increased in the presence of BAP but the level of growth was similar over all BAP concentrations (Figure 6.1).

Apex explants generally produced more buds and shoots than leaf explants with the response of explants within a treatment highly variable (Figure 6.2, 6.3). *E. erythronema* (Plate 6.1) and *E.* 'Urrbrae Gem' (Plate 6.2) apex and leaf explants produced bud clusters, hybrid 2.5 (Plate 6.3) and *E. stricklandii* produced individual buds along the explant. Bud numbers for *E. erythronema* and *E.* 'Urrbrae Gem' were greater with 1.0 μM BAP on both apex and leaf explants while bud numbers for *E. stricklandii* and hybrid 2.5 were low (Figure 6.2). Apex explants, of all genotypes, produced greater shoot numbers than leaf explants with all levels of BAP (Figure 6.3). Bud clusters and shoot organogenesis occurred from the callus mass of apex explants (Plate 6.1 and 6.2) and at the petiole region of leaf explants (Plate 6.3 and 6.4).

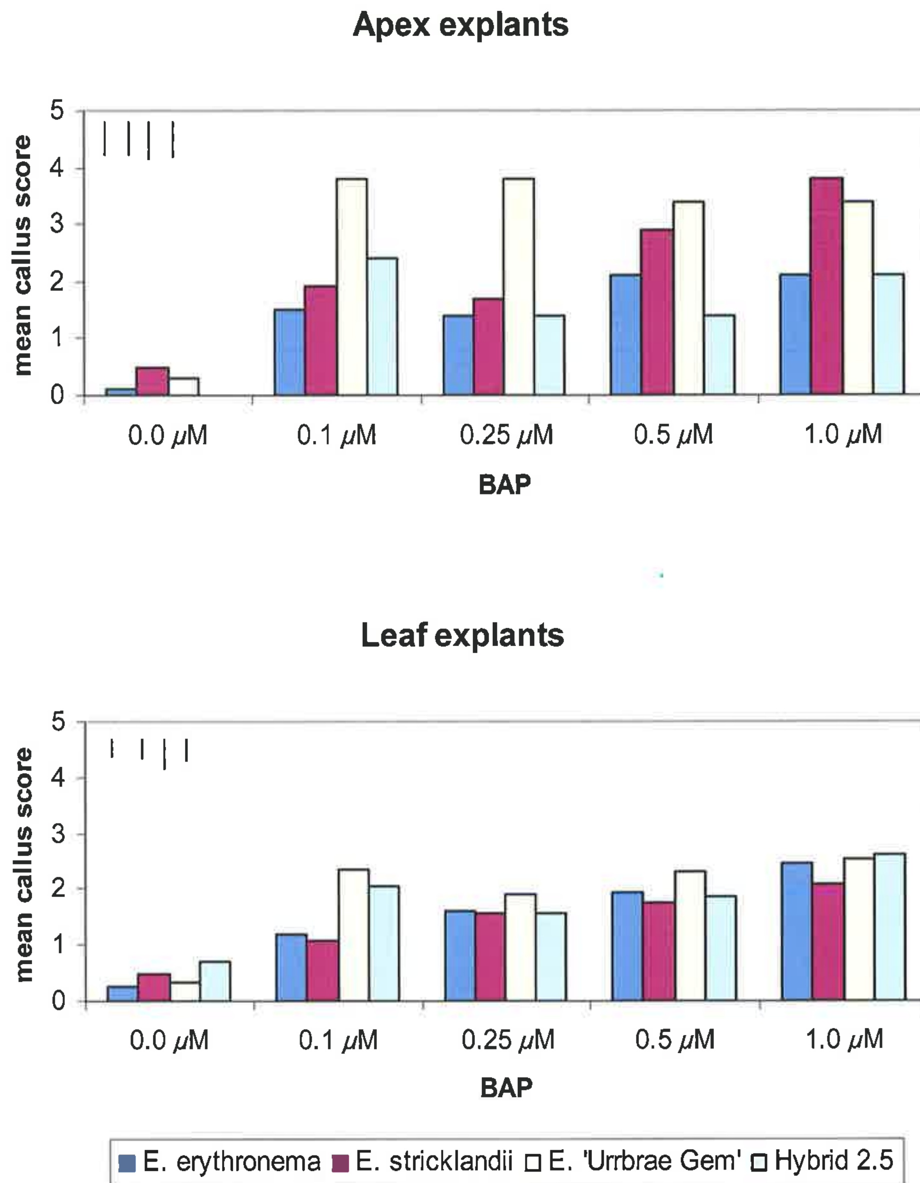
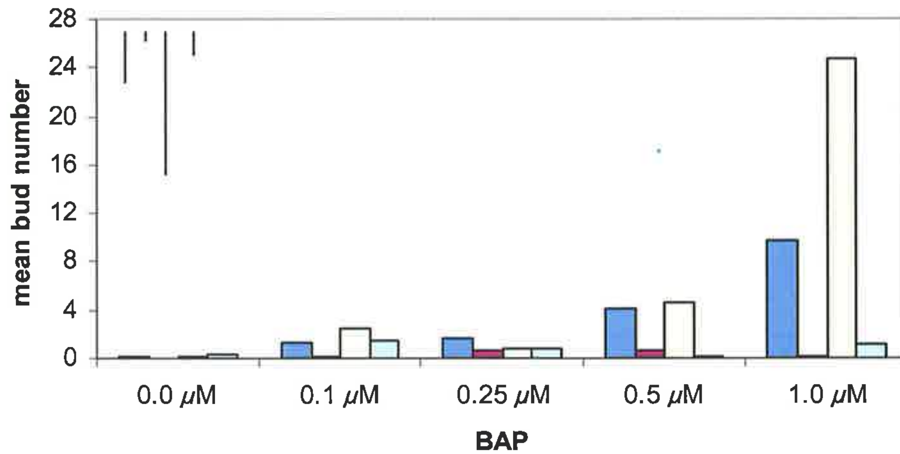


Figure 6.1 Mean callus score at 12 wk from apex and leaf explants of *E. erythronema, E. stricklandii,* and *E. erythronema* x *E. stricklandii, E.* 'Urrbrae Gem' and hybrid 2.5 on BAP. Bars represent LSDs calculated at the 0.05 probability level (*P*□0.05), (Apex: *E. erythronema,* 0.6, *E. stricklandii* 0.5, *E.* 'Urrbrae Gem' 0.7, hybrid 2.5 0.6; Leaf: *E. erythronema* 0.3, *E. stricklandii* 0.4, *E.* 'Urrbrae Gem' 0.5, hybrid 2.5 0.4)

Apex explants



Leaf explants

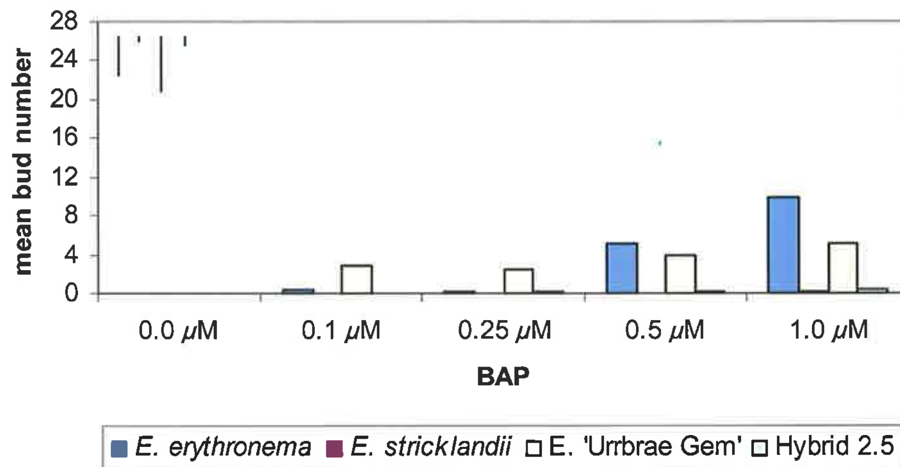
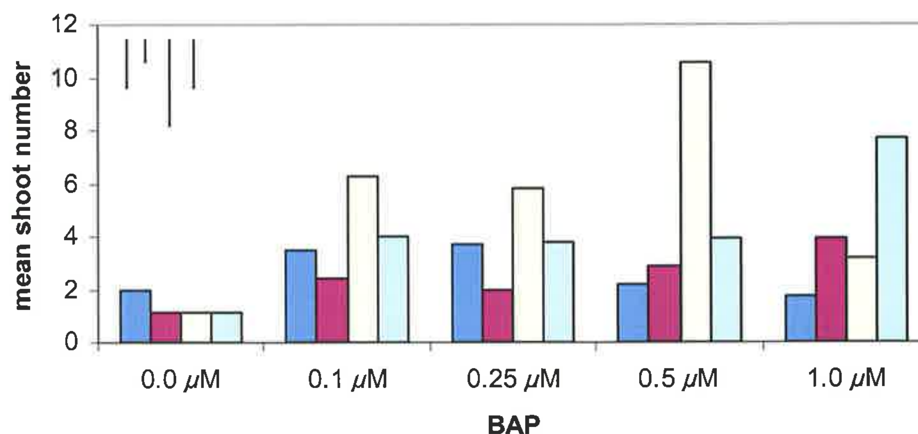


Figure 6.2 Mean bud number at 12 wk from apex and leaf explants of *E. erythronema, E. stricklandii,* and *E. erythronema* x *E. stricklandii, E.* 'Urrbrae Gem' and hybrid 2.5 on BAP. Bars represent LSDs calculated at the 0.05 probability level (*P*□0.05), (Apex: *E. erythronema,* 4.3, *E. stricklandii* 0.7, *E.* 'Urrbrae Gem' 12.0, hybrid 2.5 0.8; Leaf: *E. erythronema* 4.3, *E. stricklandii* 0.4, *E.* 'Urrbrae Gem' 5.7, hybrid 2.5 1.8)

Apex explants



Leaf explants

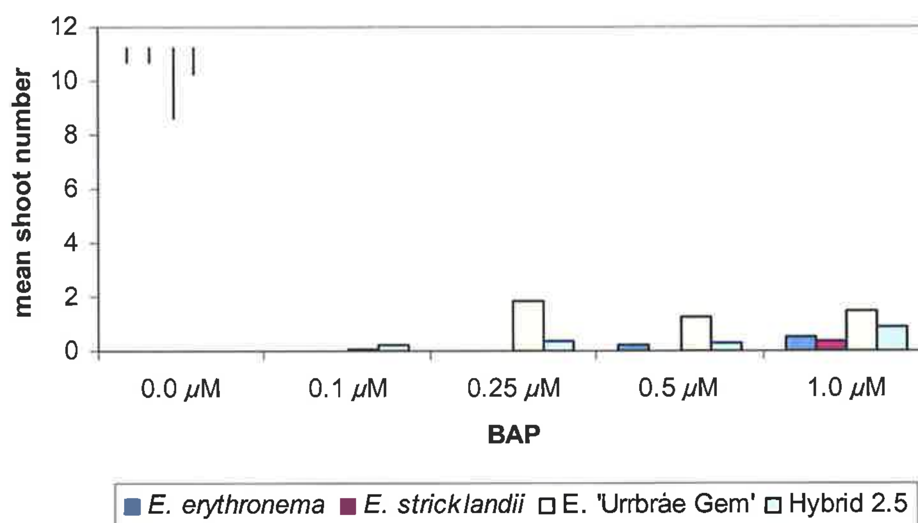


Figure 6.3 Mean shoot number at 12 wk from apex and leaf explants of *E. erythronema, E. stricklandii,* and *E. erythronema* x *E. stricklandii, E.* 'Urrbrae Gem' and hybrid 2.5 on BAP. Bars represent LSDs calculated at the 0.05 probability level ($P\leq0.05$), (Apex: *E. erythronema,* 2.1, *E. stricklandii* 1.3, *E.* 'Urrbrae Gem' 4.7, hybrid 2.5 2.0; Leaf: *E. erythronema* 0.5, *E. stricklandii* 0.5, *E.* 'Urrbrae Gem' 2.6, hybrid 2.5 1.0)

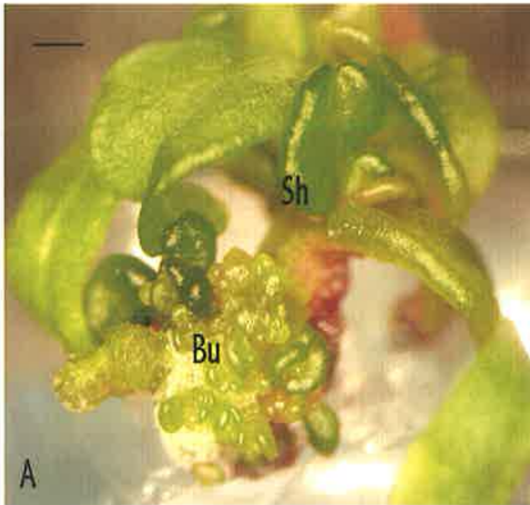
Plate 6.1 Buds and shoots on *E. erythronema* apex and leaf explants

A) Bud (Bu) and shoot (Sh) from an apex explant on 1.0 μM BAP after 6 wk. Bar = 1 mm

B) Shoots on apex explant on 0.25 μM BAP at 6 wk with very little callus at the base of the explant in contact with the medium. Bar = 1 mm

C) Callus (Ca), bud (Bu) and shoot (Sh) on leaf explants on 1.0 μM BAP at 6 wk. Bar = 1 mm

D) Bud (Bu) mass at the petiole end of the leaf (L) explant on 1.0 μM BAP at 12 wks with the explant raised off the medium by bud growth. Bar = 1 mm


Sh
Bu
A

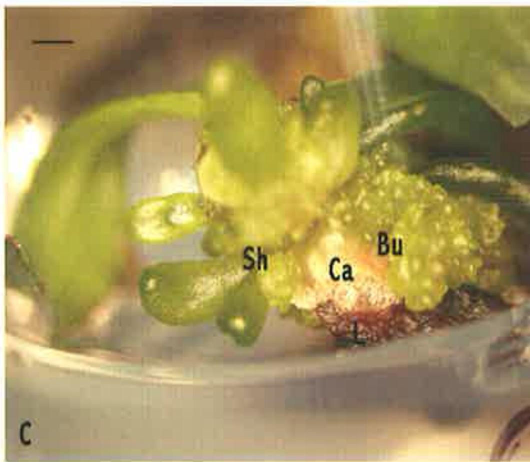
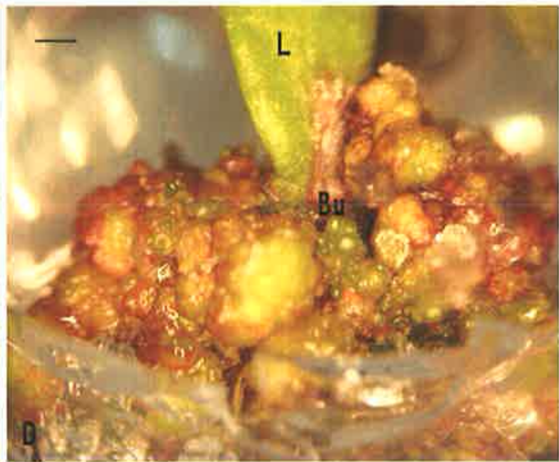
Sh
B

Sh
Ca
Bu
L
C

L
Bu
D

Plate 6.2 Callus, buds and shoots on apex and leaf explants of *E.* 'Urrbrae Gem'

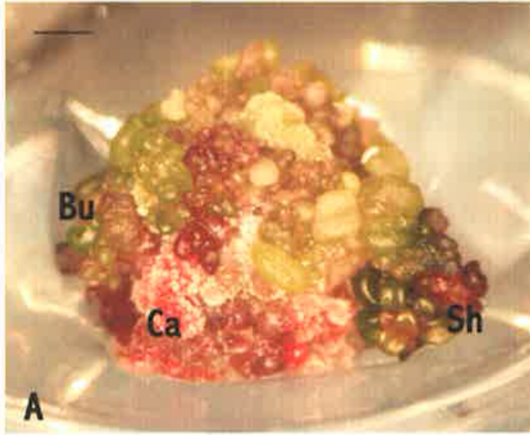
A) Callus and bud (Bu) mass with shoot (Sh) regeneration on apex explant on 1.0 μM BAP at 12 wk. Bar = 1 mm

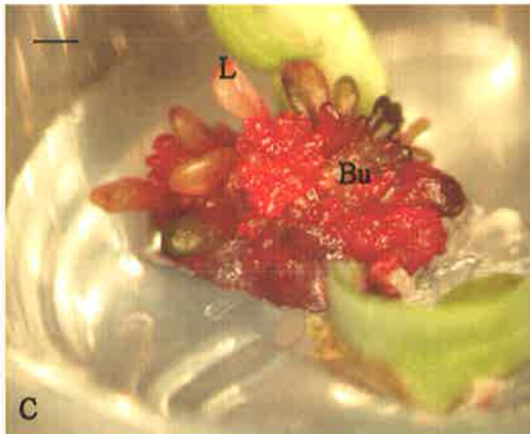
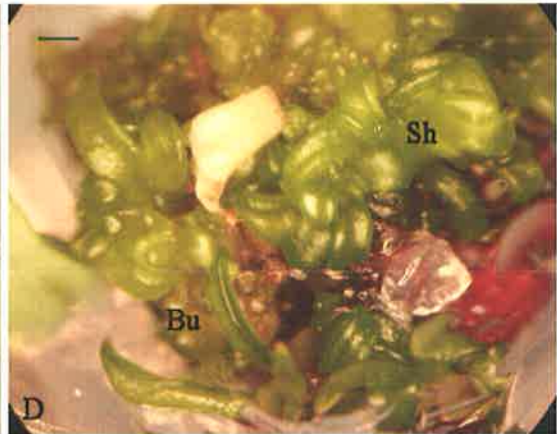
B) Shoots (Sh) on apex explant on 0.5 μM BAP at 12 wk. Bar = 1 mm

C) Buds (Bu) and leaves (L) on leaf explant on 0.25 μM BAP at 6 wk. Bar = 1 mm

D) The same explant as C at 12 wk with shoot development from buds. Bar = 1 mm

E) Shoot (Sh) growth was greater from leaf explant on 0.25 μM BAP at 12 wk that had produced fewer buds. Bar = 1 mm


Bu
Ca
Sh
A

Sh
B

L
Bu
C

Sh
Bu
D

Sh
E

Plate 6.3 Callus, buds and shoots on apex and leaf explants of *E. erythronema* x *E. stricklandii* hybrid 2.5

A) Shoots on an apex explant on 0.25 μM BAP at 12 wk. Bar = 1 mm

B) Callus (Ca), bud (Bu) and shoot (Sh) on apex explant on 1.0 μM BAP at 12 wk. Bar = 1 mm

C) Callus (Ca), bud (Bu) and shoot (Sh) on leaf explant on 1.0 μM BAP at 6 wk with shoot development from the petiole end of the leaf (L) explant showing callus growth distorting the leaf and raising it off the medium. Bar = 1 mm

D) Callus (Ca) and shoot (Sh) from the petiole end of leaf explant on 0.5 μM BAP at 6 wk. Callus growth was less than for 1.0 μM BAP. Bar = 1 mm

Sh
A

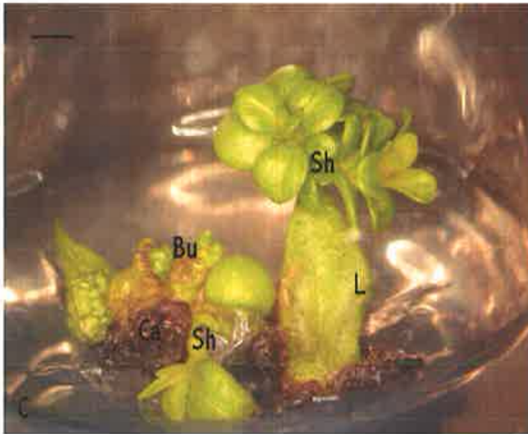
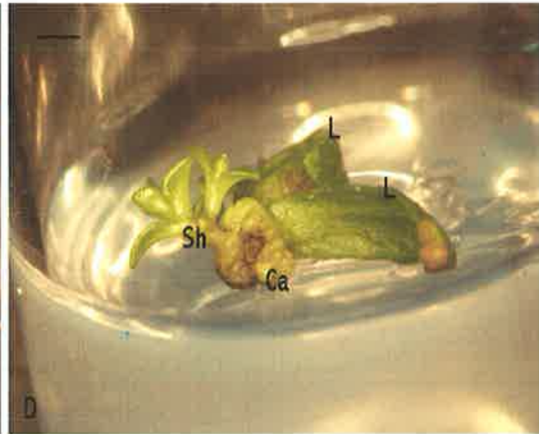
Sh
Bu
Ca
B

Sh
Bu
L
Ca
Sh
C

L
L
Sh
Ca
D

Plate 6.4 Callus (Ca) and shoot on *E. stricklandii* apex and leaf explants

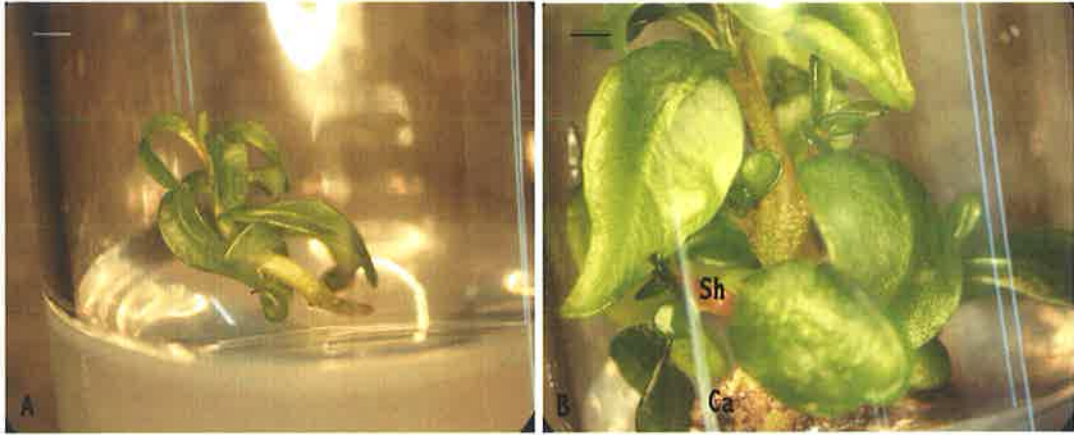
A) Apex explant on the control medium developed into a single shoot at 12 wk. Bar = 1 mm

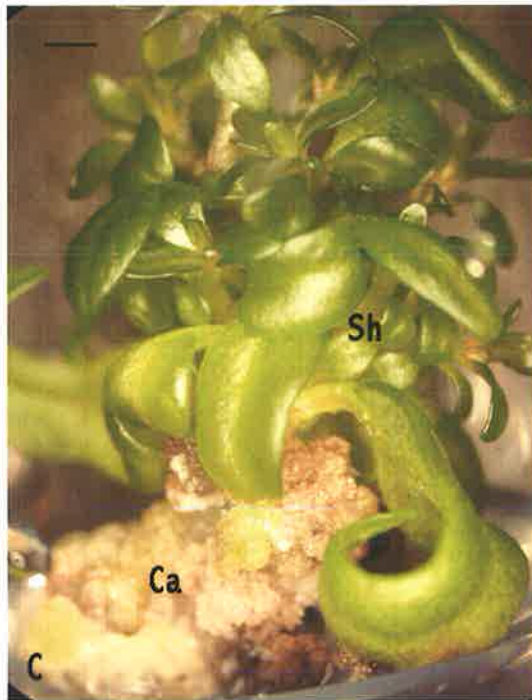
B) Apex explant on 0.25 μM BAP at 12 wk showing callus (Ca) and axillary shoot (Sh). Bar = 1 mm

