FLORAL BIOLOGY AND PROPAGATION OF BLUE-FLOWERED *CONOSPERMUM* SPP.

by

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DECLARATION

I declare that the work in this thesis is of my own research, except where reference is made, and has not previously been submitted for a degree at any institution.

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2003

ABSTRACT

Blue-flowered *Conospermum* are endemic to Western Australia, and show great potential as cut flowers. Propagation from cuttings or seed proved difficult, and root initiation *in vitro* is problematic. This thesis examines the floral biology of the species and the possibility of using somatic embryogenesis to overcome propagation problems.

A survey of explant tissue types for *C. eatoniae* and *C. caeruleum* was carried out to identify tissue that could be induced into embryogenic pathways. Vegetative, semi-floral and floral buds were initiated into culture from February to June, but were found unsuitable for embryogenesis, producing shoots, callus or dying in culture. Leaves from *in vitro* leaf cultures formed callus in the presence of 2,4-D and BAP, but were unable to differentiate into embryos in the presence of a variety of growth regulator combinations and concentrations. Immature zygotes died in culture. Direct embryogenesis and/or embryogenic callus was observed on mature zygotes of the species *C. caeruleum, C. spectabile, C. dorrienii* and *C. brownii*, and somatic embryos were maintained in culture for up to 18 months for *C. caeruleum*.

Maturation and germination of somatic embryos proved difficult; treatments of cold, ABA, desiccation or mannitol did not induce maturation. It appears that developmental pathways in *Conospermum* are well defined and are difficult to alter *in vitro*. It was concluded that somatic embryogenesis has limited commercial potential in these species.

Conospermum species have an active pollination mechanism where the style is held in a state of tension when the flower opens. When pressure is applied at the base of the style by an insect, the style flicks downwards, striking the insect pollinator and releasing pollen from the anther in a single dusty mass. However, the breeding systems of blue-flowered *Conospermum* have not previously been well explored.

Flowers on a *C. eatoniae* inflorescence opened from the basal end upwards acropetally, with the terminal two or three buds never opening. Fruit and seed set occurred only from the basal one to three buds. Isolation of *C. eatoniae* and *C. amoenum* flowers showed they were unable to self-pollinate in the absence of insect pollinators.

Experiments to determine the timing of the peak of stigmatic receptiveness were inconclusive. Pollen germinated and penetrated the stigma 0 – 6 days after anther dehiscence. Pollen loads on the stigma did not relate to the number of pollen tubes observed down the style. Controlled pollinations of cultivated *C. eatoniae* at a field station using self and cross pollen, revealed compatibility with a range of pollen genotypes, as pollen tubes were observed extending down the style. However, late-acting incompatibility could not be ruled out as controlled crosses failed to set any seed as flowers were shed from the bush.

DNA analysis of open pollinated *C. eatoniae* seed progeny from two plants from a field station and two plants in natural bushland revealed very different pollination habits. Plants from the field station showed no outcrossing, with progeny closely resembling the maternal parent, whereas plants from the wild population showed

outcrossing with several different paternal parents. These results suggest self-pollinated seed can be reliably obtained in a plantation situation using stands of ramets of the same clone. Alternatively, assuming that the required insect pollinators are present in a cultivated stand, it should be possible to obtain cross pollinated seed by surrounding the maternal plant with the desired paternal parent.

Unusual pollen behaviour was observed for many blue-flowered species, a white-flowered species of *Conospermum,* and close relative, *Synaphea petiolaris.* Up to three pollen tubes emerged from the triporate pollen *in vitro,* and at rates of up to 55 μms^{-1}. This rate was maintained for only 2 s but is greater than 20 times faster than reported in the literature for any species, *in vitro* or *in vivo.* Pollen with multiple tubes was also observed on the stigma *in vivo* in *C. amoenum* flowers.

Changing the osmotic pressure of the germination medium by altering sucrose concentration influenced the number of tubes to emerge from the pollen grain; generally the number of tubes decreased as sucrose increased. However, the rate of tube growth was unaffected. The addition of calcium channel blockers to the germination medium had no effect on *Conospermum* growth rate, nor did they eliminate pulses of tube growth.

Observation of *Conospermum* pollen ultrastructure revealed similarities to *Gramineae* pollen. The tube cytoplasm was packed with vesicles filled with material of similar electron density to the cell wall. Few golgi were identified, and the apical end of the tube contained these vesicles, smaller secretory vesicles and mitochondria. This is atypical of the tip, which is normally free of large vesicles. Distinct zones in the

cytoplasm were not identified, which is similar to *Gramineae.* Like the grasses, *Conospermum* appears to pre-manufacture cell wall material and store it in vesicles ready for rapid germination and extension.

A biological function of multiple pollen tube emergence with such rapid growth was not elucidated.

This research has shown *Conospermum* to be a complex and very interesting genus. Further investigation into the remarkable growth of multiple pollen tubes would enhance our knowledge of the biological processes involved in tube growth and the process of fast wall formation. The potential benefits to the cut flower industry of commercialising some of these species warrants further effort to find an efficient method of propagation. Introduction into horticulture may be the only means by which these threatened species will survive.

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TABLE OF CONTENTS

CHAPTER:

ABBREVIATIONS

WA: Western Australia

MS : Murashige and Skoog media (1962) including MS salts and vitamins

TDZ: thidiazuron

BAP: 6-benzylamino purine

2,4-D: 2,4-dichlorophenoxyacetic acid

IBA: indole-3-butyric acid

K: kinetin

GA_3: gibberellic acid

NAA: 1-napthaleneacetic acid

ABA: abscisic acid

FDA: fluoroscein diacetate

LIST OF PUBLICATIONS

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Stone, L., McComb, J.A., and Seaton, K.A. (2002) Propagation of blue-flowered *Conospermum* species. 10th International Association for Plant Tissue Culture and Biotechnology Congress: Plant Biotechnology 2002 and Beyond. Orlando, Florida USA, June 23-28, 2002.

Stone, L., McComb, J.A., and Seaton, K.A. (2002) Propagation of blue-flowered *Conospermum* species. International Association for Plant Tissue Culture and Biotechnology – Australian Branch: The Importance of Plant Tissue Culture and Biotechnology in Plant Sciences. Armadale, NSW, Australia, January 20-23, 2002.

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CHAPTER 1

INTRODUCTION

1.1 *CONOSPERMUM*

The genus *Conospermum* is a member of the Proteaceae family, sub-family Proteoideae (Perry and Trueman 1999). The Proteaceae are almost completely confined to the Southern hemisphere, with over 60 genera of which 37 are indigenous to Australia. The species in this family have diverse flowers, foliage, fruit, size and habit.

There are 53 species of *Conospermum* in Australia and 42 occur in Western Australia (WA). All have several genotypic variations (Bennett 1995). The genus name translates to mean "cone seed," referring to the top-shaped silky fruit (Sainsbury 1991). *Conospermum* is often referred to as smokebush, a name derived from the white flowering species, which, when seen en-masse, are said to look like smoke billowing in the wind. *Conospermum* species are perennial, sclerophyllous shrubs, ranging in size from 0.5 m to 2 - 3 m and grow in sandy heath woodland on slightly acidic soils (Seaton and Webb 1996). Leaves vary in shape from long and broad to small, crowded and heath-like. Like all Proteaceae, the perianth has four lobes, however in *Conospermum* the lobes are of unequal size, with the rear one being much larger than the other three (Bennett 1995).