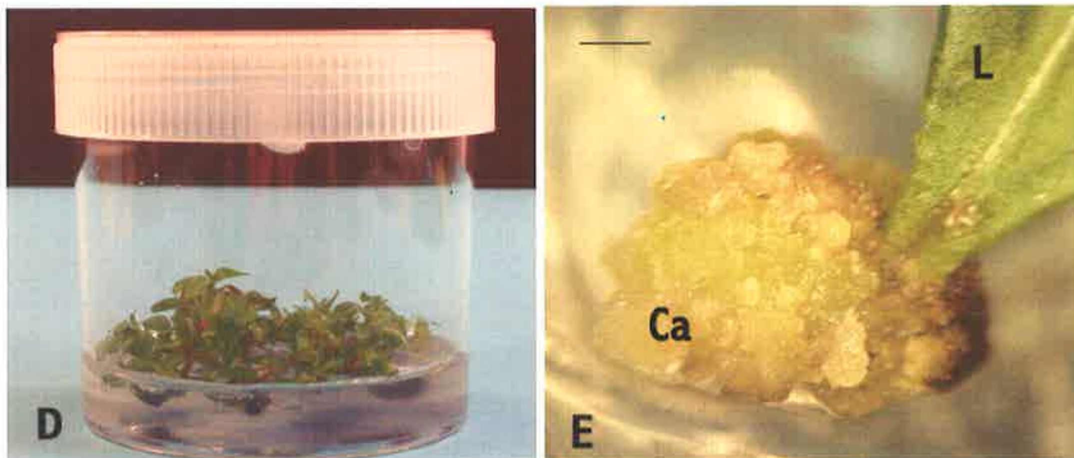
C) Apex explant on 1.0 μM BAP at 12 wk showing greater callus (Ca) growth and axillary shoot (Sh) growth than 0.25 μM BAP. Bar = 1 mm

D) Successful multiplication of axillary shoots harvested from apex explants on 1.0 μM BAP subcultured to QL medium with 1.0 μM BAP, 0.05 μM NAA, 20 g/L sucrose and 5 g/L Phytagel®. Bar = 1 mm

E) Callus (Ca) on leaf (L) explant on 1.0 μM BAP at 6 wk showing no bud or shoot development. Bar = 1 mm


Sh
Ca
A
B


Sh
Ca
C


L
Ca
D
E

6.3.2 Light microscopy

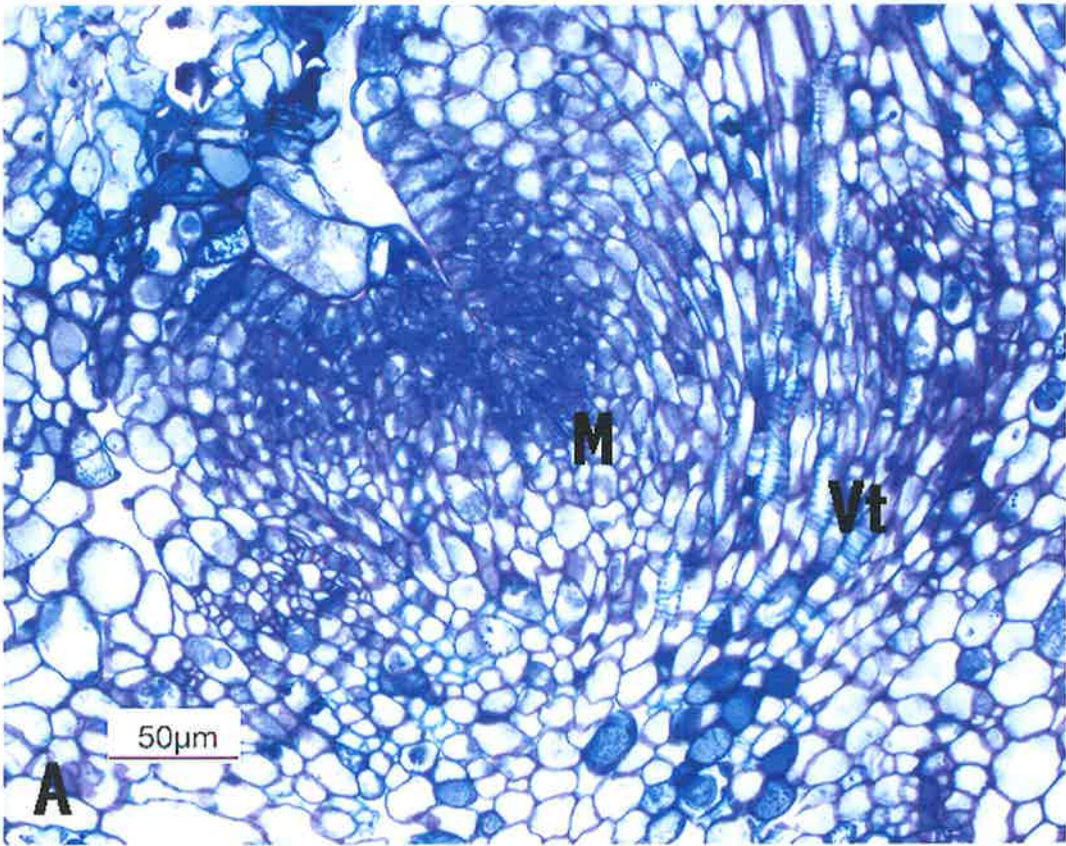
The anatomical changes observed for callus growth, bud development and shoot organogenesis within the explant were similar for all genotypes. The response to BAP was evident as a proliferation of vascular tissue and callus parenchyma cells. Within the callus of apex and leaf explants, areas of meristem development were defined by densely cytoplasmic cells with prominent nuclei, particularly on the petiole regions of *E.* 'Urrbrae Gem' leaf explants (Figure 6.5).

Macroscopically, buds were green and/or red with a smooth spherical surface compared to callus, which had a poorly defined uneven surface growing in many directions. Microscopically buds showed a single cell epidermal layer, which gave them their smooth appearance and defined outline. Buds that easily separated from their surrounding tissue had developed as a defined structure with an unbroken epidermal layer while buds firmly attached to the explant had retained cellular connections with the explant vascular tissue (Figure 6.6). Meristematic areas were observed in the buds, but these had rarely developed into apice or shoot primordium (Figure 6.6).

Plate 6.5 Meristem activity within the callus of *E.* 'Urrbrae Gem' leaf explants on 0.5 μM BAP at 12 wk

A) Group of small densely cytoplasmic cells (M) near vascular tissue (Vt) of the leaf explant (x 20). Bar = 50 µm

B) Group of small densely cytoplasmic cells (M) within the callus (Ca) of the leaf explant (x 20). Bar = 50 µm


M
Vt
50µm
A

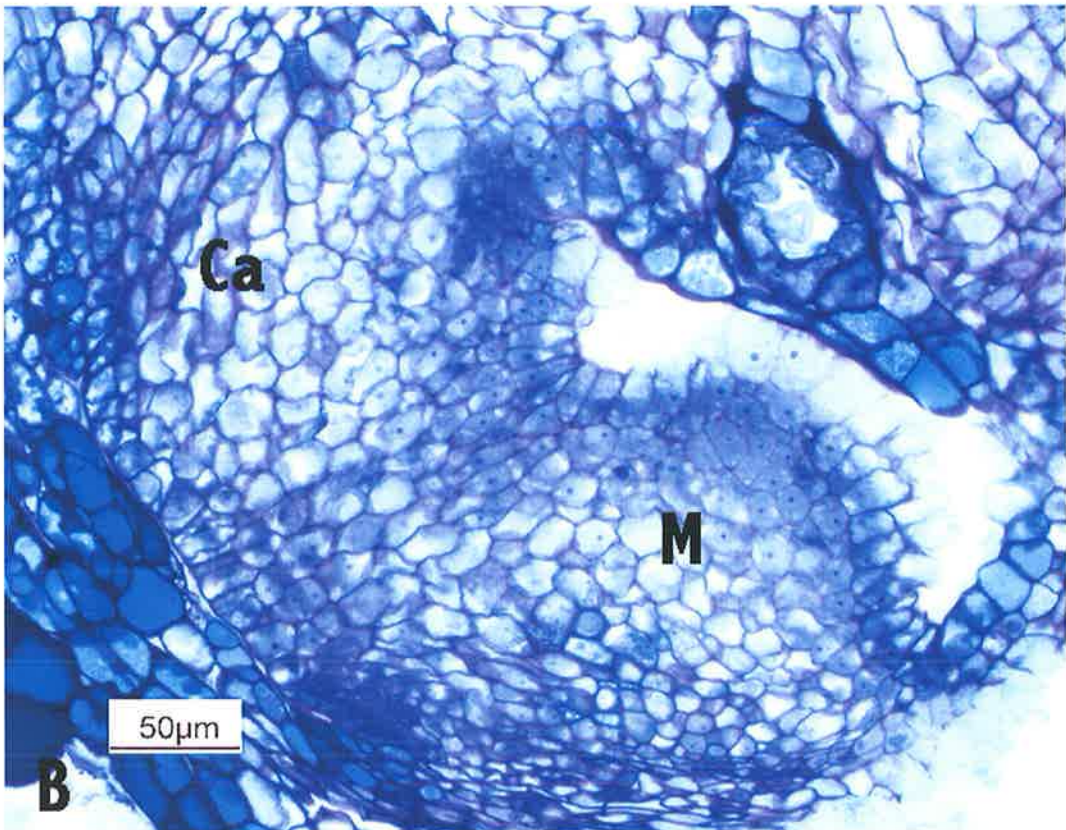

Ca
M
50µm
B

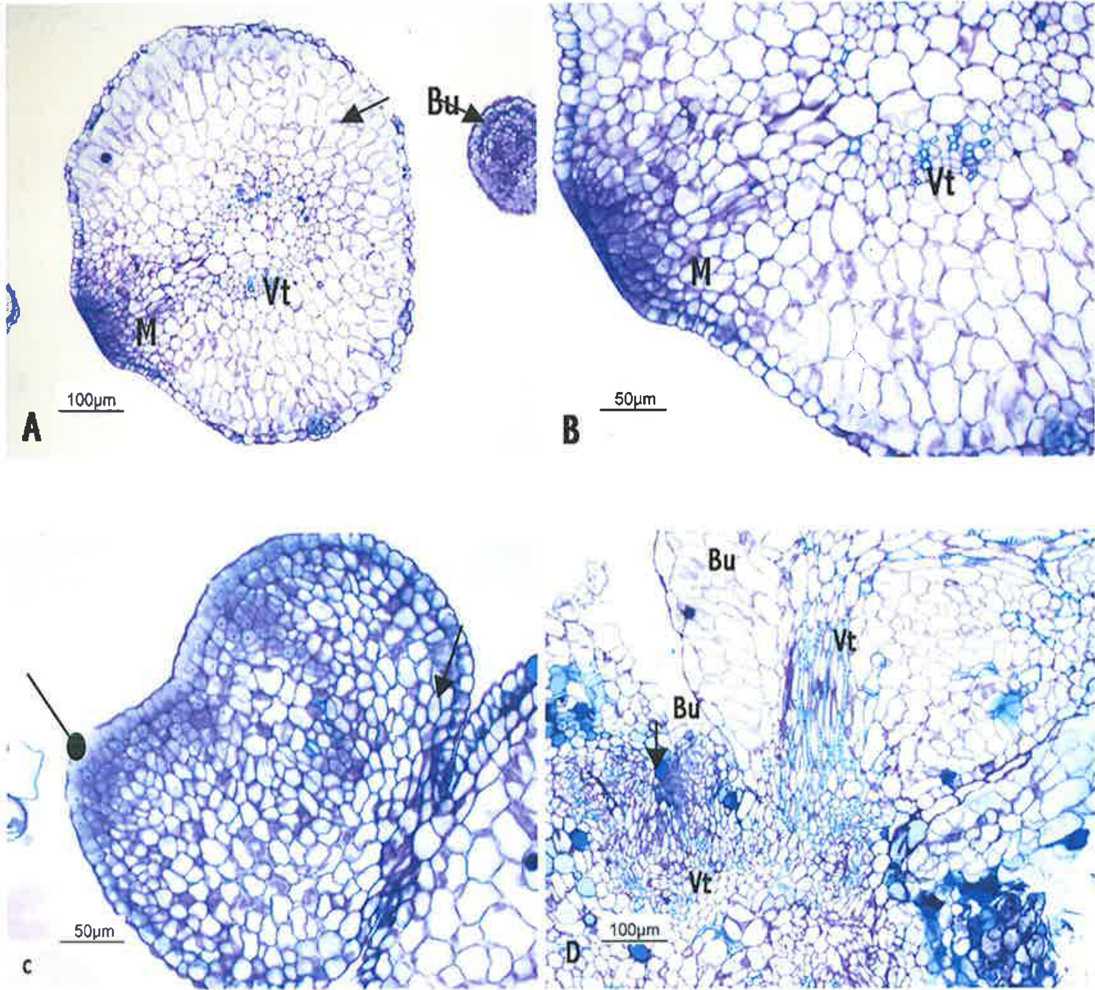
Plate 6.6 Bud development on *E. erythronema* leaf explants on 0.5 μM BAP at 12 wk

A) Buds (Bu) separated from the explant with complete epidermis. The larger bud shows vascular tissue (Vt) and an area of meristem (M) activity (a group of densely cytoplasmic cells with prominent nuclei). The smaller bud consists of densely cytoplasmic cells but no vascular tissue (x 100). Bar = 100 μm

B) Close up of the vascular tissue (Vt) and meristematic (M) region of the bud in A (x 20). Bar = 50 μm

C) Bud not completely separated from the explant (arrow) showing an area of meristem (circle) activity with a group of densely cytoplasmic cells with prominent nuclei (x 50). Bar = 50 μm

D) Vascular tissue (Vt) within the large bud (Bu) shows a connection with the vascular tissue (Vt) of the leaf explant but contains no meristem activity. There is a smaller developing bud (Bu with arrow) to the left (x 100). Bar = 100 μm


Bu
Vt
M
100µm
A
Vt
M
50µm
B
50µm
C
Bu
Vt
Bu
Vt
100µm
D

6.3.3 Multiplication

Regenerated shoots from apex and leaf explants could be successfully harvested and multiplied by axillary proliferation (Plate 6.4).

6.4. Discussion

This study has shown that BAP will induce shoot proliferation in apex explants of the selected ornamental eucalypts and that these shoots can be successfully multiplied. Organogenesis was induced in explants from both seedlings and *in vitro* shoots.

The cytokinin BAP promotes cell division, shoot multiplication and axillary bud formation while inhibiting root development (Sutter, 1996). In *E. erythronema, E. stricklandii, E.* 'Urrbrae Gem' and hybrid 2.5 it has proved difficult to induce regeneration from hypocotyl, cotyledon and leaf explants (Section 5.3) and this is consistent with past work on eucalypts where propagation was more successful from axillary meristem growth (Watt *et al.,* 2003). In this study the response of apex and leaf explants of four ornamental eucalypt genotypes to BAP showed similarities regarding callus growth, but differences in bud and shoot development.

Microscopically bud and shoot development correlated with an increase in vascular tissue, with the developing vascular tissue within the bud and shoot connecting to that of the explant. Azmi *et al.* (1997) reported that the ability of *E. globulus* hypocotyl explants to form buds and shoot meristems was related to the presence immature oil glands. Immature oil glands were observed in seedling leaf explants of *E.* 'Urrbrae Gem' but the cells of the oil gland did not give rise to shoot meristems. The origin of the developing buds on leaf and node explants of *E. gunnii* was shown by Hervé *et al.* (2001) to occur from either pre-existing meristems in nodes or in

association with vascular tissue in leaves. Ito *et al.* (1996) reported that buds developing on callus from shoot tip explants of *E. botryoides* contained small cells rich in cytoplasm at the periphery and large vacuolated cells in the interior.

Bud cluster formation as observed in *E. erythronema* and *E.* 'Urrbrae Gem' has been reported in other eucalypt species (Hervé *et al.,* 2001; Nugent *et al.,* 2001; Arezki *et al.,* 2000; Azmi *et al.,* 1997; Ito *et al.,* 1996; Niccol *et al.,* 1994; Subbaiah and Minocha, 1990) and in *E. globulus* it was shown to be genotype dependant (Trindade and Pais, 2003). The influence of genotype was observed within seedling leaf explants of *E. erythronema* and *E.* 'Urrbrae Gem' where one leaf explant could produce a large cluster of buds while other leaf explants on the same treatment produced no buds. Genotypic differences were also observed between taxa as *E. stricklandii* and hybrid 2.5 explants did not produce bud clusters compared to *E. erythronema* and *E.* 'Urrbrae Gem'.

Bud development using BAP alone did not prove a necessary requirement for shoot growth from apex and leaf explants of *E. stricklandii* and hybrid 2.5 with shoot development occurring in the absence of buds. Shoot organogenesis in these taxa may arise from within the callus or dedifferentiated cells within the explant. *E. erythronema* and *E.* 'Urrbrae Gem' apex explants produced large bud clusters but had a low frequency of buds developing into shoots. Light microscopy showed that while meristem development was occurring, may buds contained no meristematic cells.

BAP alone is suitable for the induction of callus, meristem and bud development but to improve shoot regeneration other stimuli may be necessary such as subculture to a medium with higher levels of BAP or other plant growth regulators and media additives. For example, Hervé *et al.* (2001) used picloram in combination with BAP on leaf and nodal explants of *E. gunnii* to improve shoot development, while Subbaiah and

Minocha (1990) used coconut water on *E. tereticornis.* Changes in environmental conditions may also improve shoot regeneration, as found by Ito *et al.* (1996), who placed shoot tip explants of *E. botryoides* under continuous high light intensity in a vertical rotating incubator for greater shoot development.

Other important factors for shoot regeneration can include the explant age and tissue type. Apex explants of *E. microcorys* (Niccol *et al.,* 1994) and hypocotyl explants of *E. tereticornis* (Subbaiah and Minocha, 1990) could be induced to form multiple shoots with BAP alone. In the present study, apex explants of *E. erythronema, E. stricklandii, E.* 'Urrbrae Gem' and hybrid 2.5 produced more and longer shoots, which could easily be separated for multiplication, and potentially for root initiation, compared to leaf explants where shoots were small and required removal from the explant to encourage further growth before multiplication.

From these results, future work can focus on improving the frequency of bud and shoot organogenesis from shoot tip and/or leaf explants through chemical and environmental stimuli. The development of successful protocols to outline a regeneration method for mature plants, will establish clonal propagation methods for superior ornamental hybrid eucalypts.

Chapter 7

Effect of auxins on organogenesis and somatic embryogenesis from juvenile explants of *Eucalyptus erythronema, E. stricklandii,* and their interspecific hybrids.

7.1 Introduction

Somatic embryogenesis can be used as a large scale, rapid form of clonal propagation. However, few eucalypt species produce somatic embryos (McComb *et al.*, 1996), with reported success limited to juvenile tissue from forestry species, and no reports from ornamental eucalypts.

Somatic embryogenesis has certain advantages compared to micropropagation and organogenesis. In somatic embryos, both a root and shoot meristem is developed forming a new plant axis with better vascular connection to the roots than adventitious roots. Root meristems result in the development of tap-roots and also eliminate the rooting stage that is required for *in vitro* shoots. Somatic embryos can be encapsulated to form an artificial seed, suitable for transportation or storage of germplasm for conservation (Razdan, 1993).

High levels of naphthaleneacetic acid (NAA) and/or 2,4-dichlorophenoxyacetic acid (2,4-D) can be used alone and in conjunction with cytokinins to initiate embryogenic callus in juvenile tissue of woody plant species (Sutter, 1996). Initiated embryogenic callus or somatic embryos are then subcultured to plant growth regulator (PGR) free medium to allow embryo development (Razdan, 1993). Somatic embryogenesis can occur directly from zygotic embryos and young *in vitro* plantlets or indirectly from callus of hypocotyl, cotyledon, leaf, and stem explants following

exposure to auxin (Razdan, 1993). Embryogenesis is reported from callus of hypocotyl, cotyledon (Cranshaw and Smith, 1995), juvenile leaves (Watt *et al.*, 1991) and zygotic embryos (Pinto *et al.*, 2002) of *E. grandis* and zygotic embryos and cotyledons of *E. citriodora* (Muralidharan *et al.*, 1989), with successful transfer of plants to the field.

In the previous chapter using hypocotyl, cotyledon and leaf explants of *E. erythronema, E. stricklandii* and their interspecific hybrid *E.* 'Urrbrae Gem' *in vitro* seedlings did not produce somatic embryos. The objective of the work reported in this chapter was to use higher levels of auxin for somatic embryogenesis and organogenesis in apex and cotyledon explants of *E. erythronema* var. *erythronema, E. stricklandii,* and *E.* 'Urrbrae Gem', and apex explants of *E. erythronema* var. *erythronema* x *E. stricklandii* hybrid 2.5 *in vitro* shoots, using the technique reported by Pinto *et al.* (2002) for *E. grandis*.

7.2 Materials and methods

7.2.1 Plant material

Seed collection, decontamination and germination of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' and maintenance of *E. erythronema* var. *erythronema* x *E. stricklandii* hybrid 2.5 were as per Section 5.2.

7.2.2 Callus, root, bud, shoot and somatic embryo induction

Apex explants of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' were excised from 2 wk old seedlings and *in vitro* shoots of hybrid 2.5, while cotyledon explants were harvested from 3 day old seedlings of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem'. Explants were placed on MS medium with 30 gL^{-1} sucrose, 3 gL^{-1} Gelrite® (Sigma), pH 5.8 which had been previously autoclaved at 121 °C for 15 min,

and poured into sterile 30 ml polycarbonate tubes, with 10 ml medium per tube, plus the following PGR, converted to μM from Pinto *et al.* (2002); control (no PGR), 5.37 μM NAA/4.5 μM 2,4-D, 16.11, 25.85, and 80.55 μM NAA.

Treatments were arranged in a randomised complete block design with one explant per tube, 10 replicates per treatment, and incubated at 24°C in the dark. Explants were subcultured to MS medium without plant growth regulators at 1 wk for 80.55 μM NAA and at 4 wk for the control, 5.37 μM NAA/4.5 μM 2,4- D, and 16.11 and 25.85 μM NAA. Explants were subcultured to MS medium without PGR at 4 wk intervals twice more. After 4 wk from commencement of the experiment explants were transferred from the dark to dim light for 16 hr per d at 11 μmoles m^{-2} sec^{-1} for 14 d, then transferred to cool white light for 16 hr per d at 75 μmoles m^{-2} sec^{-1} for the remainder of the experiment. Data were collected at each 4 wk subculture and included callus growth and appearance, and the number of roots, buds, shoots and somatic embryos. The effect of different treatments on callus growth was assessed by visual scoring as per Table 5.2. Data were analysed for significant differences using ANOVA in PlotIT 3.2 (Scientific Programming Enterprises, Haslet, MI, USA) with the least significant differences calculated at the 0.05 probability level (*P*□0.05).

7.2.3 Light microscopy

At 0, 2, 4, 7, 14 d and 12 weeks, specimens of callus 5 mm or smaller with and without roots, buds, shoots or somatic embryos from apex and cotyledon explants were fixed in 3% glutaraldehyde, dehydrated via an ethanol series, and embedded in glycol methacrylate. Sections 3 μm in thickness were stained with periodic acid-schiffs reagent and toluidine blue O for observation under a light microscope by the method of O'Brien and McCully (1981) (Appendix 2).

7.3 Results

7.3.1 Callus, root, bud, shoot and somatic embryo induction.

Callus growth on apex and cotyledon explants continued after subculture to PGR free medium but browned after 8 wk on PGR free medium. There was significantly less callus growth from the control compared to PGR treatments and less callus growth following a 1 wk exposure to high levels of NAA compared to explants that remained on PGR for 4 wk, except for *E. stricklandii* (Table 7.1). Callus growth with NAA/2,4-D was soft and spongy, while callus growth with NAA was hard, friable and of good appearance (Plate 7.1).

Shoot growth was best on apex explants with the control and following 1 wk on 80.55 μM NAA (Plate 7.2). Roots and shoots developed simultaneously at 4 wk, on apex explants exposed to 80.55 μM NAA for 1 wk in all genotypes (Table 7.1, Plate 7.2). Root and shoot growth was reduced when 2,4-D was used in combination with NAA, and 2,4-D continued to affect root and shoot development following explant subculture to PGR free medium. Root development was observed from apex and cotyledon explants with all PGR treatments (Plate 7.3), except for hybrid 2.5 apex explants on 25.85 μM NAA. Low shoot numbers were observed from cotyledon explants of *E.* 'Urrbrae Gem' on the control, and *E. stricklandii* on 16.11 and 80.55 μM NAA at 12 wk.

After subculture to PGR free medium very low bud numbers were observed on apex explants of *E. erythronema, E.* 'Urrbrae Gem' and hybrid 2.5 and cotyledon explants of *E.* 'Urrbrae Gem' (data not shown). No macroscopically visible somatic embryos were observed on any treatment for any genotype.

Table 7.1 Callus, roots, and shoots at 12 wk from apex and cotyledon explants of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' and apex explants of hybrid 2.5.

	Apex						**Cotyledon**					
	1	2	3	4	5	LSD	1	2	3	4	5	LSD
Callus												
E. erythronema	0.3^{c}	3.5^{a}	3.5^{a}	4.3^{a}	2.3^{b}	0.8	0.3^{c}	2.8^{b}	3.0^{b}	3.0^{b}	1.0^{c}	0.9
E. stricklandii	0.7^{c}	3.9^{b}	4.1^{a}	4.7^{a}	3.6^{ba}	0.7	0.2^{c}	4.4^{ba}	4.2^{ba}	3.7^{b}	3.0^{b}	0.9
E. 'Urrbrae Gem'	0.9^{e}	4.9^{a}	4.0^{cb}	3.8^{c}	2.3^{d}	0.6	0.4^{c}	3.9^{a}	3.5^{a}	3.7^{a}	1.9^{b}	0.9
Hybrid 2.5	0.05^{c}	3.9^{a}	4.0^{a}	3.7^{a}	2.2^{b}	0.5						
Roots												
E. erythronema	0.1^{c}	1.9^{cb}	6.9^{a}	4.3^{ba}	3.9^{ba}	3.3	0.0^{b}	0.6^{b}	5.1^{ab}	7.1^{a}	1.4^{b}	5.2
E. stricklandii	0.0^{c}	1.8^{b}	4.6^{b}	6.8^{ba}	6.0^{ba}	3.3	0.0^{c}	0.7^{c}	1.3^{cb}	1.1^{cb}	2.7^{b}	1.6
E. 'Urrbrae Gem'	0.3^{c}	1.7^{cb}	2.9^{b}	2.5^{cb}	4.0^{b}	2.3	0.6^{c}	2.1^{c}	7.7^{b}	3.7^{cb}	4.8^{cb}	5.8
Hybrid 2.5	0.0^{c}	0.05^{c}	0.15^{cb}	0.0^{c}	2.2^{b}	1.2						
Shoots												
E. erythronema	3.7^{a}	0.1^{c}	0.9^{cb}	2.0^{ba}	2.5^{ba}	1.8	0.0	0.0	0.0	0.0	0.0	0.0
E. stricklandii	1.3^{cb}	0.7^{c}	1.1^{cb}	1.0^{c}	1.7^{b}	0.6	0.0	0.0	0.1^{c}	0.0	0.2^{c}	0.3
E. 'Urrbrae Gem'	2.0^{a}	0.3^{c}	0.9^{cb}	0.8^{cb}	0.9^{cb}	0.8	0.2^{c}	0.0	0.0	0.0	0.0	0.2
Hybrid 2.5	2.2^{a}	0.5^{c}	1.0^{cb}	0.7^{cb}	1.2^{b}	0.5						

Numbers represent the mean callus score, as described in materials and methods, and mean root and shoot numbers of ten replicates per treatment.
Different letters within each row indicate significant differences (*P*□0.05), with a as the highest.
Plant growth regulator treatments

1 control
2 5.37 μM NAA/4.5 μM 2,4-D
3 16.11 μM NAA
4 25.85 μM NAA
5 80.55 μM NAA

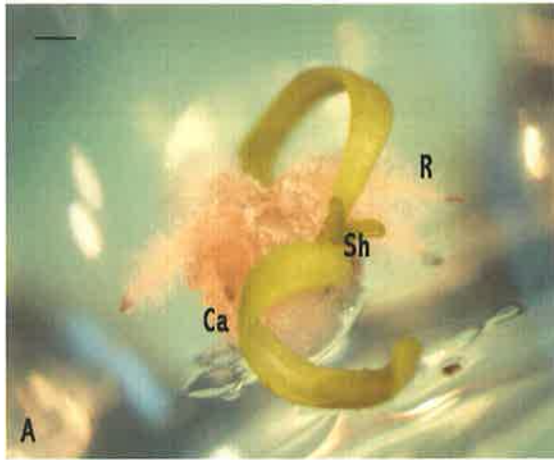
Plate 7.1 Callus, root and shoot growth on apex and cotyledon explants;

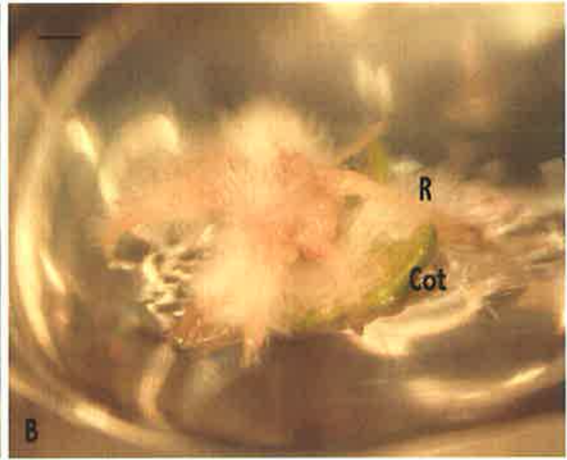
A) Callus (Ca), root (R), and shoot (Sh) on apex explants of *E.* 'Urrbrae Gem' on 16.11 µM NAA at 4 wk from commencement of the experiment. Bar = 1 mm.

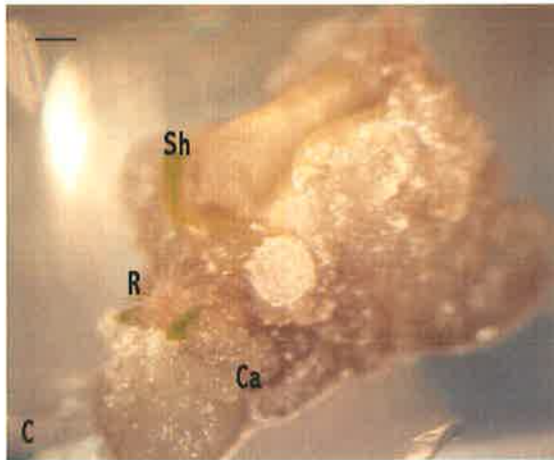
B) Root (R) on cotyledon (Cot) explant of *E.* 'Urrbrae Gem' on 16.11 µM at 4 wk from commencement of the experiment. Bar = 1 mm.

C) Callus (Ca), root (R), and shoot (Sh) on apex explant of *E. stricklandii* on 5.37 µM NAA/4.5 µM 2,4-D at 4 wk from commencement of the experiment. Bar = 1 mm.

D) Callus (Ca) on cotyledon (Cot) explant of *E. stricklandii* on 5.37 µM NAA/4.5 µM 2,4-D. at 12 wk from commencement of the experiment. Bar = 1 mm.


R
Sh
Ca
A


R
Cot
B


Sh
R
Ca
C

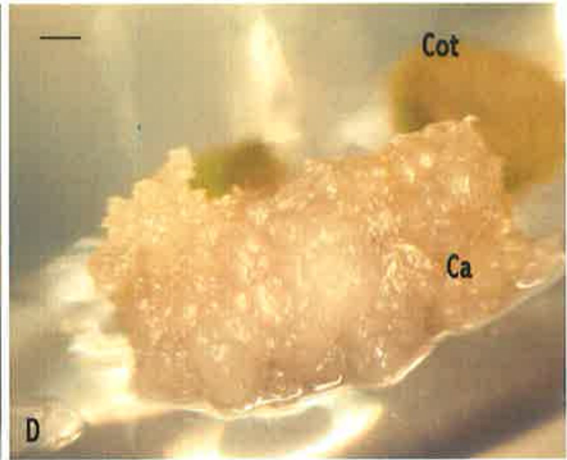
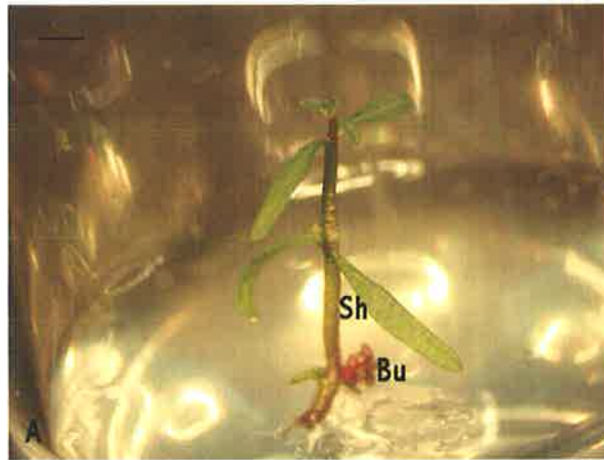

Cot
Ca
D

Plate 7.2 Callus, roots, buds and shoots on apex and cotyledon explants of *E. erythronema* at 8 wk from commencement of the experiment;

A) Apex explant on the control developed into a single shoot (Sh) with a few buds (Bu) at the basal node. Bar = 1 mm.

B) Root (R) and shoot (Sh) from apex explant, from treatment 80.55 μM NAA. Bar = 1 mm.

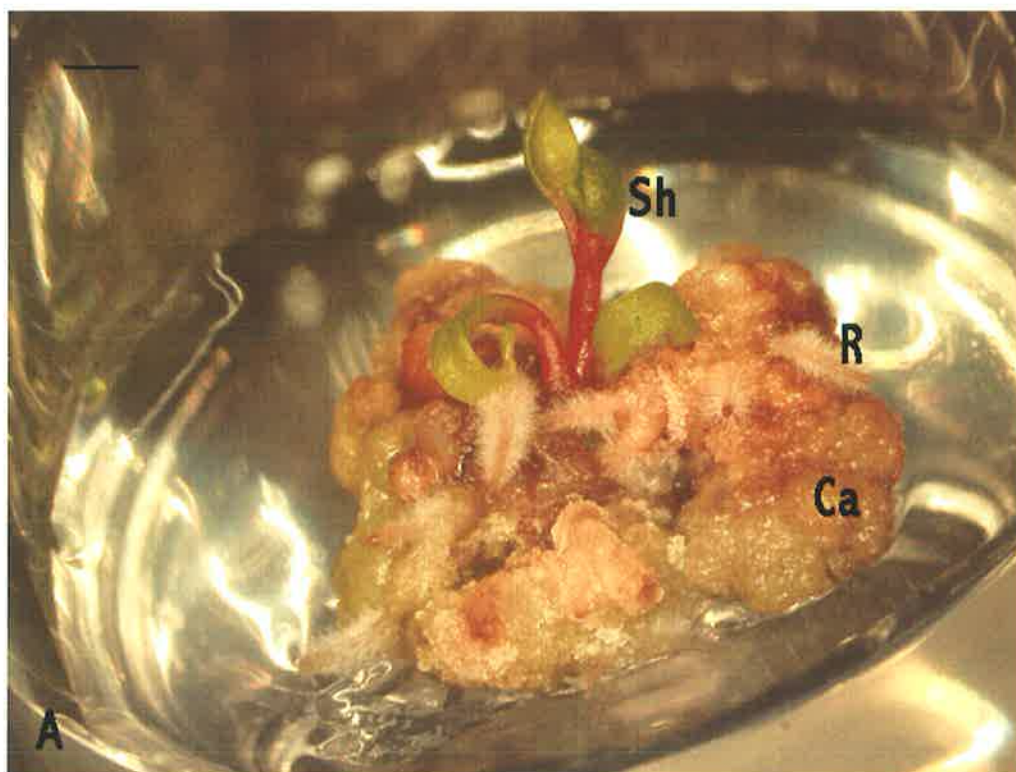

Sh
Bu
A

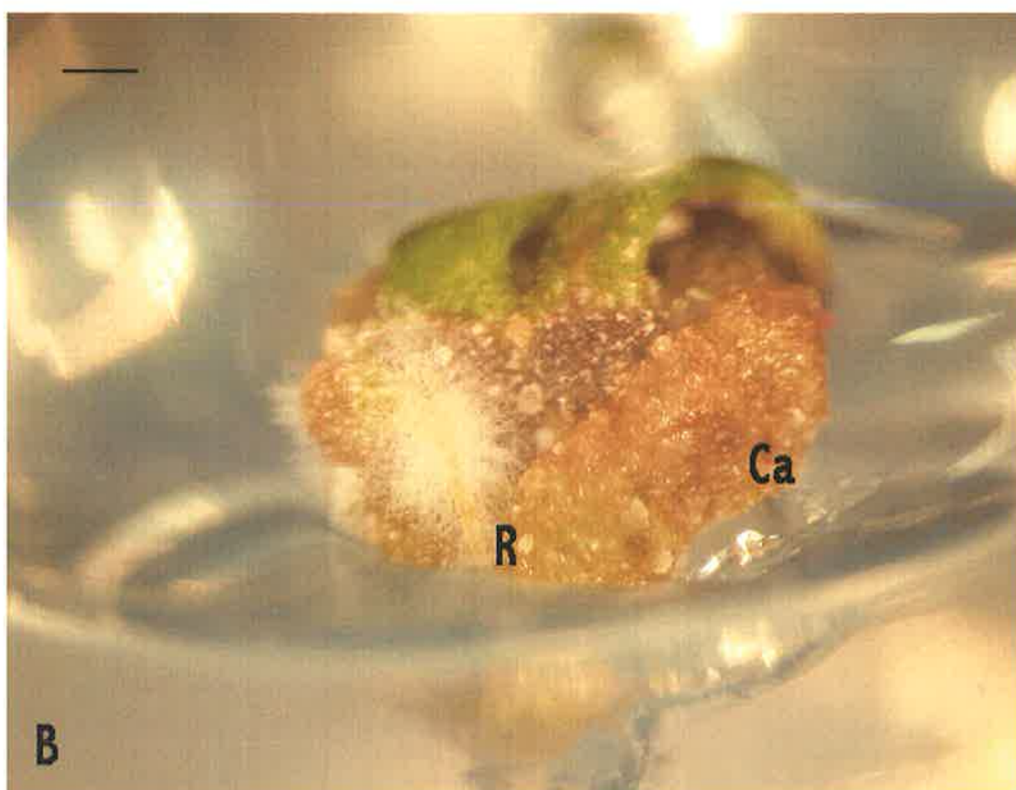
Sh
R
B

Plate 7.3 Callus, roots and shoots on apex and cotyledon explants of *E. stricklandii* at 8 wk from commencement of the experiment;

A) Callus (Ca), root (R), and shoot (Sh) on apex explant on 25.85 μM NAA. Callus was beginning to brown and shoot growth followed subculture to PGR free medium, Bar = 1mm.

B) Callus (Ca) and root (R) growth on cotyledon explant on 25.85 μM NAA, with callus growing from the cut end and lifting the explants off the medium, Bar = 1 mm.


Sh
R
Ca
A


Ca
R
B

7.3.2 Light microscopy

Callus growth and anatomical changes occurring within the explant appeared to be similar for all genotypes.

A large cell, associated with early somatic embryo development, as seen on *Olea europaea* (Benelli *et al.,* 2001), was observed from an apex explant of *E.* 'Urrbrae Gem' at 10 days on 16.11 μM NAA (Plate 7.4). Globular embryo structures were observed at 14 d on apex explants of *E.* 'Urrbrae Gem' on 16.11 μM NAA, (Plate 7.5) but not from apex and cotyledon explants at 0, 2, 4 and 7 d. Embryos did not develop beyond the globular stage. At 12 wk groups of meristematic cells were observed within the callus of apex explants with areas of circular areas of dividing cells observed within apex and cotyledon callus at 12 wk (Plate 7.5, 7.6).

Root development originated from within the callus, with the vascular tissue of the root connecting to the explant vascular tissue for both apex and cotyledon explants (Plate 7.7).

Callus development on cotyledon explants from the different genotypes was similar. At 12 wk, cotyledon explants from the no plant growth regulator treatment showed groups of cells with densely packed cytoplasmic and prominent nuclei at the explant base, where the cotyledons had been excised from the seedlings (Plate 7.7). These meristematic areas did not form somatic embryos on the selected treatments.

Plate 7.4 Apex explant of *E.* 'Urrbrae Gem' on 16.11 μM NAA at 10 days showing a large cell (arrow) (x 20). Bar = 50 μm

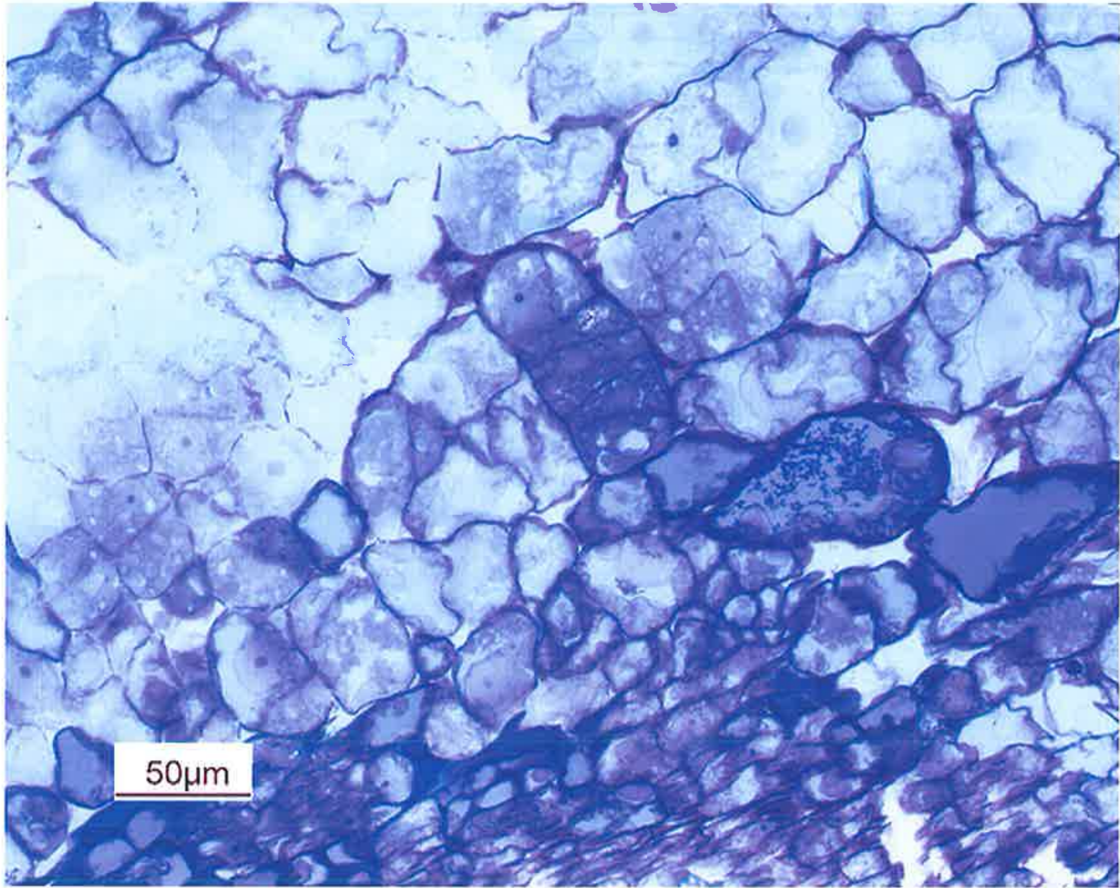

50μm

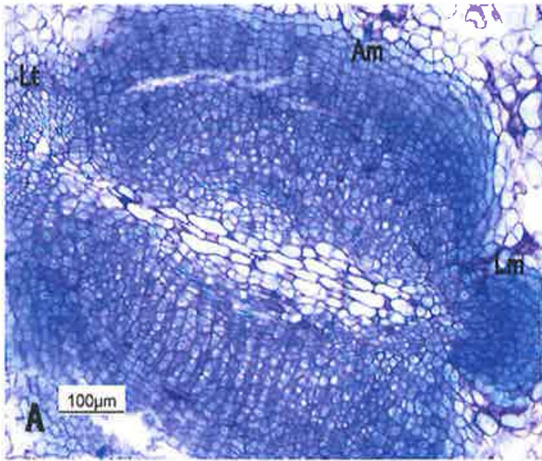
Plate 7.5 Apex explants of *E.* 'Urrbrae Gem' on 16.11 μM NAA showing

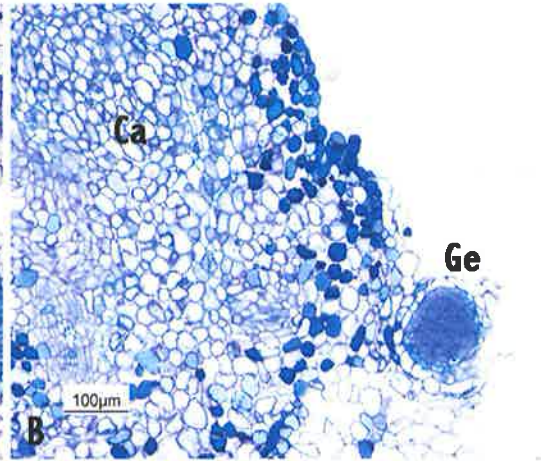
A) Transverse section of the meristem area within the explant at 7 d showing leaf trace (Lt), apical meristem (Am) and axillary leaf bud (Lm) (x 10). Bar = 100 μm

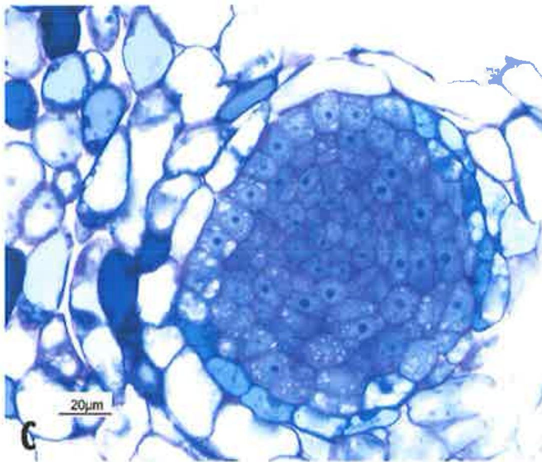
B) Callus (Ca) at 14 d with globular embryo (Ge) developing on the callus surface (x 10). Bar = 100 μm

C) The above globular embryo, showing isodimetric cells with densely packed cytoplasm and prominent nuclei (x 40). Bar = 20 μm

D) Callus (Ca) development at 12 wk with vascular tissue (Vt) and defined areas of circular growth (Cg) at the surface (x 10). Bar = 100 μm