1.1.1 Species of interest

The introduction of new Australian wildflowers to the cut flower industry is imperative to increase market strength and sustain economic growth (Seaton 2001). A

study of Australian native wildflowers by Seaton and Webb in 1995 identified *Conospermum* as having commercial potential as a cut flower, particularly the blue flowering species. Selections from wild populations were made, based on flower colour, flowering time and length, stem number and length, and vase life (Seaton 1996; Seaton and Webb 1996,1997) (Figure 1.1, 1.2).

Propagation methods were subsequently tested, revealing a need for methods other than traditional cuttings or shoot production *in vitro*, as *Conospermum* were unreceptive to these at levels acceptable for commercial production. For example, Seaton (2001) found seed germination of *C. eatoniae* and *C. caeruleum* to be less than 1 %, and root strike for *C. eatoniae* reached a maximum of 57 % of shoots on MS media supplemented with NAA and IBA. Survival after deflasking was a maximum of 65 % of those with roots (Seaton 2001). For *C. amoenum*, embryos rescued from seed germinated at 28 - 100 %. Production time using these methods was an average of two years; 15 months to establish a stable line in culture, three months to bulk up the material, three months to produce roots and another three months to harden off the plants ready for field planting (Seaton pers. comm.).

Although market demand is high, the number of *Conospermum* stems sold as cut flowers has declined over recent years due to unreliable supply and quality from wild populations. The blue flowered species are particularly popular in the Japanese ikebana and wedding trade and have a higher market value (Seaton 1996). Blue-flowered *Conospermum* makes up a very small percentage of harvested stems, mostly due to the conservation status of many of the species (Table 1.1) (Seaton 2002).

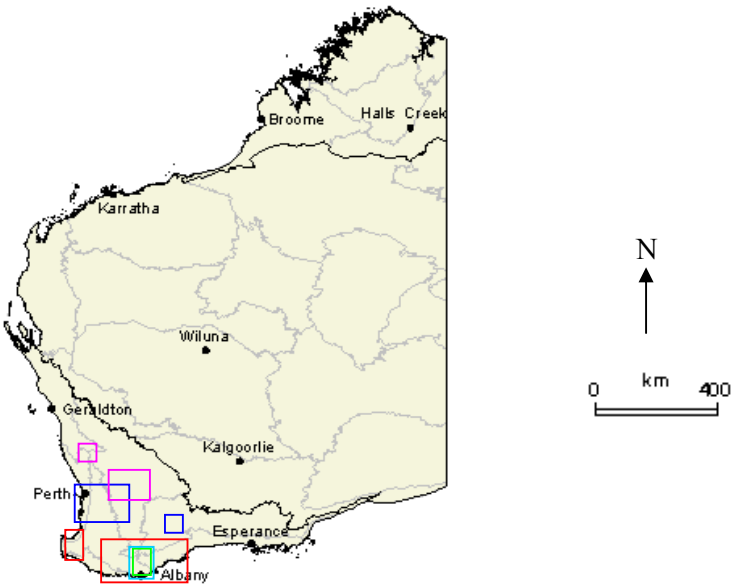

Figure 1.1 Distribution of blue-flowered *Conospermum* in Western Australia. *C. caeruleum.* *C. amoenum* *C. dorrienii* *C. eatoniae* *C. spectabile.* (Florabase, 2003)

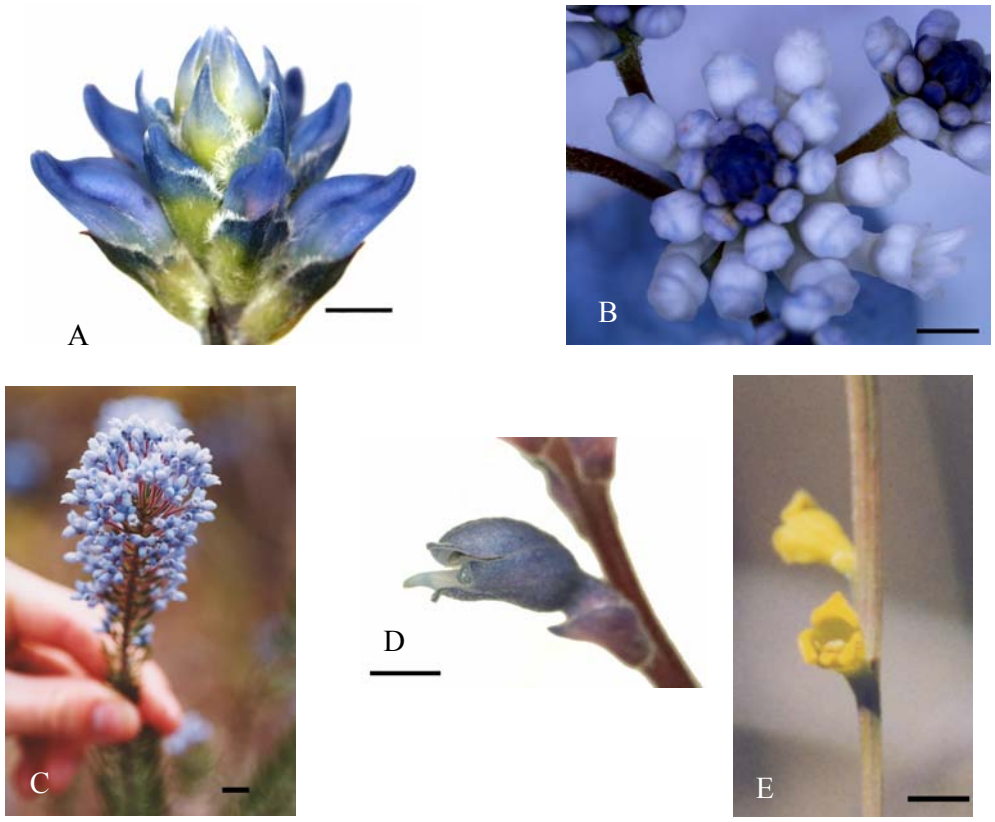

Figure 1.2 A. *Conospermum caeruleum*. Bar = 2 mm B. *C. brownii.* Bar = 2 mm C. *C. dorrienii*. Bar = 5 mm D. *C. amoenum.* Bar = 2 mm E. *Synaphea petiolaris.* Bar = 2 mm

Table 1.1. Percentage blue-flowered *Conospermum* of total *Conospermum* stems harvested. (Adapted from Department of Conservation and Land Management (CALM) statistics on wildflower picking).

Year	1997	1998	1999	2000	2001
Percentage	0.41	0.5	1.8	0.12	0.52

The focal species of this research are *C. eatoniae* E. Pritz.; *C. amoenum* Meisn. subsp. *amoenum; C. caeruleum* R.Br. subsp. *caeruleum; C. coerulescens* subsp. *dorrienii* (Domin) E.M.Benn. and *C. spectabile* E.M.Benn.