Lt
Am
Lm
100µm
A


Ca
Ge
100µm
B


20µm
C

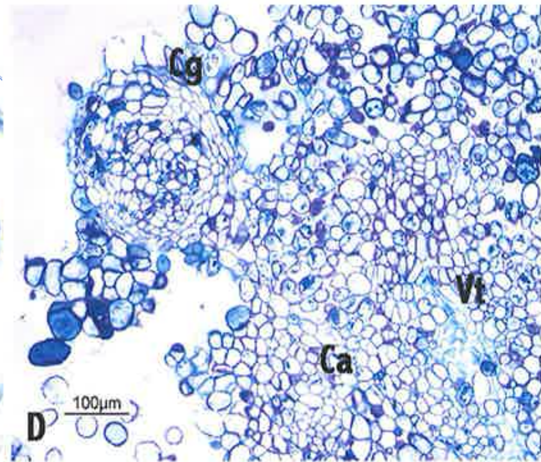
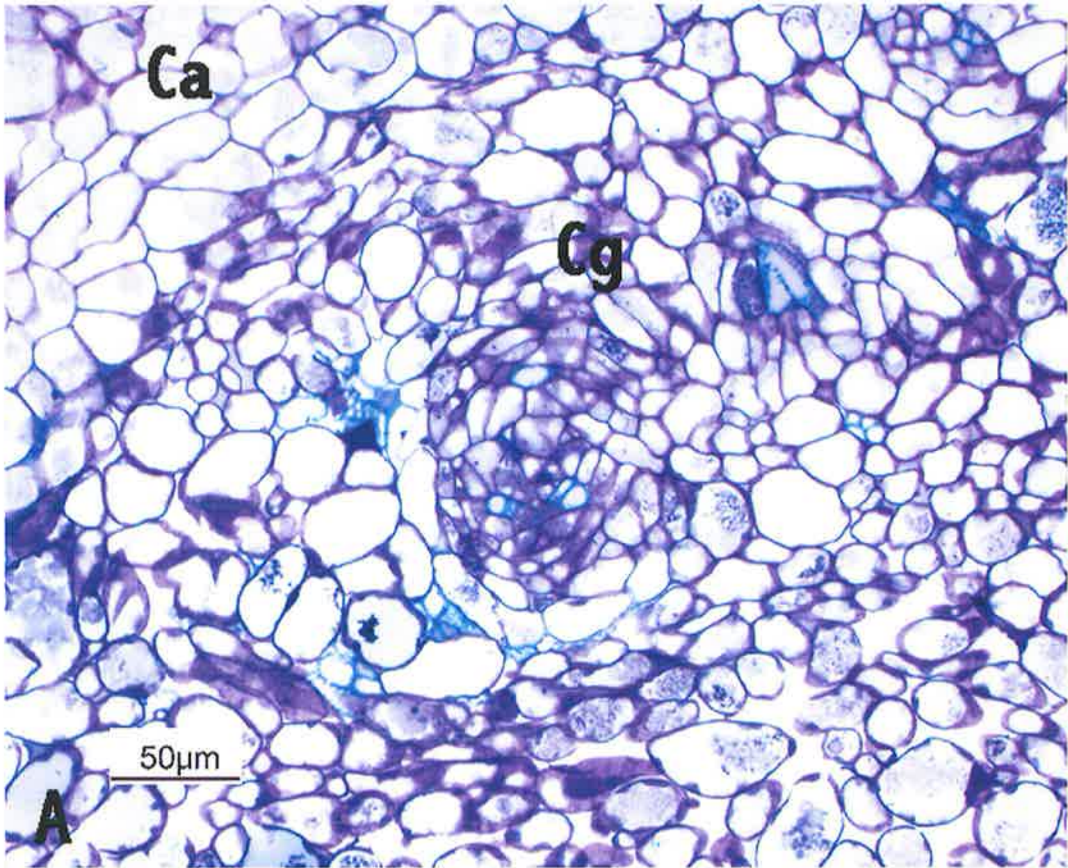

Cg
Vt
Ca
100µm
D

Plate 7.6 Comparison of cell growth between cotyledon and apex explants and with and without 2,4-D;

A) Callus (Ca) on cotyledon explant of *E. erythronema* on 5.37 μM NAA/4.5 μM 2,4-D at 12 wk, with circular areas (Cg) of dividing cells within the callus (x 20). Bar = 50 μm
B) Callus (Ca) on apex explant of *E.* 'Urrbrae Gem' on 16.11 μM NAA at 14 d with developing meristem (M) at the surface of the explant (x 20). Bar = 50 μm


Ca
Cg
50µm
A

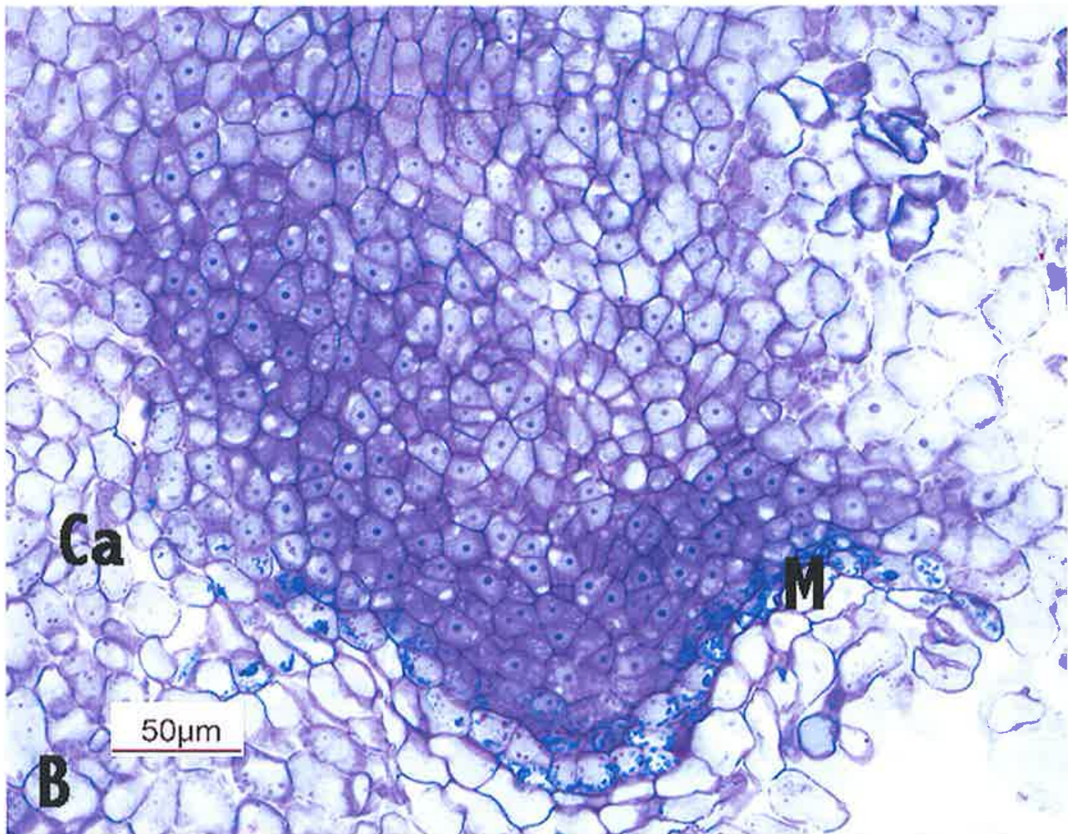
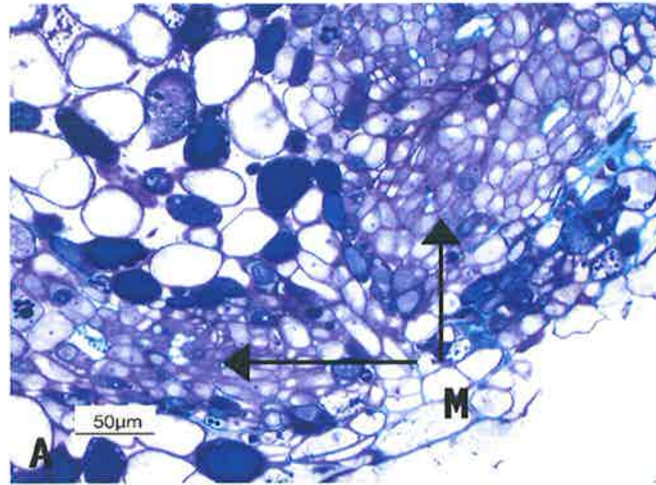
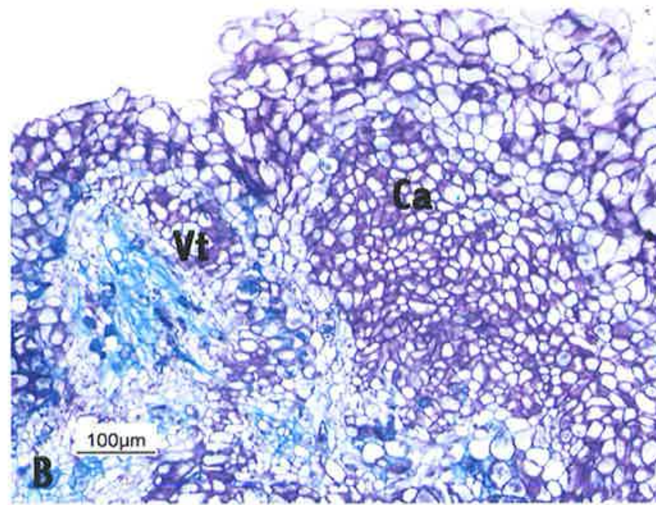
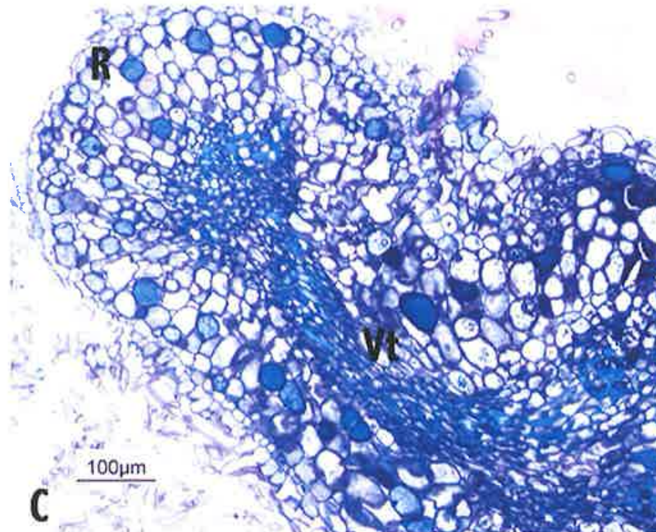

Ca
M
50µm
B

Plate 7.7 Callus and root development on cotyledon explants of *E. erythronema* showing

A) The control at 7 d with groups of dividing cells with densely packed cytoplasm (M, arrows) (x 20). Bar = 50 μm
B) Callus (Ca) and vascular tissue (Vt) on 5.37 μM NAA/4.5 μM 2,4-D at 12 wk, no somatic embryogenesis was observed (x 10). Bar = 100 μm
C) Root (R) on 80.55 μM NAA at 12 wk with vascular connection (Vt) to the explant (x 10). Bar = 100 μm


M
50µm
A


Ca
Vt
100µm
B


R
Vt
100µm
C

7.4 Discussion

This study has shown that NAA alone or in combination with 2,4-D will induce root organogenesis in both seedling and *in vitro* shoots and globular embryo like structures with NAA alone. When comparing somatic embryogenesis with root development, somatic embryo growth is generally reported from the callus or explant surface layers (Kumar, *et al.,* 2002, Benelli *et al.,* 2001) whereas root growth, in this case, was associated with the explant vascular tissue. Factors that inhibit, or are required to stimulate somatic embryogenesis in ornamental eucalypts need to be identified. The addition of various chemicals to the medium has proved successful in a range of woody plants. The addition of activated charcoal to the medium has been shown to improve embryogenesis in carrot (Razdan, 1993), *Olea europaea* (Benelli *et al.,* 2001) and *Vitis* sp. (Motoike *et al.,* 2001). Activated charcoal is known to bind toxins produced by the explant, or developing callus, and this may improve explant health and subsequently the ability to regenerate somatic embryos. Other additives reported to improve somatic embryogenesis include polyamines with *Panax ginseng* (Kevers *et al.,* 2000), polyethylene glycol with loblolly pine (Li *et al.,* 1998), and silver nitrate with both date palm (Al-Khayri and Al-Bahrany, 2001) and *Coffea canephora* (Fuentes *et al.*, 2000). Following initiation of somatic embryos, culture on medium containing cytokinin may be required to further development, as found in acacia (Quoirin, 2003), olive (Shibli *et al.*, 2001), fox grape (Motoike *et al.,* 2001), and oak (Wilhelm, 2000).

NAA and 2,4-D are commonly used to induce callus and plant regeneration through somatic embryogenesis in a range of woody and herbaceous plants (Mamiya and Sakamoto, 2001; Wilhelm, 2000; Edwald, 1998; Zaerr and Mapes, 1982). Hard, friable callus is preferred for organogenesis, somatic embryogenesis or suspension cultures (Doran, 1996), which have proved successful for globular somatic embryo

development with *E. nitens* (Ruaud *et al.,* 1997). However, such callus was not produced using 2,4-D in this study. Root development was observed on all explants treated with PGR and was lowest when 2,4-D was used in combination with NAA, as was shoot development from apex explants. 2,4-D does not appear a suitable auxin for callus development, somatic embryogenesis, or organogenesis in these genotypes.

The maintenance of viable callus cultures has proved difficult with certain *Eucalyptus* species particularly because of callus browning, which appears to be related to several factors. Callus of *E. microcorys* (Niccol *et al.,* 1994), and *E. tereticornis* (Subbaiah and Minocha, 1990) required frequent subculturing, otherwise browning and cell death occurred after 2-4 weeks. For *E. grandis* the choice of explant was critical as callus from immature inflorescences browned and died (Warrag *et al.,* 1991). The type of PGR can be detrimental to callus growth as found with *E. grandis* x *E. urophylla* explants on thidiazuron (Cid *et al*., 1999). In the present study, callus browned and died at 8 wk following subculture to PGR free medium. Explants of *E. erythronema, E. stricklandii,* and their hybrids appear to require frequent subculture to medium with PGR to maintain callus growth and viability.

High auxin exposure for 7 d proved to be a suitable rooting method for shoot tips as there was no basal callus, good root growth and strong shoot growth, with the resulting plantlets suitable for hardening off to the natural environment. Shoot tip explants taken from pea and treated with 1 μM NAA were regenerated to whole plants (Kartha, 1981), and the author suggested that the presence of endogenous cytokinins, plus the exogenous auxin, provided the correct cytokinin:auxin balance for shoot and root development.

Mature somatic embryos have yet to be induced from the hybrids *E.* cv. 'Urrbrae Gem' and 2.5, or the parental species *E. erythronema* and *E. stricklandii* and further

work is required to develop a protocol. This could be approached by looking at a range of factors such as plant growth regulator type, concentration and exposure time, and nutrient salt combinations. Other possibilities include the role of media additives, the technique of liquid culture, and the use of cytokinins in embryo expression media for their effect on somatic embryo development. Environmental conditions, and stress treatments such as starvation and chilling, could also be factors in successful plant regeneration from somatic embryos.

Chapter 8

Micropropagation of *E.* 'Urrbrae Gem' seedlings, and *E. erythronema* var. *erythronema* x *E. stricklandii* hybrids 35.2 and 2.5

8.1 Introduction

The most common method of micropropagation for eucalypts is enhanced axillary shoot proliferation whereby axillary shoots are subdivided either for further proliferation or for root induction and subsequent hardening off to the external environment (Kane, 1996). de Fossard (1976, 1983) investigated various plant media and concluded that many plants have a broad tolerance to certain inorganic elements of the medium but not to others. Complicating this are interactions over time between the minerals, gelling agents, available water and the mode of ion uptake (Williams, 1993). These studies show the importance of mineral nutrient type and concentration in relation to plant nutrient uptake and growth. The majority of eucalypts are grown on Murashige and Skoog (1962) (MS) or modified MS media (Le Roux and van Staden, 1991b). However, woody plant medium is also suitable for *E. regnans* (Blomstedt *et al.,* 1991) and *E. citriodora* (Ostrowska *et al.,* 1998), while de Fossard *et al.* (1978) developed a medium specific for *E. ficilolia* and Gribble *et al.* (2002) used tissue analysis of potted glasshouse seedlings to develop a medium for *E. urophylla* x *grandis.* Clearly, each new species introduced into culture requires a suitable growth medium.

Following shoot multiplication, shoot elongation may be required prior to rooting. Gibberellic acid (GA_3) in the multiplication medium can be used to elongate shoots of *E. grandis* (Lain and David, 1994). However, in *E. gunni* (Curir *et al.,* 1990) and *E. ficifolia* (de Fossard *et al.,* 1978), GA_3 was found to inhibit rooting. Shoot

elongation can also be achieved by adding activated charcoal to the medium (Curir *et al.,* 1990), and reducing medium nutrients (Sharma and Ramamurthy, 2000; Cortezzi Graca and Mendes, 1989; de Fossard *et al.,* 1978) but at the cost of reduced shoot multiplication.

Successful rooting of *in vitro* shoots appears dependent on a particular combination of explant age, time in culture, concentration of and exposure time to plant growth regulators and nutrient salt concentration. Juvenile explants of woody plants can show a greater rooting ability than mature explants, as found in *E. nitens* (Gomes and Canhoto, 2003) and *E. sideroxylon* (Burger, 1978). Improved rooting can be achieved with many eucalypt species by lowering the nutrient salt concentration of the medium (Watt *et al.*, 2000; Sharma and Ramamurthy, 2000; Bennet *et al.,* 1994; Le Roux and van Staden, 1991b; Franclet and Boulay, 1982; Cortezzi Graca and Mendes, 1989). Pelosi (1995) found BAP in the multiplication medium to inhibit rooting in *E. globulus,* however, this problem was overcome by Bennett *et al.* (1994, 2003) when kinetin was used prior to the rooting phase.

This chapter describes shoot and root development on different media and plant growth regulators from *in vitro* axillary shoots of open-pollinated seedlings of *E.* 'Urrbrae Gem' and nodal explants of *E. erythronema*, *E. stricklandii* and *E. erythronema* x *E. stricklandii* hybrids 2.3 and 35.2.

8.2 General materials and methods

The following methods were consistent throughout the experiments unless otherwise stated. Open pollinated seeds of *E.* 'Urrbrae Gem' (tree 1867 planted in the Waite Arboretum) were sterilised and germinated as described in Section 5.2. Two vigorous seedlings, termed lines 1 and 3, were multiplied by harvesting axillary shoots

at 3-4 wk intervals and subculturing shoots on ½ MS (Murashige and Skoog, 1962) with full strength MS vitamins, 20 g^{-1} sucrose, 4.4 μM 6-benzylamino purine (BAP), 1 μM α-naphthaleneacetic acid (NAA) and gelled with 7 g^{-1} Phytagel®.

E. erythronema x *E. stricklandii* hybrids 35.2 and 2.5 were growing as potted plants under glasshouse conditions. Stems were harvested and nodal explants initiated into culture as described in Section 4.2. These were multiplied by harvesting axillary shoots at 3-4 wk intervals with subculture to QL medium, containing 20 g^{-1} sucrose, 2.2 μM BAP, 0.1 μM NAA, 0.5 μM GA_3, gelled with 7 g^{-1} Phytagel®.

All media were adjusted to pH 5.7 before autoclaving for 20 min at 121°C, then poured into 250 mL polycarbonate pots with vented lids, with 40 mL of medium per pot. Heat sensitive chemicals were filter sterilised using a 0.45 μM filter and added after autoclaving when the basal medium had cooled to 55°C. Explants were placed onto medium and incubated at 24°C with 16 hr light and 8 hr dark, with a cool white light reading of 75 µmoles m^{-2} sec^{-1}. Data were analysed for significant differences using ANOVA in PlotIT 3.2 (Scientific Programming Enterprise, Haslett, MI, USA) with least significant differences (LSD) calculated at the P=0.05 level.

8.3 Micropropagation of *E.* 'Urrbrae Gem' seedlings line 1 and 3

8.3.1 Effect of nutrient media and gibberellic acid on axillary shoot growth

8.3.1.1 Materials and methods

In vitro shoots of *E.* 'Urrbrae Gem' seedling lines 1 and 3 which had been in culture for 7 months as described in Section 8.2 were harvested with 2-3 nodes each and allocated to the following media (Appendix 1): MS, Murashige and Skoog (1962); B5, Gamborg *et al.* (1968); WPM, Lloyd and McCown (1980); AP, Almehdi and Parfitt (1986); TK, Tabachnik and Kester (1977); QL, Quoirin and Lepoivre (1977). All media

were supplemented with 2.2 μM BAP, 1.0 μM NAA, 20 g^{-1} sucrose, 0.0 and 1.5 μM gibberellic acid (GA_3) and gelled with 7 g^{-1} Phytagel®. Shoots were placed 5 per pot (replicate) and treatments were arranged as a completely randomised design (CRD) with 5 replicates per treatment. Data were collected at 4 wk and included axillary shoot number, leaf colour, general appearance and browning in the medium (Table 8.1).

Table 8.1 Scoring method for leaf colour, exudates in the medium and general shoot appearance

Leaf colour	
Score	**Description**
1	dark green
2	green
3	pale green
4	yellow, with or without leaf tip browning
Medium browning	
0	No brown staining around the explant
1	Very slight browning around the explant
2	Light brown staining around the explant
3	Browning extending away from explant
4	Medium in container is brown
5	Dark brown staining of medium in the container
General appearance	
good;	healthy, green leaves, axillary shoot growth and shoot tip growth
fair;	pale green to green leaves, axillary shoot growth, thin spindly stems
poor;	pale green leaves, some leaf abscission, short internodes
very poor;	yellow to pale green leaves with brown tips, leaf abscission, no axillary shoot growth, very short internodes giving rosette appearance, stem and leaf callusing.

8.3.1.2 Results

8.3.1.2.1 Shoot number, leaf colour, media browning and general appearance

Axillary shoot numbers on all media were lower with the addition of GA_3 except for WPM and QL, where shoot numbers were higher (Table 8.1). WPM and QL with GA_3 showed the highest number of axillary shoots and MS with GA_3 the lowest, although there were no significant differences Shoots on QL medium with and without GA_3 were deflasked and length measured, while shoots on other treatments were kept for further multiplication. The mean shoot length without GA_3 was 3.3 mm, (SE ± 0.18) and with GA_3 was 6.5 mm, (SE ± 0.31). There was a significant increase in shoot length with the addition of GA_3 to QL medium.

Leaf colour on all media was paler with the addition of GA_3, except for WPM where leaf colour was similar and QL where leaf colour was greener (the higher the number, the paler the leaf). QL showed a significantly greener leaf compared to MS, B5, AP, and TK at 12 wk. (Table 8.1). Overall, leaf colour was mostly pale green to green, with leaf tip browning observed on yellowing leaves, and leaves often developing callus on the underside of the leaf.

Browning scores for all media were darker with the addition of GA_3 except for WPM where the media scores were lower with GA_3. Browning on media with GA_3 was lowest on MS and highest on TK media (Table 8.2).

The general appearance varied across the different media and was generally better in the absence of GA_3. Shoots on TK medium were poor to very poor with no shoot elongation when GA_3 was added to the medium. Shoots on MS, B5, and AP media produced poor to fair shoots, although there was an occasional healthy green shoot. Shoots on WPM medium varied from poor, to good and shoots on QL medium were fair to good, although leaf callusing was observed (Table 8.1).

8.3.1.3 Conclusions

Axillary shoot number was highest on WPM and QL with GA_3 and lowest on MS, AP and TK with GA_3. Leaf colour remained the same or improved with the addition of GA_3 to WPM and QL respectively, while GA_3 was detrimental to leaf colour on the other media. Media browning score increased with the addition of GA_3 to the media with the exception of WPM, probably indicating greater exudation of polyphenols. WPM with GA_3 was chosen for subsequent subcultures of *E.* 'Urrbrae Gem' seedlings as it gave good axillary shoot growth but did not increase the medium browning. The concentration of GA_3 was later reduced to 0.5 μM as this was found sufficient for shoot elongation.

Table 8.2 Shoot number, leaf colour (the higher the number the paler the leaf colour and media browning) of *E.* 'Urrbrae Gem' seedling *in vitro* shoots on twelve different media at 4 wk.

	Media						
	MS	WPM	B5	AP	Tk	QL	LSD
Shoot number							
No GA_3	1.0^{b}	1.4^{ab}	1.8^{a}	1.1^{ab}	1.4^{ab}	1.6^{ab}	0.9
With GA_3	0.8^{b}	2.1^{a}	1.7^{ab}	0.8^{b}	0.9^{b}	2.3^{b}	0.9
Leaf colour							
No GA_3	2.5^{a}	2.5^{a}	2.6^{a}	0.6^{a}	2.4^{a}	2.2^{a}	0.3
With GA_3	2.7^{ab}	2.5^{b}	2.9^{a}	2.8^{ab}	2.7^{ab}	2.0^{c}	0.3
Media browning							
No GA_3	1.0^{b}	1.8^{a}	1.5^{ab}	1.5^{ab}	1.5^{ab}	1.0^{b}	0.6
With GA_3	1.1^{c}	1.4^{cb}	1.9^{ab}	2.0^{a}	2.2^{a}	1.3^{c}	0.6

8.3.2 Effect of different concentrations and pulsing times of the plant growth regulator indole butyric acid on root development of *E.* 'Urrbrae Gem' seedling *in vitro* shoots line 1 and 3

8.3.2.1 Materials and methods

Following experiment outlined in 8.2 *in vitro* shoots of *E.* 'Urrbrae Gem' seedlings line 1 and 3 were maintained on WPM with 2.2 μM BAP, 0.5μM NAA, 0.5 μM GA_3, 20 g^{-1} sucrose, gelled with 7 g^{-1} Phytagel®. Shoots were harvested and placed on the following treatments; ½ WPM with 10 g^{-1} sucrose, 0.0, 2.5, 5.0 and 10 μM indole-butyric acid (IBA), gelled with 4 g^{-1} Phytagel® for 4 wk; ½ WPM, 10 g^{-1} sucrose with 0, 10, 20, and 40 μM indole-butyric acid (IBA) for 1 and 2 wk. At 1 and 2 wk shoots were subcultured to either ½ WPM, 10 g^{-1} sucrose, without plant growth regulators (PGR) or to peat pots (Jiffy Pot-Pack, product number 30051590) filled with sphagnum peat moss (Nu-Earth), coarse river sand (Waikerie sand), and perlite at a ratio of 0.5:2:2, with 6 peat pots per plastic disposable food container (Glad Ware, 946 mL container), sterilised for 40 min at 121°C.

Shoots were placed in the dark for one week then at 16 h light and 8 h dark, with a cool white light reading of 75 µmoles m^{-2} sec^{-1}. Data were collected at 4 wk and 8 wk for continuous exposure to IBA and at 4 wk following IBA pulse. Data included callus growth, root number and root length. The treatments were arranged in a randomised complete block design with one shoot per 30 mL polycarbonate tube, 10 mL medium per tube, and 10 replicates per treatment.

8.3.2.2 Results

8.3.2.2.1 Callus growth, root number and length for continuous IBA exposure

Callus growth was greater at 8 wk compared to 4 wk with a significant increase at levels over 2.5 μM IBA (Figure 8.1). Callus growth was significantly greater at 8 wk with IBA compared to the control. Basal callus made removal of plants for hardening off difficult as callus with emerging roots readily broke off when handled.

Root numbers were greater at 8 wk compared to 4 wk with the highest number on 2.5 μM IBA, although there was no significant difference. No roots were observed on the control (Figure 8.2). There was high variation between explants, with some explants developing large numbers of roots and others few.

Root length was greater at 8 wk compared to 4 wk, although there were no significant differences (Figure 8.2) (Plate 8.3). Roots were short and thick, and did not elongate, or elongated and grew throughout the medium. Both types of roots were observed on the same explant with all concentrations of IBA.

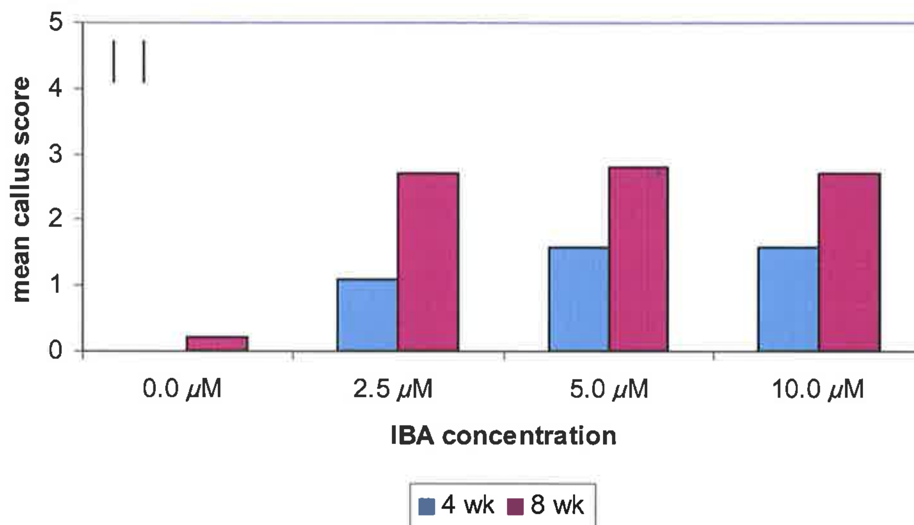


Figure 8.1 Callus growth on *E.* 'Urrbrae Gem' seedling shoot explants from five IBA treatments at 4 and 8 wk. LSD bars at the P=0.05 level (callus 4 wk, 0.6; 8 wk, 0.7)

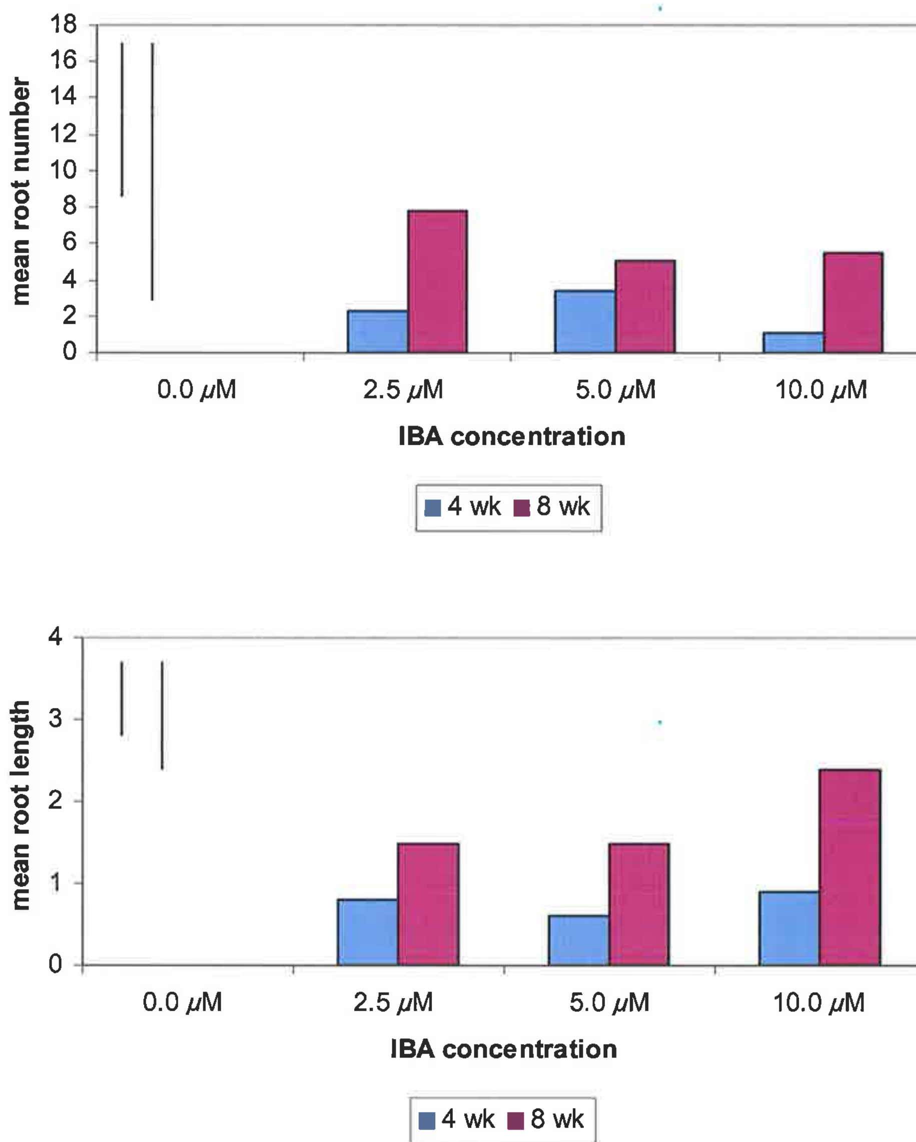


Figure 8.2 Root number and length on *E.* 'Urrbrae Gem' seedling shoot explants from five IBA treatments at 4 and 8 wk. LSD bars at the *P*=0.05 level (root number, 4 wk, 4.7; 8 wk, 8.0) (root length, 4 wk, 0.9; 8 wk, 1.3)

8.3.2.2.2 Callus growth, root number and length for IBA pulse

Basal callus growth was observed on all treatments and was significantly greater with a 2 wk pulse on 10 and 20 μM IBA followed by subculture to ½ WPM (Figure 8.3). Lowest callus score with IBA was observed following a 1 wk pulse on 20 μM IBA with subculture to peat pots.

Root development was observed following 1 wk on 10, 20, 40 μM IBA with subculture to ½ WPM with the greatest root number on 20 μM IBA. No roots were observed with 2 wk IBA treatment, shoots subcultured to peat pots, or from the control (Figure 8.4).

Root length was longest at 1 wk on 20 μM IBA followed by subculture to ½ WPM and significantly greater than 1 wk at 40 μM IBA (Figure 8.4). Roots grew directly from the stem and from the basal callus. Roots were either, short and thick and did not elongate, or elongated and grew throughout the medium. Both types of roots were observed on the same explant with 10 and 20 μM IBA treatments.

8.3.2.3 Conclusions

Continuous IBA exposure had a positive effect on callus and root development on *in vitro* shoots of *E.* 'Urrbrae Gem' line 1 and 3 with 2.5 μM IBA producing highest level of root growth. For IBA pusle successful rooting was achieved with 20 μM IBA over 7 days followed by subculture to ½ WPM with no PGR. Pulsing was the preferred method as lower callus growth was produced, making it easier to deflask plants without disturbing the roots.

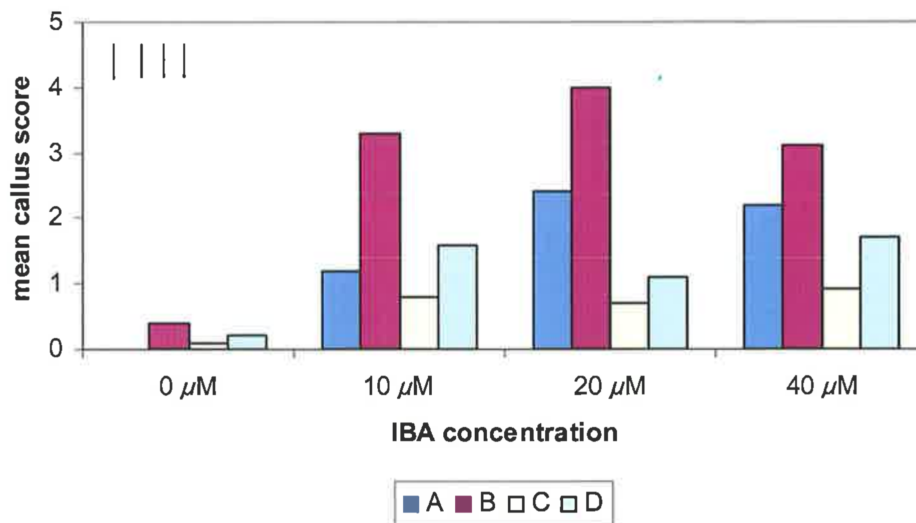


Figure 8.3 Callus on *in vitro* shoots of *E.* 'Urrbrae Gem' seedling explants from five IBA concentrations, pulsed for 1 and 2 wk before subculture to either ½ WPM or peat pots. Data collected 4 wk after subculture. LSD bars at the *P*=0.05 level (callus A, 0.9; B, 0.5; C, 0.5; D, 0.5)

A) 1 wk pulse subcultured to woody plant medium
B) 2 wk pulse subcultured to woody plant medium
C) 1 wk pulse subcultured to peat pots
D) 2 wk pulse subcultured to peat pots

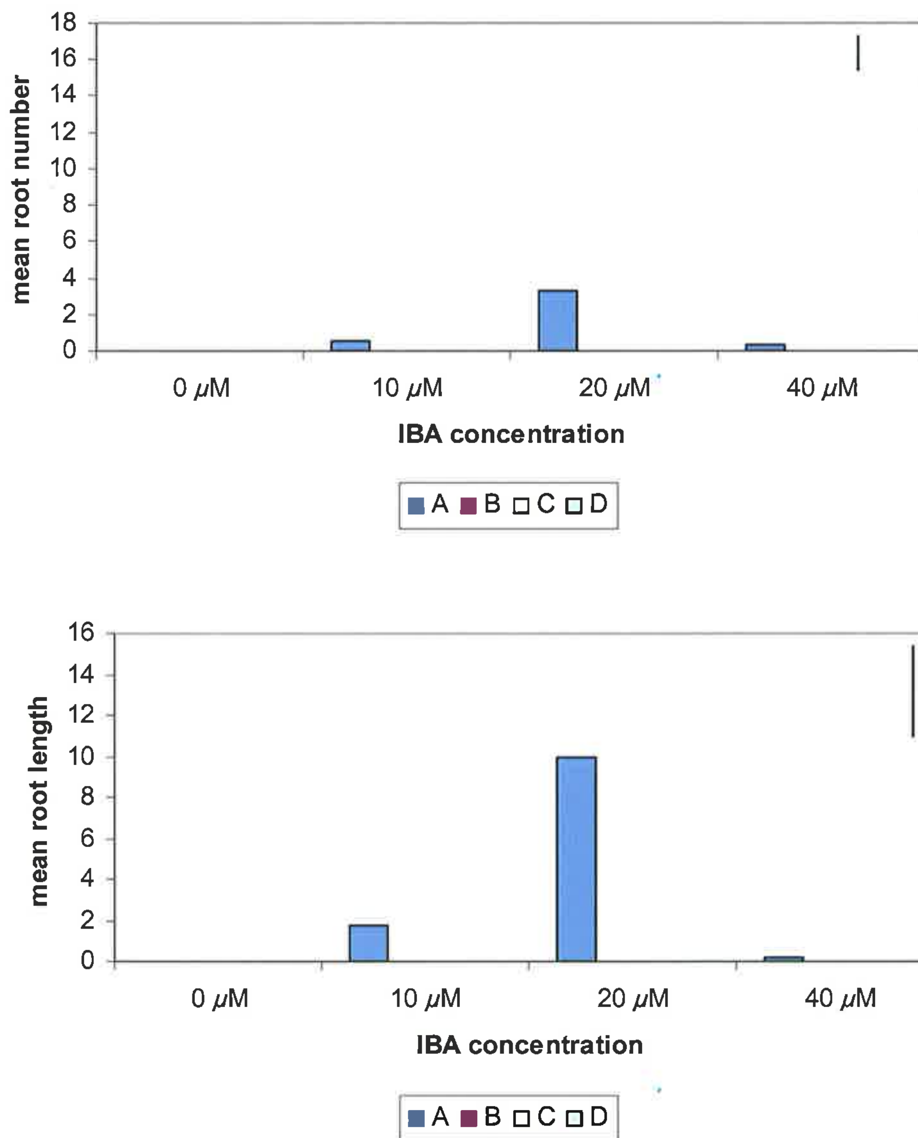


Figure 8.4 Root number and length on *in vitro* shoots of *E.* 'Urrbrae Gem' seedling explants from five IBA concentrations pulsed for 1 wk and 2 wk before subculture to ½ WPM or peat pots. Data collected 4 wk after subculture. LSD bars at the *P*=0.05 level (Root number A, 1.9) (Root length A, 4.4)

A) 1 wk pulse subcultured to woody plant medium
B) 2 wk pulse subcultured to woody plant medium
C) 1 wk pulse subcultured to peat pots
D) 2 wk pulse subcultured to peat pots

8.3.3 Hardening off of *in vitro* plants of *E.* 'Urrbrae Gem' seedlings

Root initiation and growth was achieved as per Section 8.3. Following root emergence plants were preconditioned by placing culture pots in the glasshouse for 2 days. This was to reduce stress following removal from the culture pots. Pots were prepared by pre-wetting Jiffy pots (Jiffy-strips, 4x5 cm, without hole, Jiffy product no. 30051590) then filling with Nu-Erth cutting mix. Using forceps the plant was removed from the jar and the agar gently washed off with sterile water and a paint brush. The plants were placed in Jiffy pots, watered with 20 mL/L Seasol (Rezitech,Vic), and placed in the glasshouse in a fogger at 67% humidity, 21°C for 3 days. Trays were placed on benches in trays with vented lids to control the humidity and watered daily. The vents were slowly open over 7 d before removál of the lid (Plate 8.1). Following the growth of new leaves and when roots had emerged from the peat pot, plants were potted into large tubes with Nu-Erth premium potting mix and watered in. Percentage of root development was 62% with 58% hardened off to the natural environment.

Some plants were lost due to damping off disease while those plants with short, thick roots also died. Successful hardening off occurred from plants whose roots elongated prior to deflasking.

Plate 8.1 Plants of *E.* 'Urrbrae Gem' seedling line 1, multiplied in tissue culture by axillary shoot proliferation, successfully rooted and hardened off to the external environment;

A) Plantlets in peat pots in tray. Bar = 2 cm

B) Plantlets in peat pot with Nu-Erth cutting mix. Bar = 1 cm

C) Plants at 6 months from hardening off.

A

C

8.4 Micropropagation of *E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5

8.4.1 Effect of benzylamino purine, zeatin, 2iP, and kinetin on axillary shoot growth

8.4.1.1 Materials and methods

In vitro shoots of *E. erythronema* x *E. stricklandii* hybrid 35.2 and 2.5 were maintained as per Section 8.2. Shoots were subcultured to QL medium supplemented with 30 g^{-1} sucrose, 4 g^{-1} activated charcoal (Sigma) for 4 wk prior to the experiment, to reduce any carry over effects of BAP.

In vitro shoots were harvested with 2-3 nodes and placed on WPM, 20 g^{-1} sucrose, gelled with 7 g^{-1} Phytagel® with the following plant growth regulator treatments; 2 μM of either: 6-benzylamino purine (BAP); 6[4-hydroxy-3-methylbut-2-enylamino] purine (zeatin); dimethylallyl amino purine (2iP); 6-furfuryl amino purine (kinetin) There were 5 replicates per treatment. Data were collected at 4 wk and included leaf colour, shoot number and medium browning (as per Table 8.1).

8.4.1.2 Results

8.4.1.2.1 Shoot number, leaf colour, medium browning and general appearance

Shoots on BAP showed the highest number of axillary shoot growth for both genotypes, and this was significantly greater than shoot numbers on medium with kinetin. There were no significant differences with BAP, zeatin or kinetin. Hybrid 35.2 developed more axillary shoots compared to hybrid 2.5 with the exception of BAP. Within each medium there were no significant differences in shoot number between genotypes (Figure 8.5). Both hybrids developed greater axillary shoot numbers than *E.* 'Urrbrae Gem' line 1 and 3 shoots on WPM and BAP without GA_3 in Section 8.3.

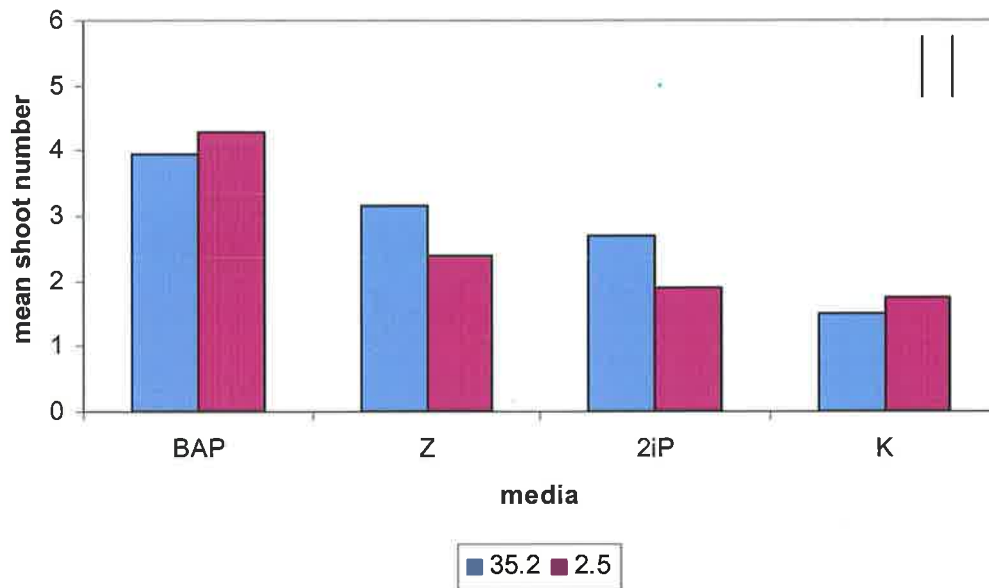
For hybrid 2.5, leaves were significantly greener on BAP and 2iP compared to zeatin and kinetin (the higher the score the paler the leaf colour). There were no significant differences between treatments for hybrid 35.2 (Figure 8.5). Overall leaf colour of hybrids 35.2 and 2.5 was greener than *E.* 'Urrbrae Gem' line 1 and 3 leaves on the same medium in Section 8.3.

Medium browning score for hybrid 35.2 were highest with kinetin and lowest with BAP, while for hybrid 2.5 browning scores were also highest with kinetin but lowest with zeatin (Figure 8.6).

Short internodes, which would require elongation for successful multiplication and rooting, were observed with BAP, 2iP and kinetin for both genotypes. Zeatin provided shoot growth with sufficient elongation but the leaves were paler. Leaf callus was a problem for both genotypes and was less with BAP compared to the other cytokinins (Table 8.1) (Plate 8.2).

8.4.1.3 Conclusions

The cytokinins BAP and zeatin were suitable for the multiplication of hybrids 35.2 and 2.5 by axillary shoot development, although shoots from BAP treatment would require elongation.