Conospermum eatoniae E. Pritz. is found in sandy heath woodland in the Avon district, WA, pH ranging from 4.5 to 5.5, (1:5, $CaCl_2$). An upright shrub growing to approximately 75 cm tall and 1 m across, its juvenile form is quite different, beginning as a rosette of basal leaves approximately 7 cm long, with leafless stems rising from the base of the plant (Figure 1.3 A) The glabrous stems form a somewhat tangled flower system from dichotomous branching, the inflorescence is paniculate with up to 10 blue flowers (Bennett 1995) (Figure 1.3 B) The flowering period is August to September. Population numbers have declined from 14 to just 3 (Seaton, pers. comm.) and the species is currently under consideration for listing as rare and endangered (Sainsbury 1991). This is attributed to the expansion of agriculture in the regions North East of Perth, weed infestation and increasing areas of salt affected soil (Seaton, pers. comm.). One selection is commercialised as “Blue Lace” (Seaton 2002).

Conospermum. caeruleum R.Br. is suited to cooler conditions, found from Denmark to east of Albany, WA, in heavier, free-draining soils subject to inundation (Bennett 1995; Sainsbury 1991). With a prostrate habit, this perennial herb is capable of covering a 2m area, and a thick woody stem can grow to 60 cm high (Figure 1.3 D). There are a number of flowering stems, which are long, narrow and leafless. The dense blue inflorescences are paniculate with up to 18 flowers each (Figure 1.2 A). The flowering period is from July to October. It remains relatively common in its natural range, and a few pink flowered genotypes have been recorded (Bennett 1995).

Conospermum amoenum Meisn. occur in lateritic soil on the Darling Scarp and east toward York, W.A. It is an erect or spreading shrub to about 1m tall spreading to 1.5 m wide, with glabrous foliage, leaf length approx 2 cm (Figure 1.3 C). The blue inflorescence spikes of 4 - 8 flowers increase in number along the upper branches (Figure 1.2 D). The flowering season is from August to September. White flowered individuals have been observed (Bennett 1995). Once a common species along the Darling Scarp, it is now seriously under threat from farming and mining.

Conospermum corulescens subsp. *dorrienii* (Domin) E.M.Benn. (hereafter referred to as *C. dorrienii)* occurs on rocky slopes of the Stirling Range, W.A. It is an upright shrub with dense leafy stems (Figure 1.3 E). Distinguished from other subspecies by a bright blue perianth (Bennett 1995), the flowers are clustered at the top of the stems and have long peduncles (Sainsbury 1991) (Figure 1.2 C). This species flowers in October and November, and cannot be bush picked as it only occurs in a National Park. Population numbers have remained steady, however it is under threat from the spread of *Phytopthora* spp. (jarrah dieback) in the Stirling Range National Park.

Figure 1.3 Growth habit of some *Conospermum* species. A. Juvenile *C. eatoniae* with basal leaves and small leafless stems. Bar = 3 cm. B. Mature *C. eatoniae.* Bar = 25 cm. C. Mature *C. amoenum.* Bar = 25 cm. D. Mature *C. caeruleum* supported with wire mesh. Bar = 10 cm. E. Mature *C. dorrienii.* Bar = 10 cm.

Conospermum spectabile also grows in the sandy soils of the Stirling Ranges, WA. An erect shrub to 80 cm tall, it has an inflorescence with terminal panicles of spikes in white, purple or blue. The woolly perianth is blue and white. This species flowers in October and November, and like *C. dorrienii,* cannot be bush picked (Bennett 1995). Population numbers are in decline due to the spread of dieback, as is the size of each population.

1.1.2 Reproductive biology

The reproductive biology of *Conospermum* has not been well documented; infact the breeding and mating systems of Proteaceae in general requires further research, as highlighted by Goldingay and Carthew (1998). Cross-pollination of *Conospermum* spp. offers an opportunity to increase species diversity and introduce new cut-flower forms. Research into this area will significantly improve the commercial viability of these flowers, and perhaps improve the chance of survival of natural populations.

1.1.3 Pollination mechanism

The flowers of *Conospermum* species have an active pollination mechanism, which utilises insects as pollinators (Figure 1.4). Of the four anthers present, two are infertile. The style is bent, the lower bend towards the fertile anthers, and the upper bend to the infertile ones. The flower opens in a state of tension, but when pressure is applied at the base of the style by the head of an insect, the style flicks away from the fertile anthers and strikes the pollinator. This causes the anthers to dehisce explosively, releasing the pollen in a single dusty mass. The cup shaped stigma is forced down onto the pollinator when triggered, to pick up pollen carried by the insect (Bennett 1995; Carolin 1961; Morrison *et al.* 1994; Morrison D.A. *et al.* 1994).

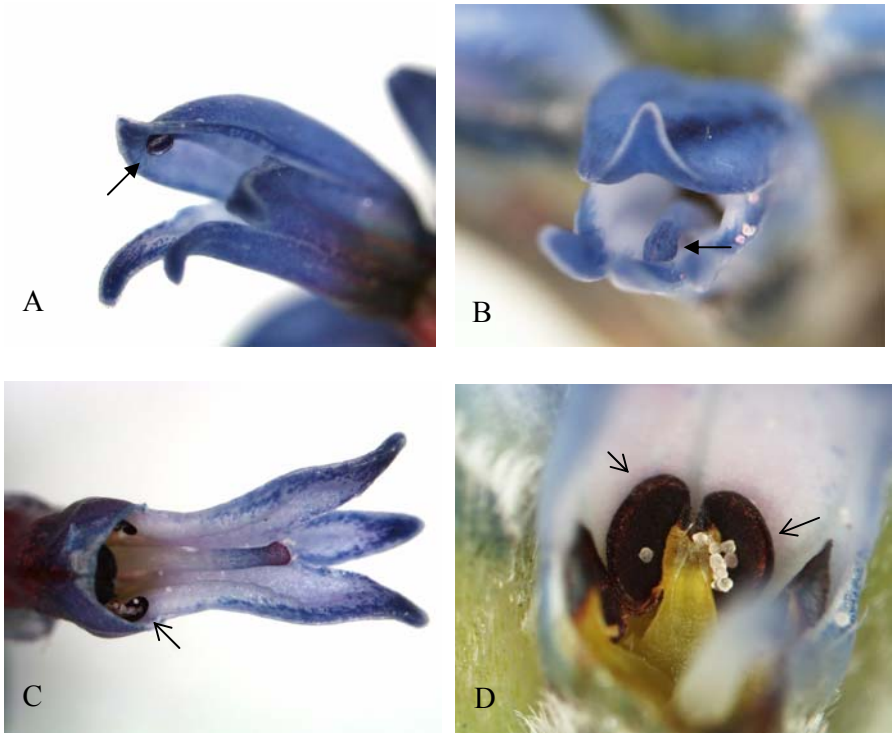

Figure 1.4 Trigger mechanism of the style and anthers in *Conospermum eatoniae.* A. Open flower with style in untriggered position (arrow). B. Open flower with triggered style (arrow). C. View of triggered style and dehisced anthers (arrow) from above, with large petal removed. D. Two dehiscent kidney-shaped anthers (arrows). Flowers are approx 6 mm in length.