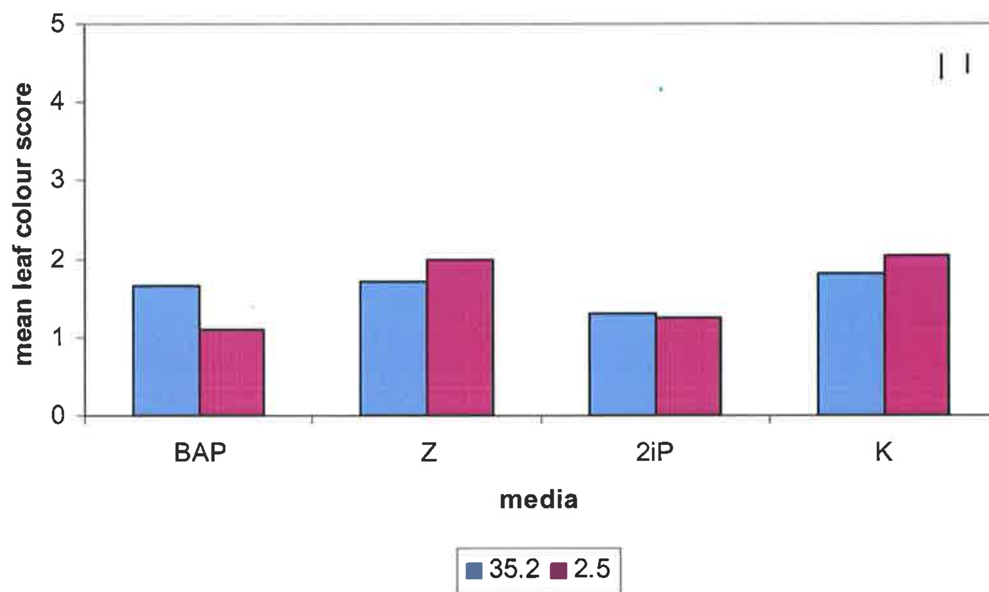


Figure 8.5 Shoot number and leaf colour for *E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5 on WPM with four different cytokinins at 4 wk (the lower the value the greener the leaf). LSD bars at the *P*=0.05 level (shoot number, 35.2, 0.94; 2.5, 0.92) (leaf colour, 35.2, 0.35; 2.5, 0.24)

BAP	6-benzylamino purine
Z	6[4-hydroxy-3-methylbut-2-enylamino] purine
2Ip	dimethylallyl amino purine
K	6- furfuryl amino purine

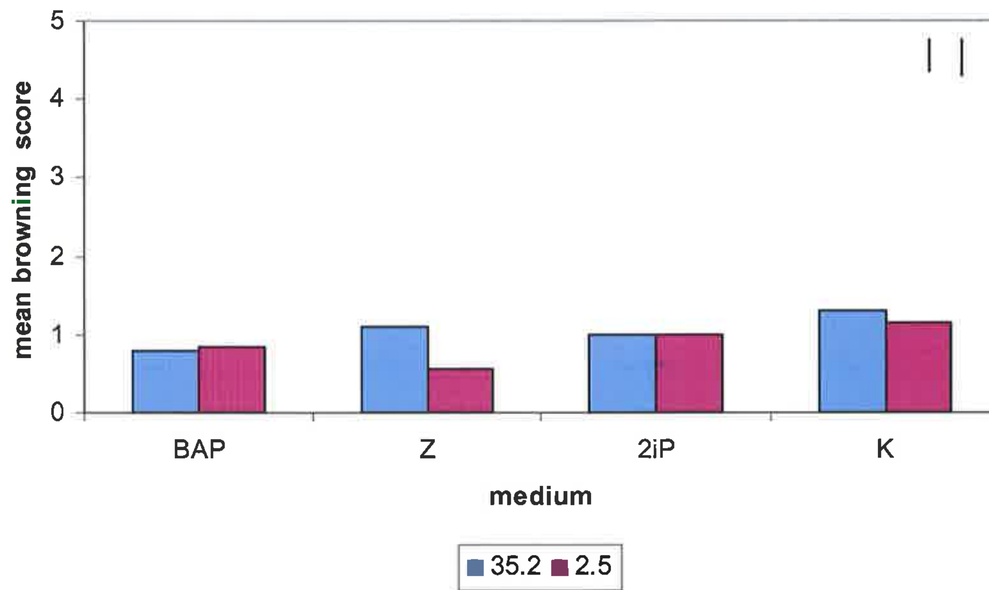


Figure 8.6 Medium browning score of *E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5 on WPM with four different cytokinins at 4 wk. LSD bars at the P=0.05 level (medium exudates, 35.2, 0.41; 2.5, 0.44)

BAP	6-benzylamino purine
Z	6[4-hydroxy-3-methylbut-2-enylamino] purine
2iP	dimethylallyl amino purine
K	6- furfuryl amino purine

Plate 8.2 *In vitro* shoots of *E. erythronema* x *E. stricklandii* hybrid 2.5 on WPM, containing 2 μM zeatin, 20 g^{-1} sucrose, gelled with 7 g^{-1} Phytagel®. Bar = 1 cm

8.4.2 Effect of kinetin on root initiation and growth of *E. erythronema* x *E. stricklandii* hybrid 35.2 and 2.5 shoots

8.4.2.1 Materials and methods

In vitro shoots of *E. erythronema* x *E. stricklandii* hybrid 35.2 and 2.5 were maintained as per Section 8.2 with subculture on QL medium containing 2 μM kinetin, 20 g^{-1} sucrose, 4 g^{-1} activated charcoal (Sigma), and 7 g/L Phytagel® for 3½ wk prior to root initiation. Shoots were placed on rooting medium of ½ WPM, 20 μM IBA, 10 g^{-1} sucrose, gelled with 4 g^{-1} Phytagel® for 1 wk in the dark, followed by subculture to ½ WPM, 10 g^{-1} sucrose, gelled with 4 g^{-1} Phytagel® and returned to 16 h light and 8 h dark, with a cool white light reading of 75 μmoles m^{-2} sec^{-1}. Data were collected at 2 and 4 wk following IBA pulse and included callus growth, root number and root length.

8.4.2.2 Results

8.4.2.2.1 Callus growth, root number and length

Callus growth was greater on hybrid 2.5 compared to 35.2 prior to subculture on peat and perlite. Much of the nodular callus gave the appearance of root primordia. There was no further callus growth on the peat and perlite mixture at 4 wk following subculture.

Shoots of hybrid 2.5 showed 5% rooting at 2 wk, compared to shoots of hybrid 35.2 where 84% of shoots showed root development. There was no further root development or root elongation at 4 wk. Shoots placed onto peat and perlite after IBA pulse did not develop roots.

Roots were short, thick and 1-4 mm in length at 2 wk (Plate 8.2). There was no increase in root length at 4 wk on plants transferred to peat and perlite mixture or those remaining on ½ WPM in culture pots.

8.4.2.3 Conclusions

Kinetin in the multiplication medium prior to rooting did not improve root development on *E. erythronema* x *E. stricklandii* 35.2 and 2.5. Roots remained short and thick without elongation. Root morphology was not improved by transferring plants to a peat and perlite mixture. The use of peat and perlite for root development following IBA pulse was not successful.

8.4.3. Effect of zeatin and activated charcoal on root initiation and growth of *E. erythronema* x *E. stricklandii* hybrid 35.2 and 2.5 shoots

8.4.3.1 Materials and methods

In vitro shoots of *E. erythronema* x *E. stricklandii* hybrid 35.2 and 2.5 were maintained as per Section 8.2 with subculture prior to rooting on QL medium containing 2 μM zeatin, 20 g^{-1} sucrose, and gelled with 7 g^{-1} Phytagel®. Shoots remained on this medium for 5 wk. Shoots were placed on rooting medium of ½ WPM, 20 μM IBA, 10 g^{-1} sucrose, gelled with 4 g^{-1} Phytagel® for 1 wk in the dark, followed by subculture to ½ WPM, 10 g^{-1} sucrose, 4 g^{-1} activated charcoal gelled with 4 g^{-1} Phytagel® and returned to 16 h light and 8 h dark, with a cool white light reading of 75 µmoles m^{-2} sec^{-1}. There were 20 shoots per genotype. Data were collected at 4 wk and included root number and root length and the percentages calculated.

8.4.3.2 Results

8.4.3.2.1 Root number and length

Elongated roots were observed in the bottom of the vessel three weeks after the IBA pulse with a mean length of 4.75 cm. On removal of shoots from the medium the

majority of shoots grew short, thick roots while others produced long, thin roots with root hairs (Table 8.2) (Plate 8.2).

8.4.3.3 Conclusions

Zeatin in the multiplication medium prior to rooting and activated charcoal after the IBA pulse was successful in producing elongated roots on *E. erythronema* x *E. stricklandii* hybrid 35.2 and 2.5 *in vitro* shoots suitable for the hardening off process.

Table 8.3 The percentage of *E. erythronema* x *E. stricklandii* hybrid 35.2 and 2.5 *in vitro* shoots that developed short and/or elongated roots when zeatin was used in the multiplication medium prior to rooting and activated charcoal in the medium after the IBA pulse

	Percentage on	
Root type	hybrid 35.2	hybrid 2.5
None	19	20
Short and thick	71.5	60
Long, thin, root hairs	9.5	20

Plate 8.3 Root development on *in vitro* shoots;

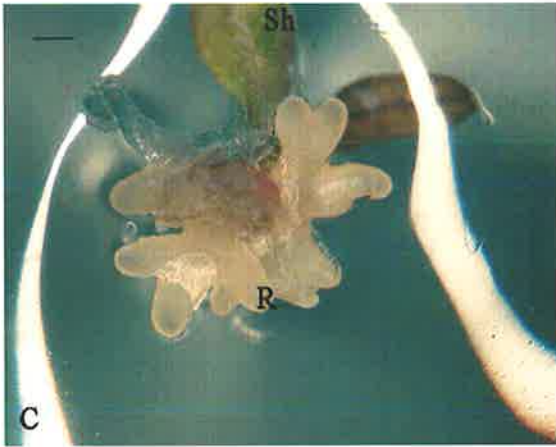
A) Roots (R) on multiplied *in vitro* shoot of *E.* 'Urrbrae Gem' seedling line 1 on ½WPM containing 10 μM IBA, 10 g^{-1} sucrose at 8 wk. Bar = 1 cm

B) Shoots of *E. erythronema* x *E. stricklandii* hybrid 2.5 on QL medium containing 2 μM kinetin, 20 g/L sucrose, 4 g^{-1} activated charcoal, gelled with 4 g^{-1} Phytagel® ready for harvest and sub-culture to rooting medium. Bar = 1 cm

C) Shoots of *E. erythronema* x *E. stricklandii* hybrid 2.5 multiplied with either kinetin or BAP and placed on ½ WPM containing 20 μM IBA, 10 g^{-1} sucrose gelled with 4 g^{-1} Phytagel® grew short, thick roots (R) that failed to elongate. Bar = 2 cm

D) Shoots of *E. erythronema* x *E. stricklandii* hybrid 35.2 multiplied with zeatin were successfully rooted (R) on ½WPM containing 20 μM IBA, 10 g/L sucrose followed by ½WPM containing 10 g sucrose, $4g^{-1}$ activated charcoal gelled with 4 g^{-1} Phytagel®. Bar = 1 cm

R
A

B

Sh
R
C

R
D

8.6 Discussion

Successful micropropagation was achieved for the ornamental eucalypts *E.* 'Urrbrae Gem' and hybrids 35.2 and 2.5 following manipulation of media components, plant growth regulator type and concentration and culture environment.

On introducing a new species into culture, different approaches to finding a suitable growth medium include the comparison of different media (Ostrowska *et al.,* 1998), a broad spectrum approach to identify required mineral nutrient concentrations (de Fossard, 1976, 1983), tissue analysis (Gribble *et al.*, 2002), and investigating media found suitable for similar genotypes (McComb *et al.,* 1996). In this study WPM and QL were shown to provide healthier shoot growth than MS, AP, B5, and TK media. Compositions vary regarding the nitrate, sulphur, sodium and growth factor content with iodine found in MS, B5, AP, and TK in similar amounts, but in QL the iodine concentration is 10 times less, with no iodine in WPM. The media (MS, B5, AP, TK), with similar levels of iodine produced poorer shoot growth compared to WPM and QL, and while iodine is not considered essential for plant growth it can improve root growth and callus cultures (Sutter, 1996). The role of iodine in eucalypt shoot growth is uncertain, as iodine may not be the limiting or toxic factor. It is difficult to establish by visual observation why a particular medium produces better shoot growth compared to another due to the complex interactions of mineral nutrients, growth factors, PGRs, gelling agents, and the plant's nutrient requirements and uptake mechanisms (Williams, 1993), together with the environmental factors of light, temperature and culture vessel type (Williams, 1999).

A number of strategies can be applied for successful root initiation and growth of *in vitro* shoots. Strategies include lowering and/or modifying medium nutrients, placing shoots in the dark and type, concentration and exposure time of plant growth

regulators in both the multiplication and root initiation medium. Improved rooting can be achieved with many eucalypt species by lowering the nutrient salt concentration of the medium (Watt *et al.*, 2000; Sharma and Ramamurthy, 2000), which also proved successful in this study. IBA has been shown to be an effective auxin for root development in eucalypts. However, improved root development was found for *E. graniticola* with a combination of IBA and NAA (Bunn and Dixon, 1997), for *E. torelliana* with IBA, IAA and NAA (Gupta *et al.,* 1983) and for *E. camaldulensis* where NAA alone was sufficient (Gupta *et al.,* 1983).

Initial experiments on root initiation with IBA produced short, thick roots that failed to elongate in culture or following transfer to pots in the glasshouse. The development of a fibrous root system consisting of short, thick roots was reported in *E. ficifolia* by de Fossard (1978). Gorst *et al.* (1983) showed that root morphology was linked to concentration of available exogenous IBA over a 4 wk period. Successful root development on *in vitro* eucalypt shoots can be achieved by treating shoots with low levels of IBA until roots of normal morphology grew, usually 2-3 wk, then harvesting plants for hardening off (Sharma and Ramamurthy, 2000; Le Roux and van Staden, 1991b). Alternatively, pulsing shoots on high levels of IBA for up to 2 wk (Blomstedt *et al.,* 1991; Cid *et al.,* 1999), with subculturing to an auxin free medium for root development. Pulsing also proved more successful for root initiation of *E.* 'Urrbrae Gem' shoots and less basal callus was produced, with root emergence direct from the stem, allowing easier removal of plants from the culture pot. Pulsing shoots on IBA proved unsuccessful for hybrids 35.2 and 2.5 as roots remained short and thick.

BAP and GA_3 are commonly used for shoot multiplication and elongation but both are associated with root inhibition (Razdan, 1993). Shoot elongation can be achieved without GA_3 by reducing mineral salts (Niccol, 1994; Cortezzi Graca and

Mendes, 1989), reducing the cytokinin concentration (Jasra, 1999; Niccol, 1994), adding activated charcoal to the multiplication medium (Curir *et al.,* 1990; McComb and Bennett, 1986; Franclet and Boulay, 1982) and the use of an enriched CO_2 culture environment (Sha Valli Khan *et al.,* 2002). Ostrowska *et al.* (1998) compared BAP, zeatin and 2iP and found that elongated shoots developed on zeatin for *E. ficifolia* and *E. citriodora*. A comparison of BAP, zeatin, 2iP and kinetin on *E. erythronema* x *E. stricklandii* 35.2 and 2.5 showed that BAP produced the greatest axillary shoot numbers but with zeatin the shoot internodes were longer, however, shoots would still require elongation prior to rooting.

Bennett *et al.* (1994, 2003) improved root growth on *E. globulus* by alternating BAP and kinetin in the multiplication medium and used kinetin prior to the rooting medium. Newell (2002) used subculture of shoots to a sterile peat:sand:perlite mixture following the auxin pulse, to significantly increase root number and length for a range of Australian native species including *Chamelaucium, Verticordia, Conospermum, Pimelea* and *Eucalyptus*. However, the above two processes were unsuccessful for root development on hybrids 35.2 and 2.5 as was the addition of activated charcoal to the multiplication medium prior to the auxin phase.

The addition or removal of specific substrates in the medium prior to the rooting phase or after the auxin pulse can improve rooting. For example Bennett *et al.* (2003) removed ammonium nitrate from the multiplication medium while Trindade and Pais (1997) added riboflavin and choline chloride prior to the rooting phase to improve root development for *E. globulus*. Blomstedt *et al.* (1991) found a significant improvement in root development of *E. regnans* when shoots were subcultured to medium with activated charcoal after the auxin pulse. This may act by maintaining the shoot base in the dark to encourage root growth and elongation while the shoot top is given light

(Sharma and Ramamurthy, 2000); or by the ability of activated charcoal to adsorb toxins such as quinones, which can be detrimental to plant growth. Successful rooting was achieved with hybrids 35.2 and 2.5 by using zeatin in the multiplication medium prior to rooting and activated charcoal after the IBA pulse.

The age of the mother plant can also influence root development of *in vitro* shoots. For shoots from mature explants of *E. marginata* (McComb *et al.,* 1999), *E. rudi,* and *E. citriodora,* (Gupta *et al.,* 1981), root development improved after a certain number of subcultures. However Gomes and Canhoto (2003) found reduced root growth in juvenile *E. nitens* shoots after 3 mth in culture and no roots with explants taken from 1 yr old mother plants. In this study plant growth regulators influenced root initiation, while time in culture did not appear to affect root initiation of either, seedling shoots of *E.* 'Urrbrae Gem', or shoots from the 5 yr old hybrids 35.2 and 2.5.

Following root growth, the transfer of *E.* 'Urrbrae Gem' seedling line 1 and 3 micropropagated plants from the *in vitro* environment to the glasshouse was successful. Hardened off plants grew well and were later potted into 15cm pots and transferred to a bush house with 50% shade cloth. The reported procedures for hardening off of eucalypt plants varies for pot type, soil mixture and fertiliser regime, but one important factor was consistent. Plants require high humidity, as found in the culture pots, which is slowly reduced as new leaves grow. This is important as *in vitro* leaves have thin wax deposits and cuticle layers, with many stomata unable to close, reducing the leaves' ability to prevent water loss (Louro *et al.,* 1999). The study by Warrag *et al.* (1989ab) of *in vitro* shoots of *E. grandis* showed reduced photosynthesis, chlorophyll content, nitrogen concentration and dry matter accumulation compared to potted seedlings. However, these differences quickly decreased as *in vitro* plants grew new leaves and roots with no significant differences in growth habit after 3 months (Warrag *et al.,*

1989ab), demonstrating that micropropagation is a successful method of clonal propagation for eucalypts.

The mineral nutrients, growth factors, and PGR must provide a balance between nutrient uptake, axillary shoot growth and shoot elongation with any carry over effects to root development and hardening off to the external environment taken into consideration and adjusted for. A successful clonal propagation protocol using tissue culture techniques was established for *E.* 'Urrbrae Gem' seeedlings line 1 and 3. However, further work was required for *E. erythronema* x *E. stricklandii* 35.2 and 2.5 which were successfully initiated into culture and multiplied, but root growth of normal morphology for successful hardening off required the use of zeatin in the multiplication medium rather than BAP. Future work is required on *E. erythronema* x *E. stricklandii* hybrids growing in the Laidlaw Plantation to establish them into culture for clonal propagation using the methods established in this chapter.

Seedling material of hybrid *E.* 'Urrbrae Gem' has been successfully used to establish a clonal propagation method to use as a basis for micropropagation of other ornamental eucalypts.

Chapter 9

Micropropagation of *E.* 'Urrbrae Gem' and *E. erythronema* var. *erythronema* x *E. stricklandii* hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, and 21U

9.1 Introduction

Selection criteria for suitable species or hybrids with commercial potential include not only physical traits such as bud, flower, fruit and tree characteristics, but also the ability for clonal propagation (Delaporte and Sedgley, 2004). Clonal propagation through organogenesis, somatic embryogenesis and axillary shoot multiplication, have been investigated in previous chapters. Using juvenile explants organogenesis from leaf explants and axillary shoot multiplication from nodes was successful in the parental species *E. erythronema, E. stricklandii* and seedlings of *E.* 'Urrbrae Gem' (Chapter 5, 6). The approach of somatic embryogenesis using juvenile tissue (Chapter 7) and callus growth from mature explants (Chapter 4) is not yet successful and was therefore, not tested in this chapter.

The aim of experiments outlined in this chapter was to apply results from previous organogenesis and axillary shoot proliferation experiments to *E.* 'Urrbrae Gem' and *E. erythronema* x *E. stricklandii* hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, and 21U (Section 2.4, 2.5) that show commercial potential and test for their propagation ability via organogenesis and micropropagation.

9.2 Initiation of leaf and nodal explants of 20E, 20P, 20R, 20T, 20V, 21A, 21G, 21U and *E.* 'Urrbrae Gem' into culture

9.2.1 Materials and Methods

Initiation of nodal and leaf explants of *E.* 'Urrbrae Gem' and *E. erythronema* x *E. stricklandii* hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, and 21U were as per Section 3.1.2 with the addition of dipping explants in 2 mL^{-1} Sporkill (Hygrotech) (personal communication F. Blakeway, 2003) after washing and leaving them to air dry before decontaminating with 1% sodium hypochlorite.

Data were collected at 10 d after decontamination and included contamination percentage, media browning and explant score (Table 8.1), which represents the level of explant deterioration. For medium browning it was assumed that an increase in the browning of the medium represented either an increase in polyphenols, or an increase in oxidation, or both.

9.2.2 Results

9.2.2.1 Explant contamination

Dissected leaf explants of all genotypes showed lower contamination percentages than their nodes. Nodal explants of 20P, 20R, 20V and EUG had lower contamination percentages than 20E, 20T, 21A, 21G and 21U (Table 9.1). Contamination was from fungal growth with no bacterial colonies observed.

9.2.2.2 Explant score

There was significantly more leaf blackening on 20E, 21A, 21G and EUG compared to 20P, 20R, 20T, 20V and 21U. Nodal explants showed more blackening than leaf explants except for 21A and 21G with significantly more blackening between

leaf and nodal explants of 20R, 20T, 20V, 21U and EUG. Nodal explants of EUG had significantly greater blackening compared to all other explants and genotypes (Figure 9.1).

9.2.2.3 Media browning

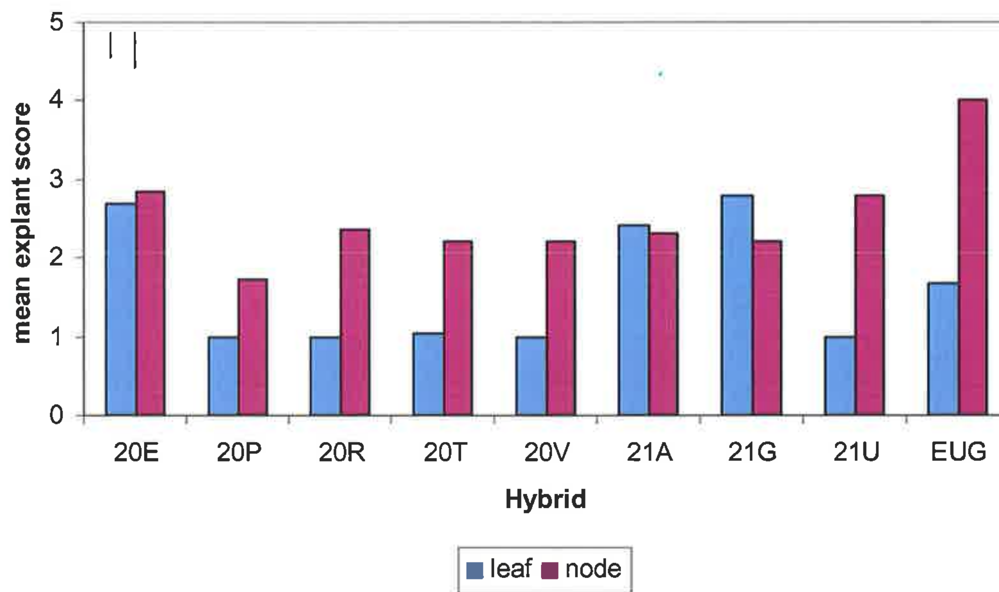
All explants from all genotypes displayed media browning around the explant base during the first week in culture. After regular subcultures over 8 d, leaf and nodal explants of 20E, 20P, and leaf explants of 20R, 21A and 21U displayed no further media browning while further media browning was observed from nodes and leaves of 20T, 20V, 21G and EUG, and nodes of 20R, 21A and 21U (Figure 9.1).

9.2.3 Conclusions

The amount of contamination, leaf and nodal blackening, and medium browning varied between genotypes and explants. Leaf explants showed less contamination with generally less blackening than nodal explants of the same genotype. EUG had high explant and media browning scores with no surviving nodal explants after ten days, while 20P showed low contamination percentages, minimal explant blackening with no further media browning after ten days.

Table 9.1 Percentage of contamination, 10 days after decontamination.

Hybrid **%contamination**	**20E**	**20P**	**20R**	**20T**	**20V**	**21A**	**21G**	**21U**	**EUG**
Leaf	3	4	0	14	3	26	20	2	4
Nodes	63	20	5	60	22	41	53	62	0



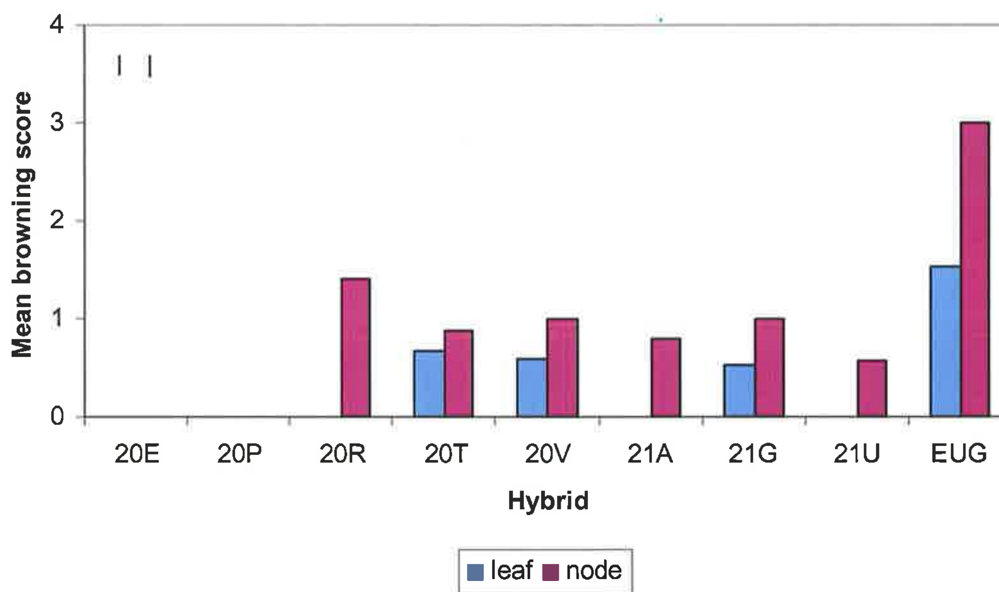


Figure 9.1 Mean explant score and medium browning of leaf and nodal explants from hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, 21U and EUG 10 days after initiation into culture. LSD bars at the P=0.05 level (explant score, leaf 0.3, node 0.6) (media browning, leaf 0.18, node 0.22)

9.3 Callus and shoot development in *E.* 'Urrbrae Gem' and *E. erythronema* x *E. stricklandii* hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, 21U

9.3.1 Materials and methods

Leaf and nodal explants initiated into culture as described in Section 9.2 were transferred to ½ strength MS (Murashige and Skoog, 1962) medium with full strength MS vitamins, 20 g^{-1} sucrose, with 5 μM BAP and 10 μM NAA for callus production on dissected leaf explants, while nodal explants were subcultured to QL with 2.2 μM BAP, 0.1 μM NAA, 20 g^{-1} sucrose, and gelled with 7 g/L Phytagel®, all media at pH 5.7. Data were collected at 4 and 8 wk following subculture to ½ strength MS or QL and included callus growth, bud and shoot development.

Sections 5 mm or smaller of callus with leaf explant from 20P on 5 μM BAP and 10 μM NAA were fixed, embedded and stained for observation under light microscopy using the method outlined in Section 5.2.

9.3.2 Results

9.3.2.1 Callus, bud and shoot development in leaf explants

Callus growth was observed on dissected leaf explants of all hybrids except 21U. Callus first developed from the mid-vein region and spread along the cut edges. Callus growth on hybrid 20T began to brown and die at 8 wk, so although callus was readily initiated it was difficult to sustain in culture. Poor callus growth, which was greyish and soft grew on hybrids 21A, 20E and 20R, but good callus growth which was yellow or white, hard and friable, grew on hybrids, 20T, 20V, 21G and EUG, with white nodular callus on 20P (Table 9.2, Plate 9.1). No callus was observed on 21U. Bud or shoot development was not observed on callus of any genotype.

9.3.2.2 Shoot development in nodal explants

Shoot development was observed from one node each of 21A and 21G, however, the nodes were browning and continued to die. Four nodes of 20P produced shoots at 8 wk, one was healthy (Plate 9.1), one died, the other two shoots were yellowing with the nodes continuing to brown. Nodes continued to deteriorate after the 8 wk assessment with occasional bacterial contamination evident.

9.3.2.3 Light microscopy

Microscopy of 20P leaf explants showed no meristem activity within the callus or explant (Plate 9.2).

9.3.3 Conclusions

A difference was observed between the hybrids in callus growth and appearance. Callus that grew well tended to be yellow, hard and friable or nodular while poor callus was grayish, white and soft. There was no organogenesis of buds or shoots from callus and little shoot development from nodal explants of the genotypes initiated.

Table 9.2 Percentage of leaf explants from hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, 21U and EUG that developed callus and callus appearance

Hybrid	% leaf explants with callus	description
20E	31	small, greyish, soft
20P	88	yellow, hard, nodular at first then friable
20R	71	small, greyish, soft
20T	94	yellow, hard, friable, overgrown explant
20V	82	yellow, hard, friable
21A	45	small, greyish soft
21G	73	yellow, hard, friable
21U	0	-
EUG	50	small, white, red, friable

Plate 9.1

A) Nodular callus (Ca) on leaf (L) explant of hybrid 20P on ½ MS medium with 5 μM BAP and 10 μM NAA at 4 wk. Bar = 1 mm

B) Axillary shoot (Sh) on nodal explant (N) of hybrid 20P on QL medium with 2.2 μM BAP, 1.0 μM NAA, 4 g/L activated charcoal at 8 wk. Bar = 1 cm

C) Axillary shoot (Sh) 2 wk following subculture from above medium (B) to QL medium with 2 μM zeatin, with callus (Ca) on the underside of the leaves. Bar = 2 mm

Ca
L
A

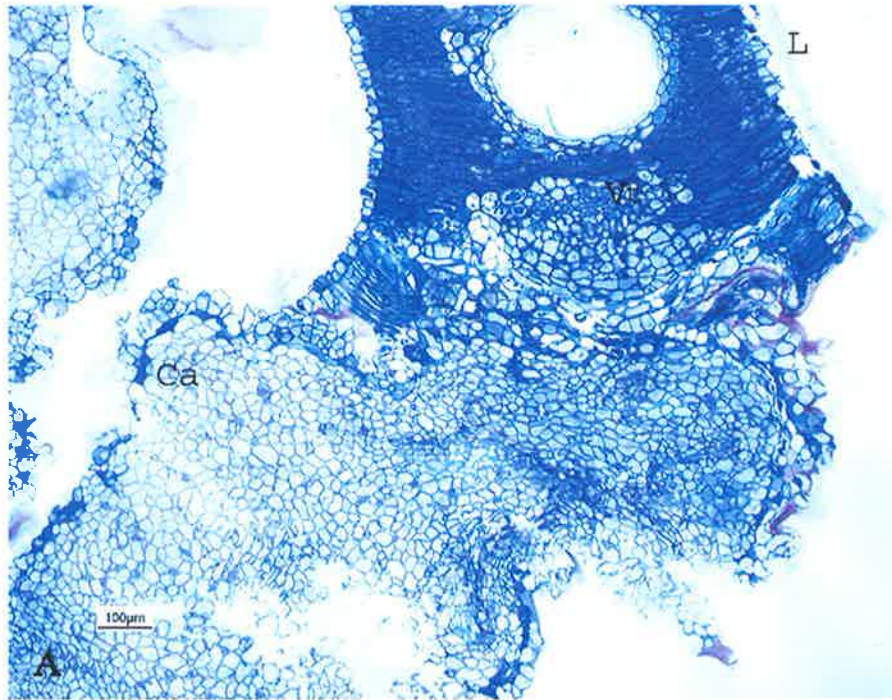
N
Sh
B

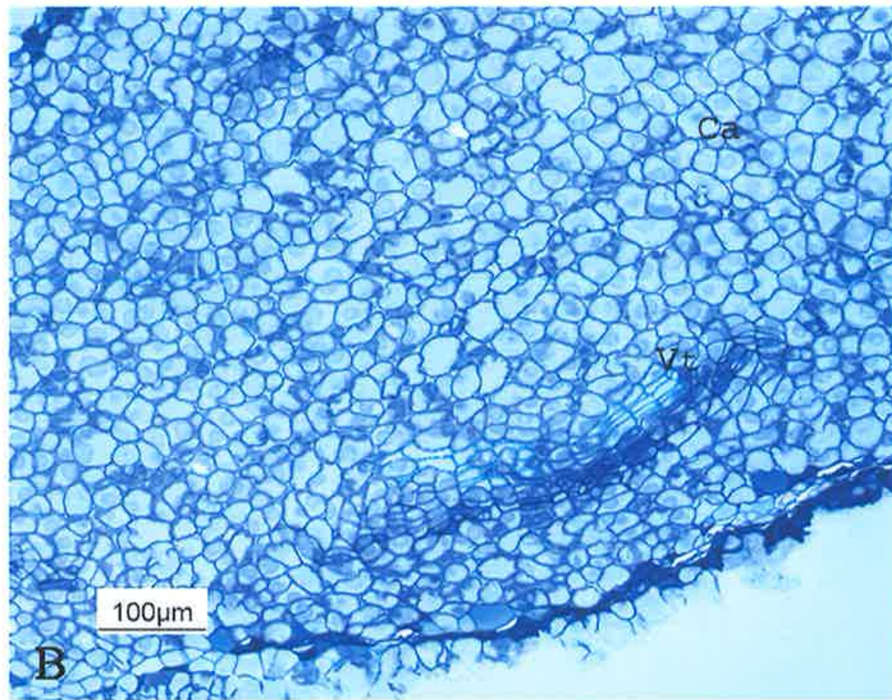
C
Sh
C

Plate 9.2 Light microscopy of nodular callus on leaf explants of hybrid 20P on ½ MS medium with 5 μM BAP and 10 μM NAA at 8 wk

A) Leaf explant (L) with vascular tissue (Vt) and callus (Ca) (x 5). Bar = 100 μm

B) Callus (Ca) with vascular tissue (Vt) but no meristematic cells (x 10). Bar = 100 μm


L
Vt
Ca
100μm
A


Ca
Vt
100μm
B

9.4 Effect of cytokinins auxin combinations on callus growth, bud and shoot development in leaf explants of *E. erythronema* x *E. stricklandii* 20E, 20P, 20R, 20T, 20V, 21A, 21G, and EUG

9.4.1 Materials and methods

Callus generated from leaf explants of hybrids 20P, 20T, 20V, and 21G as described in Section 9.3, were harvested and placed on ½ MS medium, 20 g^{-1} sucrose at pH 5.7 and the following plant growth regulators: 1 μM BAP, 2 μM zeatin, 2 μM 2iP, 5 μM BAP/5 μM NAA and gelled with 7 g^{-1} Phytagel®. Callus of 20E, 20R and 21A were only placed on 5 μM BAP/5 μM NAA due to the small amount of callus available. Callus was subcultured to the same media at 3, 6 and 10 wk. Data were collected at 3, 6, and 10 wk and included callus growth, bud and shoot development.

9.4.2 Results

9.4.2.1 Callus growth, bud and shoot development

Callus growth continued with all PGR treatments with callus browning occurring at 10 wk for all genotypes except for 20U on 2 μM zeatin and 20P, 20T and 21G on 1 μM BAP while 20P and 20V showed vigorous callus growth 5 μM BAP/5μM NAA.

Bud growth was observed at 10 wk on ½ MS medium with 5 μM BAP/5μM NAA for hybrids 20P, 21U and EUG and on ½ MS medium with 1 μM BAP for hybrid 20P (Figure 9.2, Plate 9.3). Buds were not observed from callus on other PGRs of the other genotypes.

No shoots were observed from any treatment for any genotype during the 10 wk, however buds of 20P on 5 μM BAP/5μM NAA later developed shoots.

9.4.3 Conclusions

The response of genotypes to BAP, BAP/NAA combinations, which were the most successful for organogenesis in juvenile leaf explants (Sections 5.3.2, 6.3.1), and zeatin and 2iP, varied as regards callus growth and appearance, and bud development. No shoots were observed on any callus from any genotype or PGR treatment.

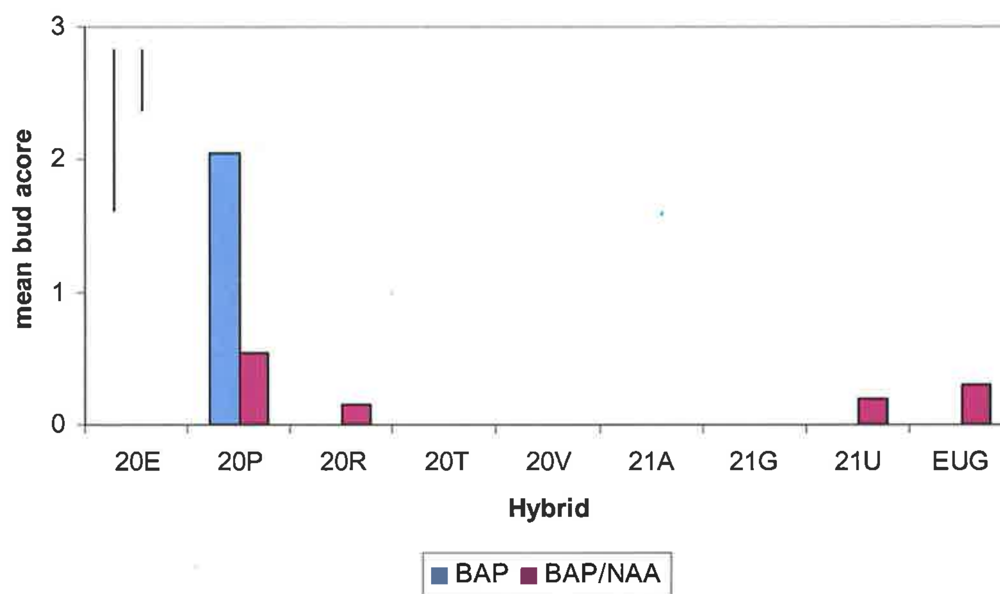
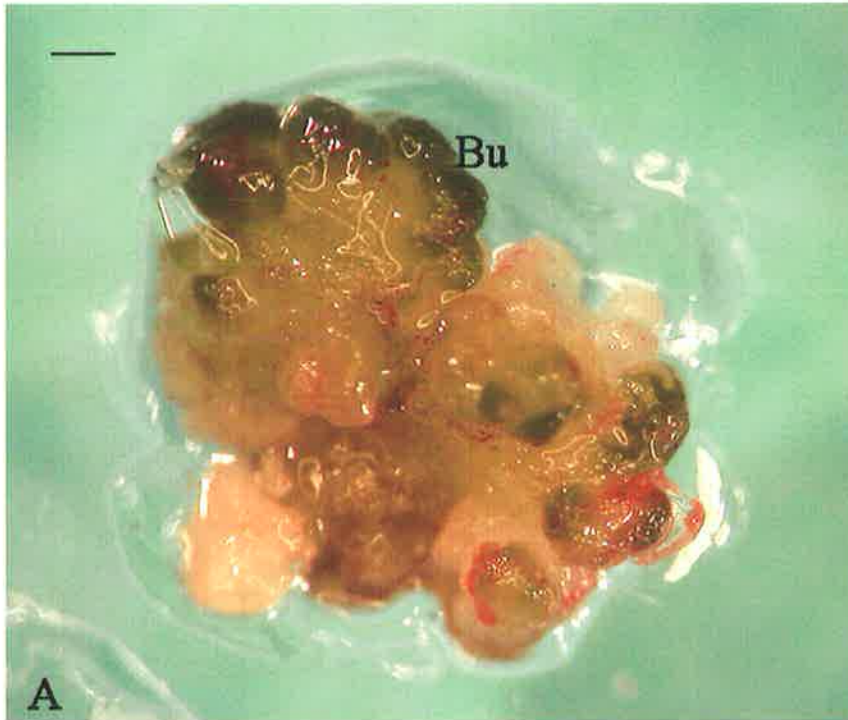


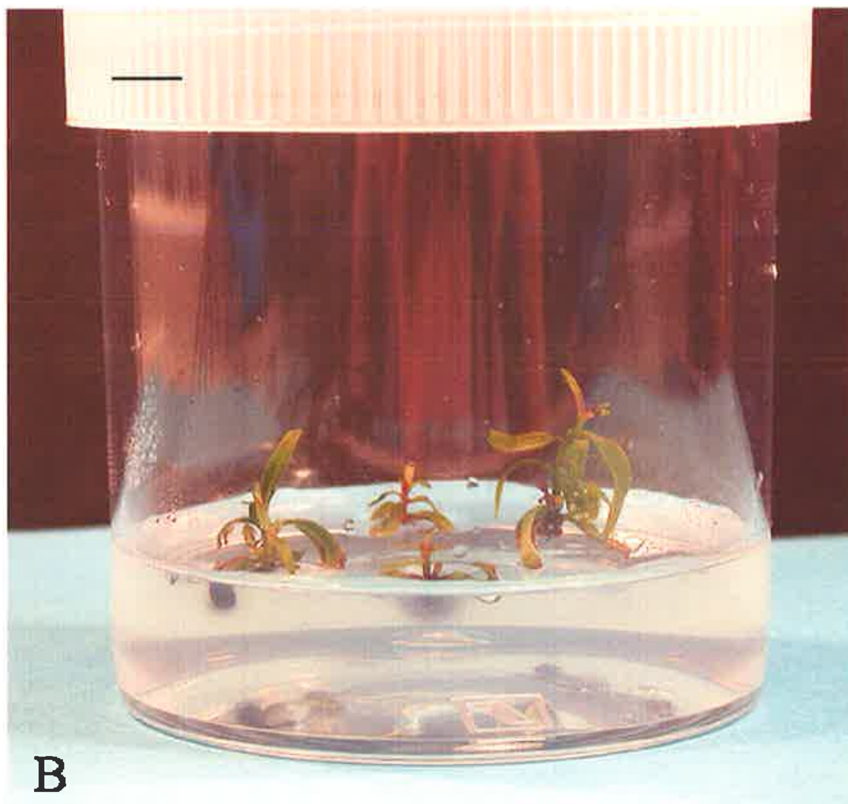
Figure 9.2 Buds on hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, 21U and EUG at 10 wk after treatment with 1 μM BAP and 5 μM BAP/5 μM NAA. LSD bars at the P=0.05 level (BAP, 1.2) (BAP/NAA, 0.5)

Plate 9.3

A) Buds (Bu) from leaf callus of hybrid 20P subcultured with ½ MS medium with 1 μM BAP after 10 wk. Bar = 1 mm

B) *In vitro* shoots from *E.* 'Urrbrae Gem' grafted plant growing under controlled conditions in the glasshouse, on WPM, 1.0 μM BAP, 0.05 μM NAA and 0.5 μM GA_3, after 3 mth in culture. Shoots did not sustain in culture and died after 14 mths. Bar = 1 cm


Bu
A


B

9.5 Initiation into culture of *E.* 'Urrbrae Gem' grafted plant growing under controlled conditions in the glasshouse

9.5.1 Materials and methods

Stems of *E.* 'Urrbrae Gem' growing on *E.* 'Urrbrae Gem' seedling rootstock were harvested and initiated into culture as described in Sections 3.1.2, and multiplied by harvesting axillary shoots at 3-4 wk intervals with subculture on WPM (Lloyd and McCown, 1980), 20 g^{-1} sucrose, 1 μM BAP, 0.05 μM NAA, 0.5 μM GA_3, pH 5.7, gelled with 7 g^{-1} Phytagel®, with every fourth subculture containing 1.5 g^{-1} activated charcoal. After 6 months medium was changed to QL, in an attempt to revive cultures.

9.5.2 Results

Initial axillary shoot growth was good with shoots harvested and multiplied (Plate 9.3). After several subcultures shoots deteriorated with reduced axillary shoot growth. At 12 months axillary shoots growth diminished and shoots could not be multiplied. Shoot tips blackened and wilted, and stems within the media also blackened.

9.5.3 Conclusions

Initiation into culture of explants from grafted plants in glasshouse conditions was successful. However, repeated subculture of axillary shoots did not induce reinvigouration of mature tissue and micropropagation was not successful.

9.6 Discussion

For a hybrid plant to become commercially successful one of the selection criteria is clonal propagation ability. Two major obstacles to overcome with clonal propagation of ornamental eucalypts include decontamination of field material and poor axillary shoot growth in culture. Field material was more difficult to introduce into culture than potted

plants grown under glasshouse conditions, as is commonly found in other eucalypts (Le Roux and van Staden, 1991; McComb and Bennett, 1986).

The ability of a woody plant to be clonally propagated diminishes with increasing plant maturity and this is often observed when both juvenile and mature explants from the same species are used, as observed in *Fagus* spp (Vicitez *et al.,* 2003) and the ornamental *E. sideroxylon* (Burger, 1987). However, selection criteria for ornamental hybrids cannot be applied until after first flowering. Subsequently explants are more difficult to establish into culture. Manipulation of environmental factors, plant growth regulators, plant rejuvenation or reinvigouration can successfully overcome these obstacles (von Aderkas and Bonga, 2000). Rejuvenation can be induced by coppicing, harvesting explants from rooted cuttings, repeated grafting of mature scions onto juvenile rootstock and repeated subculturing in tissue culture (Le Roux and van Staden, 1991). The comparison of field and potted plants in this study has shown that grafting and management under controlled conditions will improve initiation into culture and subsequent multiplication. Explants harvested from *E.* 'Urrbrae Gem' grafted plant were initiated into culture and initially grew well, compared to field harvested nodes of *E.* 'Urrbrae Gem' that rapidly deteriorated on initiation and failed to develop axillary shoots. The *E.* 'Urrbrae Gem' tree is 69 yr old and will require further work such as regular re-grafting to induce vigour and improve clonal propagation ability.

E. erythronema x *E. stricklandii* field hybrids 20E, 20P, 20R, 20T, 20V, 21A, 21G, 21U, EUG showed differences between genotype and explant with regards to successful initiation into culture, callus growth, organogenesis, and axillary shoot growth.

Organogenesis through callus from mature *Eucalyptus* explants has proven difficult, as many species produce callus but fail to develop buds or shoots (Watt *et al.,* 2003; Le Roux and van Staden, 1991). Once established, callus growth itself was

difficult to maintain, with frequent subculture of actively growing callus essential and removal of all browning callus. Browning of *Eucalyptus* callus has been reported in other taxa including *E. microcorys* (Niccol *et al.,* 1994), *E. grandis* x *E. urophylla* (Cid *et al.,* 1999) and *E. tereticornis* (Subbaiah and Minocha, 1990). Of the 9 field hybrids selected for clonal propagation *E. erythronema* x *E. stricklandii* 20P showed potential through axillary shoot proliferation and shoot organogenesis from leaf callus. For a clonal propagation method to be commercially viable a minimum 50% success rate is required (Delaporte and Sedgley, 2004). The establishment of grafted plants of hybrid 20P under glasshouse controlled conditions could greatly improve its clonal propagation ability via micropropagation.