Houston (1989) identified a distinct species-group of small bees (5 – 9 mm long) that were collected only from white-flowered *Conospermum*. These bees were observed standing on the outside of the flower and releasing the trigger mechanism with their tongues, the pollen being trapped on their hairy bodies. Three groups from the species *Leioproctus conospermi* appeared to have evolved structural adaptions for foraging on *Conospermum*, such as the females having sparse scopa for carrying the coarse pollen, and the males having camouflage modifications to avoid detection whilst visiting the flower.

1.1.4 Stigma receptivity

There is much variation between Proteaceae species in stigma receptivity timing and duration. Some are partially receptive at anthesis, others 2 - 4 days later. The relationship between stigma receptivity, anthesis and pollen removal is not known for *Conospermum*. Physical changes in the stigma can indicate receptiveness. In *Grevillea* the stigmatic papillae enlarge as receptivity increases, whereas Banksia species have wet stigmas where secretion increases with receptivity, or dry stigmas where grooves open with increased receptivity (Goldingay and Carthew 1998). Preliminary investigations show that *Conospermum* appears to have a wet stigma.

A study of several white-flowered *Conospermum* species in the eastern states of Australia concluded that they were cross-compatible to varying degrees (Morrison *et al.* 1994). No similar study has been done on the Western Australian species. Assessment of pollen tubes is commonly used to determine if a species is self compatible or not, but Goldingay and Carthew (1998) proposed that this might not be conclusive. Incompatibility may be late acting, and as such may go undetected using

this method. Pollen tube rejection in other species has been documented in the lower style. Tubes reaching the ovary may still be unable to penetrate the ovules for fertilisation. An example of late-acting incompatibility is in *Eucalyptus globulus*, where the number of zygotes observed at four weeks after fertilisation was significantly more than two and four weeks later, when many ovules had degenerated in both self- and cross-pollinations (Pound *et al.* 2002). Similarly, fruit set as a measure of self-compatibility was considered inconclusive due to the possibility of inbreeding depression. To detect whether incompatibility is pre or post-zygotic the only conclusive test is through sectioning initial embryo stages.

1.2 REPRODUCTION

The ovule type in *Proteaceae* varies, but for *Conospermum*, there is a single orthotropous ovary. The embryo sac is of the *Polygonum* type (Davis 1966) consisting of seven cells: the haploid egg cell which is fertilised to produce the zygote; two synergids; the diploid central cell which forms the endosperm when fertilised, and two antipodal cells.

Conospermum pollen is triporate with large circular apertures. The grains are large, up to 80 - 90 μm in diameter and have a thick exine. When shed from the tetrasporangiate anther, the pollen grains are 2-celled and contain starch (Davis 1966).

Embryos in the Proteaceae family are of the Aster type, however no suspensor is formed (Davis 1966).

1.3 SOMATIC EMBRYOGENESIS

Somatic embryogenesis is the process by which haploid or diploid cells that are not naturally embryogenic are induced to form embryos. Development proceeds through characteristic embryological stages, leading to the development of a differentiated plantlet (Williams and Maheswaran 1986). Somatic embryogenesis can occur via two pathways, direct or indirect. Spontaneous somatic embryos have rarely been observed *in vivo* (Taylor 1967) but they can be produced *in vitro* (Rugkhla and Jones 1998; Choi *et al.* 1999; Murthy and Saxena 1998).

The first report of somatic embryo production was by Steward *et al.* (1958) in carrot, which has become a model species for studying embryogenesis. He observed that carrot suspension cells could be triggered to develop in stages similar to those seen in zygotic embryo formation. Since then many herbaceous and woody plant species have been regenerated via the somatic pathway *in vitro* (Murthy and Saxena, 1998).

1.3.1 Medium requirements

The media used in tissue culture to induce embryogenesis depends upon the plant species, and several media steps may be necessary. Early experiments using different nitrogen sources on various explants revealed an apparent requirement for reduced nitrogen at a critical time of development. A higher frequency of embryogenesis in carrot was observed in the presence of NH_4^+ than on NO_3^- (Reinert *et al.* 1967; Sharp *et al.* 1980). However, Reinert (1968) found the opposite when using White's medium. The effects of nitrogen source appear to be influenced by the other media components. More recent studies by Elkonin and Pakhomaova (2000) found high NH_4^+ produced compact embryogenic sorghum calli, whereas high NO_3^- produced friable embryogenic calli.

Other mineral salts also play a crucial role in promoting embryo formation. In the case of carrot, raising the K^+ levels can increase embryo formation (Tazawa and Reinert 1969).

Calcium plays numerous vital roles in plant function, many of which are still not understood. *In vitro* medium concentrations can influence embryogenic response. Changing the calcium content in the culture medium modified embryo development, texture, water and mineral status of *Hevea brasiliensis* (Mull.) Arg. calli. Concentrations above 9 mM resulted in friable calli that were unable to undergo embryogenesis (Montoro *et al.* 1995). Ettiene *et al.* (1997) also reported somatic embryogenesis and an increase in culture regeneration capacity, quality and quantity at 9 mM calcium for *H. brasiliensis*.

Sucrose is the most commonly used source of carbohydrates. Mannitol is also used, and in the case of *Zea mays* L., was required to maintain an embryogenic rather than rhizogenic culture (Emons *et al.* 1993). The concentration of sucrose in the culture medium varies, but is commonly around 20 gL^{-1}. Samoylov *et al.* (1998) found growth of soybean embryogenic cultures declined as sucrose concentration increased.

1.3.2 Explant

The choice of explant can be influential in achieving somatic embryogenesis. Pedroso *et al.* (1997) reported differences in morphogenic competence in leaf regions of *Camellia japonica* L. Direct embryo formation occurred only in marginal leaf regions, roots formed in the midrib area, and callus on the leaf base. This is not the same for all species however, as Gill and Saxena (1992) reported the formation of somatic embryos on the midrib section of peanut. A review by Raemakers *et al.*

(1995) found that direct somatic embryogenesis occurred most frequently from zygotic embryos, whereas vegetative explants produced indirect somatic embryos.

Not only is the explant tissue type critical, the age of the explant can also influence success. In general, the more juvenile the tissue the greater the chance of inducing somatic embryos *in vitro*. A precise age of tissue may be needed, for example, Murthy and Saxena (1998) found somatic embryogenesis could be induced in young peanut seedlings up to 9 days old, but seedlings older than 21 days produced no embryos at all. This supports the view that in many species the ability to express embryogenesis is restricted to a small developmental window.

A review of dicot species producing somatic embryos by Raemakers *et al.* (1995) revealed 51 % used zygotic embryos as the primary explant, followed by 23 % floral explants and 3 % vegetative explants. The remaining explants were a combination of the above. The use of zygotes as explants is often one of the problems associated with somatic embryogenesis; frequently the objective is to obtain clonal plants from a superior genotype rather than from a zygote with unknown properties.

1.3.3 Gelling agents

The medium used to support embryogenic cultures varies and may differ for embryo induction and maturation. Solid, semi-solid and liquid suspension cultures have been used, often in combination (Murthy and Saxena 1998). Liquid cultures give a more uniform dispersal of cells, but in some species there are problems associated with their use. In *Zea mays* L. somatic embryos derived from suspension cells are blocked

in stem meristem formation. On transfer to agar, roots grow abundantly from these embryos before a stem meristem forms (Emons and Kieft 1991).

In some cases it is necessary to decrease the osmotic water potential at a particular stage of embryo development, such as for conifer embryos (Klimaszewska *et al.* 2000). It can be done by increasing the sucrose concentration (Lelu *et al.* 1994) or by supplementing the media with permeating (Roberts 1991) or non-permeating solutes (Li *et al.* 1998). Klimaszewska *et al.* (2000) found that increasing the gel strength was an alternative to osmotically restricting the available water, and that both increased the germination frequency of somatic conifer embryos.

1.3.4 Potential markers and cell types leading to embryogenesis

A current focus of research in somatic embryogenesis is finding markers, biochemical and molecular, that will identify embryogenic cells. It is known that embryogenic cells are unique and several methods of identifying these cells before embryogeny can been used. A good marker would be one that identified embryogenic cells very early in embryo development so that cultures could be quickly screened for embryogenic potential.

Potential markers include phosphoproteins, such as those identified in embryogenic cells of carrot but were absent in non-embryogenic cells (Tan and Kamada 2000). Isoenzyme electrofocusing has been used to differentiate between organogenic and embryogenic cells (Chibbar *et al.* 1988; Coppens and Gillis 1987; Tchorbadjieva and Odjakova 1991), and there are differences in esterase activity in barley (Coppens and

Gillis 1987) and peroxidases in different stages of carrot embryogenesis (Joersbo *et al.* 1989).

Increasing evidence exists that molecules totally different from conventional plant growth regulators can direct the transition from somatic to embryogenic cell (Schmidt *et al.* 1994). Arabinogalactan proteins (AGPs) appear to have diverse roles within the plant, such as the promotion of embryogenesis in carrot (Kreuger and Van Holst 1993). Recent research suggests that the composition of these molecules alters as a function of development (van Hengel *et al.* 2002), and hydrolytic enzymes activate these molecules in immature tissue. van Hengel *et al.* (2001) showed increased somatic embryogenesis of carrot when AGPs were exposed to the hydrolytic enzymes EP3 endochitinases. AGPs are identified using monoclonal antibodies, and as such can be detected in specific tissues. Filonova *et al.* (2000) utilised this technique to distinguish between somatic embryos and proembryogenic masses in *Picea abies*, and showed that only proembryogenic masses expressed the AGP epitope.

1.3.5 Gene expression in embryogenesis

The developing plant embryo has an estimated 20,000 to 30,000 genes expressed during embryogenesis (Goldberg *et al.* 1989). Many genes have been identified that have altered expression during embryogenesis; however most of these are in later developmental stages.

One of the most useful methods for investigating gene function and expression is the use of mutants, such as in *Arabidopsis*. Recently, a gene was discovered (WUS) that appears capable of promoting cell transition from vegetative to embryonic in *Arabidopsis.* The genetic locus PGA6 enabled the transition from vegetative to

embryogenic cells when two gain-of-function- mutations at this locus were made (Zuo *et al.* 2002).

Mutants of *Arabidopsis* enable the identification of genes that may be important in all aspects of embryo development, from polarity and asymmetrical cell division, to pattern and meristem formation and maturation and germination (Dodeman *et al.* 1997). They also allow comparison between zygotic and somatic embryo formation (Zhang *et al.* 2002).

1.3.6 Embryogenic cell types

It was originally thought that typical embryogenic cells were small in size, had dense cytoplasmic contents (Ho and Vasil 1983), large nuclei, small vacuoles and a profusion of starch grains (Williams and Maheswaran 1986). Toonen *et al.* (1994) however, developed a cell tracking system that enabled determination of the fate of single carrot cells. Single cells could be classified into five groups according to their structure, and all morphological types developed somatic embryos at a rate of 0.2 -1 %, not just the small cells with dense cytoplasm. The cell types were described as: 1. Small spherical vacuolated cells; 2. Small spherical cytoplasm-rich cells; 3. Oval vacuolated cells; 4. Elongated vacuolated cells; 5. Irregular shaped cells. In addition, three major developmental pathways were observed for specific cell types: an asymmetrical cell cluster (cell types 3 and 4); a symmetrical cell cluster (cell types 1 and 2) and aberrantly shaped cell clusters (cell type 5). Filonova *et al.* (2000) has since used a similar system for tracking *Picea abies* L. (Norway spruce) cells and cell aggregates from embryonic suspension cultures, which enabled classification of

proembryogenic masses into three types according to cellular organisation and cell number.

Although a particular cell type may be classed as embryogenic, the density of cells in the culture plays a significant role. A high density of cells can induce embryogenesis, while too few cells inhibit embryo production. Low density cultures in the presence of preconditioned growth medium (from a high density culture) can also induce embryogenesis, suggesting that there are soluble signal molecules that are involved in interactions between cells (de Vries *et al.* 1988; McCabe *et al.* 1997).

1.3.7 Comparison of zygotic and somatic embryogenesis

A direct comparison between somatic and zygotic embryogenesis can only be made from the globular stage onwards. Many similarities exist, and it is also quite possible that they share common regulatory pathways prior to the globular stage.

Two major differences exist between the zygotic and somatic embryo; somatic embryos lack an endosperm and the suspensor tissue shows little differentiation. It would appear that both the presence of endosperm, and the development of a suspensor are made redundant by culture conditions. For Proteaceae, zygotic embryos normally lack a suspensor.

Some genes identified as essential for somatic embryogenesis also have similar expression patterns in the zygotic embryo. Examples are the *knotted1 (kn1)* and *ZmLEC1* genes in maize. *Kn1* is expressed where the shoot meristem is initiating in *in vitro* maize somatic embryos, and also in the zygotic embryo (Zhang *et al.* 2002).

The maize *ZmLEC1* gene has shown similar expression patterns to the *Arabidopsis* gene LEC1 during zygotic embryo development, both expressed in early embryo development (Lotan *et al.* 1998; Zhang *et al.* 2002).

1.4 THE MALE GAMETE: POLLEN

1.4.1 Adhesion and hydration

Effective adhesion of pollen to the stigma is the first step in successful pollination. Wet stigmas such as in *Conospermum* and *Solonaceae* are less selective for pollen of the same species than dry stigmas, such as the *Brassicaceae.* Studies so far on the proteins and genes involved in pollen adhesion and interactions between the stigma and pollen have revealed a complex system, as reviewed by Wheeler *et al.* (2001). The pollen coat is essential for adhesion to the stigma, as demonstrated by the mutant *lap1* (less adherent pollen) that has a defective exine layer, and showed reduced adherence to the stigma (Zinkl and Preuss 2000). Associations between stigmatic proteins and adhesion have also been discovered in *Brassica* (Luu *et al.* 1999) and *Lilium* (Lord 2001).

Pollen undergoes rapid dehydration in the anther prior to dehiscence, and must rehydrate to germinate on the stigma. The pollen wall regulates the uptake of water from stigma to pollen grain. Long chain lipids have been implicated as signals for pollen hydration stimulation (Wolters-Arts *et al.* 1998). Ikeda *et al.* (1997) identified an aquaporin (water channel protein) in the plasma membrane essential for hydration. The wide variation in stigmatic surface between species suggests the components and mechanisms involved in hydration will also be diverse (Wheeler *et al.* 2001).