In conclusion there was variation in response between genotypes to the sterilisation procedure, plant growth regulators and nutrient medium and a greater difficulty in establishing nodal explants from the field compared to leaf explant. The initiation of explants into culture was more successful from glasshouse grown plants compared to field plants and more successful from field leaf explants than field nodal explants. Further work should address reducing explant losses on decontamination and initiation, and improving axillary shoot growth and shoot organogenesis, by aiming to develop stock plants managed under controlled glasshouse conditions and improving plant growth regulators requirements for individual hybrids at all stages of the micropropagation process.

Chapter 10
General Discussion

For commercial development of the ornamental trees *Eucalyptus erythronema, E. stricklandii* and their hybrids, clonal propagation is required to maintain genetic integrity. These species and their hybrids are new to tissue culture propagation techniques, therefore experiments were conducted to determine the requirements for explant selection, decontamination, initiation into culture, the nutrient media, and type and concentration of plant growth regulators suitable for axillary shoot proliferation, shoot and root organogenesis and somatic embryogenesis. The results obtained from this research have contributed to our knowledge of clonal propagation of the selected ornamental eucalypt species.

Experiments conducted on *E. erythronema*, *E. stricklandii* and their hybrids have shown that *in vitro* methods of micropropagation by axillary shoot proliferation, and bud, shoot and root organogenesis from leaf explants are the most successful. Levels of explant deterioration, callus growth, root, bud and shoot development differed between genotypes in their response to decontamination, nutrient medium, type and concentration of plant growth regulators.

The source and age of the explant was critical to the success of micropropagation and organogenesis. Initiation rates and regeneration were low from mature field grown explants, higher from glasshouse derived explants and greatest from seedlings sterilised and germinated in culture (Sections 8.3, 8.4, 9.2). Improvements to mature explant initiation and survival can be achieved with leaching, antioxidants, cold treatment, frequent subculture and reduced nutrient salts (Section 3.1) (Le Roux and van Staden, 1991a). Despite these treatments, leaf and nodal explants from the majority of

selected hybrids deteriorated, with only one hybrid (20P) producing low rates of shoot organogenesis from callus and axillary shoot proliferation from nodal explants (Section 9.2).

Improvements in mother plant vigour can be achieved by reinvigoration prior to explant harvest through grafting, coppicing, and intensive plant management such as watering, fertilising, pest and disease control, which is best achieved with potted plants grown under glasshouse conditions (Le Roux and van Staden, 1991a). In this study a comparison of field grown plants, and potted plants growing under glasshouse conditions (*E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5, and the grafted plant of *E.* 'Urrbrae Gem'), showed that explants from the glasshouse survived decontamination and initiation into culture, and readily produced axillary shoots (Sections 8.4, 9.5). However, while *E erythronema* x *E. stricklandii* hybrid 35.2 and 2.5 shoots proliferated in culture over a three year period, *E.* 'Urrbrae Gem' shoots lost vigour after several months and could not be sustained (Section 9.5). Hybrids 35.2 and 2.5 were 2 yr potted plants in the glasshouse, while the *E.* 'Urrbrae Gem' is a 67 yr old, field grown tree, either reinvigoration by grafting was not successful, or explant age differences, as well as genotype, influenced regeneration.

Seeds were successfully decontaminated and germinated in culture with very low levels of contamination and good germination percentages. The seedlings grew well with juvenile tissue harvested and used for shoot and root organogenesis, or for shoot multiplication by axillary shoot proliferation.

The above findings are consistent with reports on tissue culture across a wide range of genera in that; juvenile tissue is more responsive in culture than mature (Dolcet-Sanjuan *et al.,* 2004; Watt, *et al.,* 2003), field explants are difficult to decontaminate for successful initiation into culture (McComb and Bennett, 1986), there

is a genotypic effect as regards response to the *in vitro* technique, with genetic differences requiring modifications at each stage to obtain successful axillary shoot proliferation (Bonga and Pond, 1991). Greater levels of decontamination, improved organogenesis and axillary shoot proliferation can be achieved with intensive management of the mother plants (Dolcet-Sanjuan *et al.,* 2004).

MS medium supplemented with BAP and NAA is the most commonly used combination for axillary shoot proliferation of eucalypt species (Johnson, 1997). In this study shoot multiplication of *E. erythronema, E. stricklandii* and *E.* 'Urrbrae Gem' was achieved with MS, but shoot numbers and appearance were improved with either WPM or QL media (Section 8.3). This finding was then adopted for hybrids 35.2, 2.5 and 20P with successful axillary shoot proliferation, and was followed by improved rooting of *in vitro* shoots of hybrids 35.2 and 2.5 (Section 8.4).

In certain species, the use of BAP and GA_3 for shoot multiplication can later inhibit the shoots' rooting ability (Razdan, 1993) by either unknown PGR interactions or loss of shoot health (Bennett *et al.,* 1994, 2003; Graca and Mendes, 1989). Roots of normal morphology were produced on *E.* 'Urrbrae Gem' seedlings lines 1 and 3 although the number of rooted shoots was low (Section 8.3.2). However, the same multiplication method led to the development of short thick roots on hybrids 35.2 and 2.5 which were not suitable for hardening off. By using zeatin in the multiplication medium, and activated charcoal in the post-rooting medium, this problem was overcome, and elongated roots developed. BAP and/or GA_3 appear to reduce the rooting ability of these genotypes. These results should be considered for future rooting of other *E. erythronema* x *E. stricklandii* hybrids such as 20P.

Regeneration by organogenesis and somatic embryogenesis can be achieved from meristematic centres induced within callus tissue. However, the type of callus

produced and the explant source and age, contribute to the regeneration potential. In this study hard, friable callus, suitable for regeneration (Doran, 1996) was produced but organogenesis was low and no somatic embryos were observed (Sections 5.3, 7.3.1). Light microscopy observation showed the hard friable callus contained actively dividing cells, vascular tissue and meristematic cells, although hard friable callus from mature leaf explants of *E.* 'Urrbrae Gem' was devoid of meristematic cells (Section 7.3.2). Therefore, a more efficient combination of PGR, media and environmental factors is required to improve organogenesis and promote somatic embryogenesis.

Callus from eucalypt explants can become brown and inactive over time as found with *E. microcorys* (Niccol *et al.,* 1994), *E. grandis* x *E. urophylla* (Cid *et al.,* 1999) and *E. tereticornis* (Subbaiah and Minocha, 1990). Callus browning was also observed with *E. erythronema, E. stricklandii* and their hybrids, but in this study it was largely overcome through frequent subculturing of actively growing hard friable callus and the maintenance of high NAA to low BAP levels within the medium (Sections 5.3, 7.3.1, 6.3.1). Although callus could be maintained, callus age and browning could be contributing factors to the lack of regeneration.

Root, bud and shoot organogenesis through callus from mature explants has proved difficult with many eucalypts (Watt *et al.,* 2003), but has been achieved with juvenile tissue using a combination of auxins and cytokinins, most commonly BAP and NAA. IBA is most commonly used for rooting of eucalypt *in vitro* shoots but NAA alone was sufficient for *E. camaldulensis* (Gupta *et al.,* 1983), while combinations of NAA/IBA were successful for *E. graniticola* (Bunn and Dixon, 1997), and IBA, IAA and NAA for *E. torelliana* (Gupta *et al.,* 1983). In this study root organogenesis was achieved with NAA on all genotypes and tissue types using 5 and 10 µM NAA (Section 5.3). The ability of juvenile explants to regenerate roots with NAA differed from that of

root initiation and growth on *in vitro* shoots. The rooting of *E.* 'Urrbrae Gem' and *E. erythronema* x *E. stricklandii* hybrids 35.2 and 2.5 *in vitro* shoots was more successful with IBA (Sections 8.3.2, 8.4.3). The carry over effect of cytokinins in the multiplication medium influenced root development of *in vitro* hybrid 35.2 and 2.5 shoots, requiring a different approach than for *E.* 'Urrbrae Gem" (Sections 8.4).

High frequency bud development has the potential to increase micropropagation rates if buds can develop shoots. The success of shoot regeneration through bud induction ranges from low in *E. camaldulensis* (Arezki *et al.,* 2000) and *E. globulus* (Trindade and Pais, 2003; Nugent *et al.,* 2001), to high in *E. botryoides* (Ito *et al.*, 1996). In this study juvenile tissue of *E. erythronema* and *E.* 'Urrbrae Gem' had high numbers of structures that macroscopically appeared to be buds but a low level of shoot development (Sections 6.3.2). Further observation by light microscopy showed the buds contained areas of cells with densely packed cytoplasm and prominent nuclei, yet few had differentiated into shoot meristems (Sections 6.3.2). So while bud numbers were high, the potential to differentiate into shoots appears low. Further work is required to improve shoot organogenesis from buds. The relationship of specific plant tissue with regeneration is not widely reported. Azmi *et al.* (1997) reported an association of bud development and shoot meristem regeneration from immature oil gland cells in hypocotyl explants of *E. globulus*, while this study showed bud and shoot development were associated with the vascular tissue. Of the 21 hybrids selected from the Laidlaw Plantation (University of Adelaide, Waite Campus), 20P, 20R and 21U showed the ability to regenerated buds from leaf callus, as did seedling leaf explants, in which leaf explants developed high bud numbers. However, only 20P continued to develop buds, while buds on the other hybrids browned. Therefore, other methods are required to improve shoot regeneration such as those employed by Ito *et al.* (1996), who introduced

a rotating culture system for a range of eucalypts, or by the introduction of other PGR, such as TDZ, as found with *E. globulus* (Nugent *et al.,* 2001).

Juvenile explants from different eucalypts vary in their ability to regenerate shoots. Cid *et al.* (1999) found cotyledons of *E. grandis* x *E. urophylla* gave higher shoot regeneration while McComb *et al.* (1996) suggests hypocotyls to be more successful. In this study, shoot organogenesis was greatest from apex explants and seedling leaf explants compared to hypocotyl and cotyledon explants (Sections 5.3, 6.3, 7.3). Apex explants had lower levels of bud development but higher rates of shoot regeneration. From this result, young leaves and shoot apex explants from the shoot tips of field grown hybrid trees may prove suitable explants for clonal propagation by tissue culture, rather than nodal explants. However, the problems of shoot tip deterioration following decontamination need to be addressed.

Few eucalypt species have been induced to form somatic embryos and of these only *E. grandis* (Pinto *et al.,* 2002; Watt *et al.,* 1991; Cranshaw and Smith, 1995) and *E. citriodora* (Muralidharan *et al*., 1989), and have produced plants. Somatic embryos in eucalypts have been produced on explants such as hypocotyls, cotyledons and juvenile leaves, using the auxins NAA and/or 2,4D alone or in combination with the cytokinins BAP or kinetin, with a high auxin to cytokinin ratio. However, the above combinations did not produce somatic embryos on *E. erythronema, E. stricklandii* and their hybrids with the exception of apex explants from seedlings of *E.* 'Urrbrae Gem', in which embryos did not develop beyond the globular stage (Sections 7.3.1). Failure of embryos to continue development, as for *E. nitens* (Ruaud, 1997), or to mature, as for *Quercus* spp. (Wilhelm, 2000), are major limiting factors in the use of somatic embryogenesis for clonal propagation.

Non-PGR treatments have been successful in the initiation of somatic embryos in other genera. There are reports of an association between heat shock protein synthesis and somatic embryogenesis in the absence of heat (Puigderrajols *et al.*, 2002; Dong *et al.*, 1996; Misra *et al.*, 1996; Apuya and Zimmerman, 1992), with heat treatments alone capable of producing somatic embryos (Kumar *et al.*, 2002; Cordewener *et al.*, 2000; Touraeu *et al.*, 1996a; Perrotta *et al.*, 1992). Smoked water is known to improve seed germination in Australian native plants (Allan *et al.*, 2004) and was successful in inducing somatic embryos in hypocotyls of *Pelargonium hortorum* (Senaratna *et al.*, 1999). However, both heat shock and smoked water were unsuccessful in producing somatic embryos from leaf callus of *E.* 'Urrbrae Gem' (Sections 4.3). It may be that the heat and chemicals within the smoke trigger somatic embryogenesis in existing meristems rather than inducing callus or other somatic cells along the pathway to embryogenesis.

This is the first report of successful clonal propagation by axillary shoot proliferation and shoot organogenesis from the ornamental species *E. erythronema, E. stricklandii* and their hybrids. The research detailed in this thesis will contribute to the clonal propagation component of the eucalypt breeding programme by providing a method for explant initiation into culture, multiplication by axillary shoot proliferation, shoot organogenesis, root development, and hardening off to the natural environment of selected ornamental hybrid eucalypts, and providing the basis for future work on somatic embryogenesis.

Future work can focus on several areas: The development of grafted or clonal stock mother plants for care under glasshouse controlled conditions would greatly increase the success rate of introducing a selected plant into culture: To improve axillary shoot proliferation, shoot organogenesis, the development of shoots from buds,

rooting percentage of *in vitro* shoots and somatic embryogenesis, through investigation on *in vitro* environmental conditions at all stages of tissue culture, further investigation on heat shock proteins and media additives. A further point illustrated from these results is the importance of genotype and genetic predisposition to response in culture. This suggests that whole general approaches can be developed for these species and their hybrids, while specific research will be required to refine protocols for each superior hybrid.

Chapter 11
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Appendix 1

Media Composition

1) MS, Murashige and Skoog (1962)

Compound	mg/L
Ammonium nitrate (NH_4NO_3)	1,650
Boric acid (H_3BO_3)	6.2
Calcium chloride 2- hydrate ($CaCl_2 \cdot 2H_2O$)	440
Cobalt chloride 6-hydrate ($CoCl_2 \cdot 6H_2O$)	0.025
Cupric sulphate 5-hydrate ($CuSO_4 \cdot 5H_2O$)	0.025
Magnesium sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$)	360
Manganese sulphate 1-hydrate ($MnSO_4 \cdot H_2O$)	16.9
Potassium iodide (KI)	0.8
Potassium nitrate (KNO_3)	1,900
Potassium phosphate monobasic, anhydrous (KH_2PO_4)	170
Sodium molybdate 2-hydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.25
Zinc sulphate 7-hydrate ($ZnSO_4 \cdot 7H_2O$)	8.6
Ferrous sulphate 7-hydrate ($FeSO_4 \cdot 7H_2O$)	27.8
Ethylenediamineteraacetic acid, disodium salt (Na_2EDTA)	37.3
Myo-inositol	100
Nicotinic acid	0.5
Pyrioxine HCL	0.5
Thiamine HCL	0.1
Sucrose	30,000
pH	5.7

2) B5, Gamborg *et al.* (1968)

Compound	mg/L
Ammonium sulphite (NH_4SO_3)	134
Boric acid (H_3BO_3)	3
Calcium chloride 2-hydrate ($CaCl \cdot 2H_2O$)	150
Cobalt chloride 6-hydrate ($CoCl_2 \cdot 6H_2O$)	0.025
Cupric sulphate 5-hydrate ($CuSO_4 \cdot 5H_2O$)	0.025
Magnesium sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$)	250
Manganese sulphate 1-hydrate ($MnSO_4 \cdot H_2O$)	10
Potassium iodide (KI)	0.75
Potassium nitrate (KNO_3)	2,500
Sodium di-hydrogen orthophosphate (NaH_2PO_4)	130
Sodium molybdate 2-hydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.25
Zinc sulphate 7-hydrate ($ZnSO_4 \cdot 7H_2O$)	2
Ferrous sulphate 7-hydrate ($FeSO_4 \cdot 7H_2O$)	27.8
Ethylenediamineteraacetic acid, disodium salt (Na_2EDTA)	37.3
Myo-inositol	100
Nicotinic acid	1
Pyrioxine HCL	1
Thiamine HCL	10
Sucrose	30,000
pH	5.7

3) WPM, Lloyd and McCown (1980)

Compound	mg/L
Ammonium nitrate (NH_4NO_3)	400
Boric acid (H_3BO_3)	6.2
Calcium chloride 2-hydrate ($CaCl_2 \cdot 2H_2O$)	96
Calcium nitrate 4- hydrate ($Ca[NO_3]_2 \cdot 4H_2O$)	556
Cupric sulphate 5-hydrate ($CuSO_4 \cdot 5H_2O$)	0.025
Magnesium sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$)	370
Manganese sulphate 1-hydrate ($MnSO_4 \cdot H_2O$)	22.3
Potassium phosphate monobasic, anhydrous (KH_2PO_4)	270
Potassium sulphate (K_2SO_4)	990
Sodium molybdate 2-hydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.25
Zinc sulphate 7-hydrate ($ZnSO_4 \cdot 7H_2O$)	8.6
Ferrous sulphate 7-hydrate ($FeSO_4 \cdot 7H_2O$)	27.8
Ethylenediamineteraacetic acid, disodium salt (Na_2EDTA)	37.3
Glycine	2
Myo-inositol	100
Nicotinic acid	0.5
Pyrioxine HCL	0.5
Thiamine HCL	0.1
Sucrose	30,000
pH	5.6 – 5.8

4) AP, Almehdi and Parfitt (1986)

Compound	mg/L
Ammonium sulphate ($[NH_4]_2SO_4$)	270
Boric acid (H_3BO_3)	4.5
Calcium chloride 2-hydrate ($CaCl_2 \cdot 2H_2O$)	150
Cobalt chloride 6-hydrate ($CoCl_2 \cdot 6H_2O$)	0.03
Cupric sulphate 5-hydrate ($CuSO_4 \cdot 5H_2O$)	0.025
Magnesium sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$)	190
Manganese sulphate 4-hydrate ($MnSO_4 \cdot 4H_2O$)	20
Potassium iodide (KI)	0.75
Potassium nitrate (KNO_3)	2,500
Sodium molybdate 2-hydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.06
Sodium phosphate 1-hydrate ($NaH_2PO_4H_2O$)	150
Zinc sulphate 7-hydrate ($ZnSO_4 \cdot 7H_2O$)	2
Ferrous sulphate 7-hydrate ($FeSO_4 \cdot 7H_2O$)	28
Ethylenediamineteraacetic acid, disodium salt (Na_2EDTA)	37.3
Myo-inositol	25
Pyrioxine HCL	2
Sucrose	20,000
pH	5.7

5) TK, Tabachnik and Kester (1977)

Compound	mg/L
Boric acid (H_3BO_3)	6.2
Calcium nitrate 4- hydrate ($Ca[NO_3]_2 \cdot 4H_2O$)	1,140
Cobalt chloride 6-hydrate ($CoCl_2 \cdot 6H_2O$)	0.025
Cupric sulphate 5-hydrate ($CuSO_4 \cdot 5H_2O$)	0.025
Magnesium sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$)	410
Manganese sulphate 1-hydrate ($MnSO_4 \cdot 1H_2O$)	16.9
Potassium iodide (KI)	0.83
Potassium nitrate (KNO_3)	200
Potassium phosphate monobasic, anhydrous (KH_2PO_4)	200
Sodium molybdate 2-hydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.25
Zinc sulphate 7-hydrate ($ZnSO_4 \cdot 7H_2O$)	8.6
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	27.8
Ethylenediamineteraacetic acid, disodium salt (Na_2EDTA)	37.3
Glycine	2
Myo-inositol	100
Nicotinic acid	0.5
Pyrioxine HCL	0.5
Thiamine HCL	0.1
Sucrose	20,000
pH	5.9

6) QL, Quoirin and Lepoivre (1977)

Compound	mg/L
Ammonium nitrate (NH_4NO_3)	400
Boric acid (H_3BO_3)	6.2
Calcium nitrate 4- hydrate ($Ca[NO_3]_2 \cdot 4H_2O$)	1,200
Cobalt chloride 6-hydrate ($CoCl_2 \cdot 6H_2O$)	0.025
Cupric sulphate 5-hydrate ($CuSO_4 \cdot 5H_2O$)	0.025
Magnesium sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$)	360
Manganese sulphate 4-hydrate ($MnSO_4 \cdot 4H_2O$)	1
Potassium iodide (KI)	0.08
Potassium nitrate (KNO_3)	1,800
Potassium phosphate monobasic, anhydrous (KH_2PO_4)	270
Sodium molybdate 2-hydrate ($Na_2MoO_4 \cdot 2H_2O$)	0.25
Zinc sulphate 7-hydrate ($ZnSO_4 \cdot 7H_2O$)	8.6
Ferrous sulphate ($FeSO_4 \cdot 7H_2O$)	27.8
Ethylenediamineteraacetic acid, disodium salt (Na_2EDTA)	37.3
Myo-inositol	100
Nicotinic acid	0.5
Pyrioxine HCL	0.5
Thiamine HCL	0.4
Sucrose	20,000
pH	5.7

7) Knop's (Conger, 1981)

Compound	mg/L
Calcium nitrate 4- hydrate ($Ca[NO_3]_2 \cdot 4H_2O$)	500
Magnesium sulphate 7-hydrate ($MgSO_4 \cdot 7H_2O$)	125
Potassium nitrate (KNO_3)	125
Potassium phosphate monobasic, anhydrous (KH_2PO_4)	125
Sucrose	20,000
pH	5.7

Appendix 2

Fixation, embedding and staining procedure for light microscopy

Sections 5 mm or smaller of callus with and without roots, shoots, or buds, from hypocotyl, cotyledon, leaf and callus explants were fixed, embedded and stained for observation under light microscopy using the following method (O'Brien and McCully, 1981).

Fixation and embedding

- Sections placed in 2.5 % glutaraldehyde for 48 hr at 4 °C
- Dehydrated in
  - 2 hydroxy methanol 1 h and repeat once
  - Ethanol 1 h and repeat once
  - Propanol 1 h and repeat once
  - Butanol 1 h and repeat once
  - 1:1 butanol: Glycol methacrylate (GMA) 1 hr
- Embedded in
  - GMA 2 days
  - Fresh GMA for a further 2 days
  - Fresh GMA, heat at 60°C for 2 days or until set
- Embedded samples were placed in a microtome and sectioned using a glass knife at 3 μM, placed on glass slides, adhered to the slides by heating to 60°C for 48 hrs

Staining

- 2,4-dinitropheylhydrazine solution for 30 min
- Rinse for 1 h in running water
- Place slides in 1% periodic acid for 30 min
- Rinse in running water for 5 min
- Place in Schiff's reagent (Merck Pty Ltd) for 1 h
- Place in solution of 5 mL 10% sodium metabisulphite, 5 mL 1 M hydrochloric acid and 90 mL Reverse Osmosis (RO) water for 2 min, repeat twice more
- Rinse in RO water

- Place in 0.05% toludine blue O in benzoate buffer pH 4.5 for 5 min
- Rinse in water until blue colour washes out of plastic
- Dry for 20-30 min in fume hood and mount in xylene (Histoclad)

Chemicals for light microscopy
Fixative

8 ml of 2.5 % glutaraldehyde in 0.025 M phosphate buffer, pH 6.8-7.2

Phosphate buffer 0.2 M

Stock solutions (1) $NaH_2PO_4.2H_20$ - 3.12g in 100ml

(2) Na_2HPO_4 - 2.839.g in 100ml

1.6 ml of stock solution (1) and 8.4 ml of (2) + 70 ml water = 8 ml 0.025 M buffer

Embedding

2- hydroxyethyl methacrylate (Glycol methacrylate or GMA)

93 ml GMA

7 ml polyethylene glycol 400 (Sigma)

0.6 g benzoyl peroxide

Mix for 2 hr at room temperature

Store at 0 – 4 ^{0}C for up to several months

Staining

2,4-dinitropheylhydrazine solution

0.5 g 2,4-dinitropheylhydrazine (DNPH), 100 ml 15 % acetic acid, mix 1 hr and filter

1% periodic acid

3 ml 50 % periodic acid in 147 ml water

Glocke, P., Collins, G. & Sedgley, M. (2015). In vitro organogenesis from seedling explants of the ornamentals Eucalyptus erythronema, E. stricklandii and the interspecific hybrid E. erythronema x E. stricklandii cv. 'Urrbrae Gem'. *Journal of Horticultural Science & Biotechnology, 80*(1), 97-104.

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