1.4.2 Germination and penetration of the stigma.

When released from the anther, pollen is in a metabolically dormant state until rehydration occurs on the stigma. Stigmatic surfaces are classed as wet or dry, with dry stigmas considered more selective and pollen specific. *Conospermum* have a wet stigma which binds pollen easily. The stigma is the site for pollen recognition, and rejection of incompatible pollen where incompatible alleles on the pollen surface are identified. In *Brassica* and *Papaver*, the stigma can prevent germination and penetration of incompatible pollen tubes into the style immediately (Nasrallah *et al.* 1994). Self-incompatibility can be of two types: sporophytic incompatibility (SI) where the phenotype of the pollen is determined by the genotype of the parent, and gametophytic (GI) where the phenotype of the pollen is determined by its own haploid genotype.

Most *Proteaceae* are self-incompatible, or those that are self-compatible preferentially outcross. Pollen tube inhibition in the pollen presenter and upper style were reported for *Banksia coccinea* R. Br., *B. prionotes* and *B. menziesii* R. Br. (Fuss and Sedgley 1991; Sedgley *et al.* 1994), and in the lower style for *Macadamia integrifolia* (Sedgley 1983) and *Persoonia mollis* R. Br. (Krauss 1994).

1.4.3 The stylar matrix.

Once the pollen has germinated and the tube passed between the stigmatic cells, the style provides a nutrient-rich environment for further pollen tube growth. In addition, it and the ovary provide chemical signals to control the direction of pollen tube growth. The internal structure of the style is varied. The extracellular matrix (ECM) either lines a hollow tract of elongated cells that have large intercellular spaces for the

pollen tubes to grow between, or is secreted between cells of a well-defined canal down to the ovary. The composition of the matrix is a mixture of arabinogalactan proteins (AGPs), proline-rich glycoproteins and extensin-like proteins, along with lipids, carbohydrates and small molecules. Transmitting tract specific (TTS) proteins have been identified which appear to stimulate pollen tube growth *in vitro* and may also have chemotrophic properties. They have been shown to attract pollen tubes in semi-*in vivo* experiments using *N. tabacum* (Cheung *et al.* 1995). These AGPs adhered to the tube cell wall and pollen tube tip, and were found incorporated into the pollen tube cell wall, implying that ECM components can influence pollen tube growth. A major problem suggested by Lush (1999) and Lush *et al.* (2000) of the chemo attraction abilities of these proteins is the physical distance over which the chemical must diffuse. The gradient necessary for the chemical to behave as an attractant over a 1 mm distance requires a 10 000 fold difference in concentration at each end of the style. The actual gradient of the TTS protein, although higher in concentration at the ovarian end of the style, is closer to only four-fold (Wu *et al.* 1995). The average length of the style of *N. tabacum* is around 50 mm, so the actual role of these proteins remains unclear.

Lord (2001) isolated two adhesion molecules from the stylar matrix of *Lilium*, a pectic polysaccharide and a cysteine-rich protein. Pollen bound to the protein *in vitro* and *in vivo*, but was specific to lily; tobacco pollen did not bind.

1.5 THE POLLEN TUBE

1.5.1 Basic structure

Pollen tubes grow by tip extension, in contrast to most plant cells that grow over the whole cell surface. This rather unique form of growth has been compared by some to axon growth in animal cells, fungal hyphae and root hairs (Cai *et al.* 1997).

The pollen tube has a clear zone at the apical tip where growth occurs. This area, 3 - 5 μm long in lily, contains a large number of randomly arranged vesicles of two different sizes (300 nm and 50 nm diameter), which contribute to the growth of tube (Cresti and Tiezzi 1990). No organelles are present in the tip if the tubes are actively growing.

1.5.2 Cytoplasmic streaming

The cytoplasm contains the organelles essential for growth, and the vegetative nucleus and generative cell that must make their way to the micropyle for fertilisation. Organelle movement in the pollen tube is controlled by the cytoskeleton, which is made up of microtubules, actin filaments and associated proteins. The interaction between actin and myosin enables bi-directional organelle transport, such as secretory bodies that carry plasma membrane and cell wall components to the apical tip and enable tube growth. The structure of the cytoskeleton is becoming increasingly well known by using light and immunofluorescence microscopy, however the function of some of its components are yet to be fully understood.

Myosins are the main motor proteins for organelle movement, moving along actin filament "tracks" (Cai *et al.* 1997). Inhibitors of microtubules (MTs) do not affect

cytoplasmic streaming (Heslop-Harrison *et al.* 1988) and so may not be involved in organelle trafficking, however close associations with some cytoplasmic components has been observed, (Hepler *et al.* 1990), and so their function is not clear. MTs occur in bundles along the longitudinal axis of the tube, but not in the tip. However, Del Casino *et al.* (1993) identified possible short MTs in the tip using tubulin antibodies, which Cai *et al.* . (2001) speculate could be an intermediate pool between the formation of monomeric tubulin into MT bundles. Cai *et al.* (2001) also proposed a hypothetical model, based on current knowledge, to suggest the role of microtubule motor proteins, which use ATP hydrolysis as energy for transport. Among other things, the authors suggest MT motor proteins may assist the orientation of MTs around the structures they are to transport, such as the generative cell and vegetative nucleus, and may be responsible for the association and organisation between actin filaments and microtubules.

1.5.3 The pollen tube cell wall

The cell wall is made up of three main types of polysaccharides: callose, cellulose and pectin. Enzyme complexes in the plasma membrane synthesise callose and cellulose, and golgi make and secrete pectin (Delmer and Stone 1988; Hasegawa *et al.* 1998).

There are thought to be three layers of the cell wall, an outer microfibrillar pectic layer, a middle cellulosic later, and an inner callose layer (Heslop-Harrison 1987, Stepka *et al.* 2000). The exact composition and structure of the wall is not completely understood. The outer layer is continuous around the tube and tip, however the inner layer disappears at the extreme tip of the tube.

1.5.4 The role of calcium

Calcium plays a vital role in pollen tube growth. The mechanisms of calcium are complex and not yet fully understood, but some aspects are well known. Ratiometric analysis has shown that growing pollen tubes have a cytosolic Ca^{2+} tip-focused gradient whereas tubes that are not growing do not (Pierson *et al.* 1994). Direction of pollen tube growth is also influenced by calcium, as demonstrated by Malho and Trewavas (1996) who found an increase in calcium on one side of the pollen tube apex induced a change in growth direction toward the increased calcium. Calcium channels are concentrated at the apical tip of the growing tube, and it is thought possible that they are activated by stretching of the plasma membrane (Pierson *et al.* 1994).

Calcium levels in the tube oscillate, and have been shown to accompany tube growth oscillations (Holdaway-Clarke *et al.* 1997). However, it remains unclear if changes in calcium concentration in the apical region cause the oscillating growth, or whether growth causes the changes in calcium concentration (Franklin-Tong 1999).

The calcium dependency for growth of pollen tubes has enabled studies using calcium channels blockers and inhibitors to further investigate the precise mechanisms and role of calcium. Geitmann and Cresti (1998) showed that inorganic Ca^{2+} inhibitors ceased the pulsating growth of petunia pollen tubes, but did not stop growth overall. Organic inhibitors ceased all growth at high concentrations. They hypothesised that different types of Ca^{2+} channels were responsible for different aspects of pollen tube growth.

1.5.5 Guidance of pollen tube to ovule and micropyle

Once pollen tubes emerge from the transmitting tissue into the ovary, they must navigate their way to the ovule(s). This guidance is thought in part to come from the ovules themselves. Hulskamp *et al.* (1995) showed, with the use of an *Arabidopsis* mutant, that in plants with defective ovules, the pollen tubes lost their ability to enter the ovule and simply grew randomly around the ovary. Signalling between pollen tubes must also occur at this late stage, as in normal plants only one pollen tube approaches each ovule (Wilhelmi and Preuss 1996).

1.5.6 Sperm delivery to embryo sac

Upon reaching the micropyle, the pollen tube tip enters the embryo sac and bursts. The two sperm cells are rapidly released, then fertilisation of the egg and central cells occurs, resulting in formation of the endosperm and zygote respectively. Signalling during this process is not yet understood due to the rapid cell diffusion that occurs, but some chemoattraction, possibly involving the synergid cell, has been suggested (Wilhelmi and Preuss 1997). The zygote begins to divide, first yielding a small apical cell and a large basal cell, and then develops through characteristic stages to maturity. The endosperm divides and develops into seed tissue that nourishes the developing embryo, or is used by the seedling after germination.

1.5.7 Seed

Once the zygote has achieved its maximum size, the ovule differentiates into a seed coat to provide protection to the zygote during dispersal. Prior to dispersal, the zygotic embryo dehydrates, losing up to 95 % of its water content, and dormancy begins.

1.6 *IN-VITRO* POLLEN GERMINATION

Germination of pollen *in vitro* is a common technique used to assess pollen viability. It is assumed that if pollen can germinate *in vitro* to produce a pollen tube, it is viable and would have been able to grow and effect fertilisation *in vivo*. Binucleate pollen will generally readily germinate in a minimal medium containing boron, calcium and an osmoticant, such as that developed by Brewbaker and Kwack (1963), however some trinucleate pollen is recalcitrant and a universally reliable germination medium is not known. Germination can take several minutes to hours and tubes grow normally at speeds up to 2 - 3 μms^{-1} (Stanley 1971). Table 1.2 shows some recorded pollen tube growth rates both *in vitro* and *in vivo*. Pollen tubes grown in culture rarely reach the length necessary for fertilisation *in vivo*, suggesting that the medium requirements are not fully met *in vitro*.

Table 1.2 Previously recorded pollen tube growth rates *in vitro* and *in vivo*.

SPECIES	CONDITIONS	RATE μms^{-1}	REFERENCE
Ornithogalum virens	*in vitro*	0.25	Stepka et al, 2000
Alopecurus pratensis L.	*in vitro*	0.38	Heslop-Harrison, 1984
Zea mays	*in vivo*	1.14	Heslop-Harrison, 1984
Oenothera organensis L.	*in vivo*	1.8	Stanley, 1971

1.7 STUDY OBJECTIVES

The market-driven demand for new Australian native species for cut flowers has prompted further investigation into an efficient method for propagation of selected *Conospermum* species. Current methods of propagation using shoot cultures has proven difficult and inefficient, with poor root strike and high mortality of plants during the hardening off stage. In addition, our understanding of the floral biology of

these species is limited, and greater knowledge is required to assist future breeding programs.

The objectives of this study were:

To develop somatic embryogenesis techniques for micropropagation of selected blue-flowered species of *Conospermum*.

To understand the floral biology of the species.

CHAPTER 2

EXPLANT SELECTION FOR SOMATIC EMBRYOGENESIS IN *CONOSPERMUM* SPP.

2.1 ABSTRACT

A wide range of explant types of *Conospermum* species were tested on MS media supplemented with various growth regulators, for their ability to produce somatic embryos. Zygotic embryos produced either direct somatic embryos and/or embryogenic callus in all the species tested. In *C. caeruleum*, embryogenic callus was cultured for over 18 months. Other explants from field plants included vegetative buds, semi-floral buds, floral buds, stems and immature zygotic embryos. Vegetative buds produced shoots and callus on combinations of auxin and cytokinins; stems, semi-floral and floral buds mostly died in culture, and all immature zygotic embryos died. *In vitro C. eatoniae* leaf portions were also screened for embryogenic capacity on a range of growth regulators. Callus readily formed in the presence of 2,4-D, however no difference in response was found for particular leaf regions. Contamination was a significant problem for field-initiated explants, particularly for *C. caeruleum.*

2.2 INTRODUCTION

Somatic embryogenesis is the process whereby embryos are induced from cells that are not naturally embryogenic, and can occur via two pathways; directly from the primary explant, or indirectly through a callus phase. It is one of the methods for mass propagation of clonal plants.

Somatic embryogenesis has been achieved for a number of gymnosperm and angiosperm species, both monocotyledons and dicotyledons, although there are more reports of success in herbaceous, rather than woody species. There is little consistency in the culture conditions and types of explants that can be induced to produce somatic embryos. The vast majority of primary explants used are zygotic embryos (Raemakers *et al.* 1995), or parts of the embryo such as the hypocotyl or cotyledon. The response of these explants in culture is quite diverse, for example the hypocotyl gave the best production of direct somatic embryos in *Eleutherococcus senticosus* Harms (Choi *et al.* 1999), whereas the cotyledon was the most successful in producing *Eucalyptus globulus* Labill indirect somatic embryos (Pinto *et al.* 2002). For *Arachis hypogaea* L., cotyledonary sections placed horizontally in the medium produced direct somatic embryos, however sections placed vertically did not (Gill and Saxena 1992). In *Juglans nigra* L. the age of zygotic embryo (weeks after anthesis) from which the cotyledon was excised influenced the production of somatic embryos (Neuman *et al.* 1993). Culture of intact zygotic embryos have enabled the production of direct somatic embryos in *Santalum album* L. (Ravishankar Rai and McComb 2002), and indirect somatic embryos in *Acacia farnesiana* and *A. schaffneri* (Canedo Ortiz *et al.* 2000).

Zygotic embryos do not provide material with known genetic properties, whereas somatic embryos are clonal. Thus there are considerable advantages in using floral, leaf or stem explants of adult plants with known properties. This is of particular importance in breeding programs where selections have been made and superior genotypes are required.

Some success has been reported using vegetative explants, as summarised by Raemakers et al. (1995). For example, studies have reported somatic embryogenesis on the midrib of leaves of *Camellia japonica* L. (Pedroso and Pais 1993), *Cydonia oblonga* Mill. and *Arachis hypogaea* L. (Gill and Saxena 1992). In the case of *Camellia,* the region of the leaf was important; organogenic callus was observed at the leaf base and tip, direct embryo formation was observed in marginal leaf regions, and direct root formation was found on the midrib. Internode fragments from shoots in *Quercus suber* L. (Maataoui *et al.* 1990) have also successfully been used to produce somatic embryos.

Floral parts have also been successful for somatic embryo induction in some cases. For example, Li *et al.* (1998a) induced embryogenesis on staminodes of *Theobroma cacao* L., and macerated immature *Manihot esculenta* Crantz inflorescences were highly embryogenic (Woodward and Puonti-Kaerlas 2001).

As explained in Section 1.1 of Chapter 1, *Conospermum* species show great potential as cut flowers for the Australian Wildflower industry (Seaton 1996), and although lines for several species have been selected for their desirable horticultural attributes commercialisation is limited by propagation problems.

The experiments described in this chapter aim to determine possible explant sources for somatic embryo induction in *Conospermum,* focusing predominantly on *C. eatoniae*. Direct embryogenesis is likely to produce clonal lines with low levels of somoclonal variation; while indirect embryogenesis through an intermediate callus phase may be produce somaclonal variants.

2.3 MATERIALS AND METHODS

In their juvenile state *C. eatoniae*, *C. caeruleum, C. spectabile, C. dorrienii* and *C. brownii* form a rosette of basal leaves from which leafless stems extend. *C. amoenum* does not begin as a rosette, but as leafy stalks. As *Conospermum* plants mature, vegetative buds form on the leafless stems. These remain dormant until flowering season, when they develop into semi-floral buds, and then mature into flower buds in a spike or paniculate inflorescence.

2.3.1 Explants from field plants

Explants were taken from *C. eatoniae, C. caeruleum* and *C. brownii* plants growing at Agriculture Western Australia Medina field station. For *C. amoenum*, explants were taken from plants growing at Medina and also from a wild population at Armidale, Western Australia. Mature seed was collected from *C. dorrienii* and *C. spectabile* plants located in the Stirling Range National Park.

Explants tested from field plants of *C. eatoniae* included vegetative buds, semi floral buds, floral buds, immature zygotic embryos and mature zygotic embryos. Buds were placed horizontally in the media, and some semi-floral buds were dissected longitudinally and placed cut surface down in the media. Explants were initiated into culture from April to December to detect any seasonal effects. For *C. caeruleum*, explants tested were vegetative buds, stems and immature and mature zygotic embryos. For *C. amoenum,* semi floral buds and mature zygotic embryos were tested and for *C. spectabile, C. dorrienii* and *C. brownii,* mature zygotic embryos were initiated into culture.

Explants were surface sterilised in 0.1 % (v/v) sodium hypochlorite for 15 minutes, and left on damp filter paper in a Petri dish overnight at room temperature. The following day they were further sterilised with 1 % (v/v) calcium hypochlorite with a drop of Tween 80 for 30 minutes, and gently hand scrubbed with a small paintbrush. They were rinsed twice in sterile water. For immature and mature zygotic embryos, seeds were sterilised using the method described, and the seed coat either fully or partially removed using a scalpel, avoiding damage to the zygote where possible.

The basal medium in all experiments was Murashige and Skoog's (1962) medium (MS), selected for its success in somatic embryogenesis induction in other woody species (Rugkhla and Jones 1998), supplemented with 2 % sucrose, 8 % agar and growth regulators. Growth regulators used were thidiazuron (TDZ), 6-benzylamino purine (BAP), and 2,4-dichlorophenoxyacetic acid (2,4-D), concentrations ranging from 0.1 μM to 10 μM. All media were adjusted to pH 5.8 before autoclaving for 20 minutes at 121 °C. Cultures were grown in 55 mm Petri dishes with 10 mls medium and were incubated in a growth room at 24°C ± 2°C under a 16 h light photoperiod (23 - 37 $\mu molm^{-2} s^{-1}$) provided by fluorescent tubes.

For all explants except zygotic embryos, up to 10 explants were placed in each Petri dish with 2 - 4 replicates per treatment. For zygotic embryos, up to 100 from each species were initiated into culture. Initially 55 mm Petri dishes were used with 8 embryos per dish, however high rates of contamination were detected and so 10 mls media was poured into 50 ml tubes, with one zygotic embrto per tube, to avoid cross contamination. Sterile zygotic embryos were returned to Petri dishes at the first subculture, 4 - 6 weeks after initiation. Cultures were maintained for up to 18 months.

Data were analysed using an analysis of variance (ANOVA).

2.3.2 *In vitro* explants

Explants were taken from *in vitro* shoot cultures of *C. eatoniae* growing on a MS medium containing 0.1 μM indole-3-butyric acid (IBA) and 0.3 μM BAP, 2 % sucrose and 8 % agar adjusted to pH 7.0 prior to autoclaving. From these *in vitro* shoot cultures growing as rosettes, leaves were removed and sliced into 4 equal sized portions. These were placed abaxial side down and cultured on MS medium as described previously, on a range of growth regulators. The leaf portions were placed on the medium in the order they were in the complete leaf, so that the response of each segment in culture could be observed. Regional differences were observed by Pedroso and Pais (1993) in *Camellia.* Each treatment had 3 replicates of 2 leaves per plate. Cultures were subcultured every 4 weeks and scored after 6 weeks. To score cultures, a classification system based on the amount of callus observed on the original explant was designed (Table 2.1). Data was analysed using ANOVA.

Table 2.1 Classification system for scoring callus on *in vitro Conospermum eatoniae* leaves.

Rating	Description
0	No callus
1	Callus on 1 edge, less than 50 % of explant covered
2	Callus on 2 edges, less than 50 % of explant covered
3	Callus on 50 - 89 % of explant
4	Callus on 90 - 100 % of explant
5	Explant completely covered by callus

The mean rating was calculated for each treatment (Figure 2.4). The mean survival rate of explants was also calculated (Figure 2.5).

From *C. eatoniae* shoot cultures that had produced leafless 'inflorescence' stems *in vitro* the soft tips were taken for culture on MS medium supplemented with 0.1 μM kinetin and 0.5 μM TDZ.

2.3.3 Effect of growth regulator pulsing and light

Conospermum eatoniae in vitro leaf segments were placed randomly in Petri dishes as described previously, on MS media supplemented with auxin and cytokinins. Each treatment had 3 replicates, 4 explants per Petri dish. Leaf explants were pulsed on media with growth regulators for 4 days, and then subcultured onto MS basal medium in the light or dark. After 1 month the cultures were placed back on growth regulator media for 3 days, then subcultured onto MS basal medium once again, in light or dark. After 4 weeks, cultures were placed on 1/10 the original growth regulator concentration for a further month. Embryogenesis in species such as *Daucus carrota* L. (Sharp *et al.* 1980) and *Foeniculum vulgare* Miller (Anzidei *et al.* 2000) has been induced by using high followed by low concentrations of 2,4-D in particular.

2.4 RESULTS

2.4.1 Field plants

Particular explant types are only available at certain times of the year. Between March and May, buds on the leafless stems of *C. eatoniae* are vegetative (Figure 2.1). These explants were the most responsive in culture, producing shoots and callus on a range of concentrations of BAP and 2,4-D with TDZ. Semi-floral and floral explants develop in June and July, depending on seasonal variation. There was a high rate of contamination and death for these explants across the range of media types tested. Table 2.2 shows callus growth and differentiation observed on explants. TDZ in

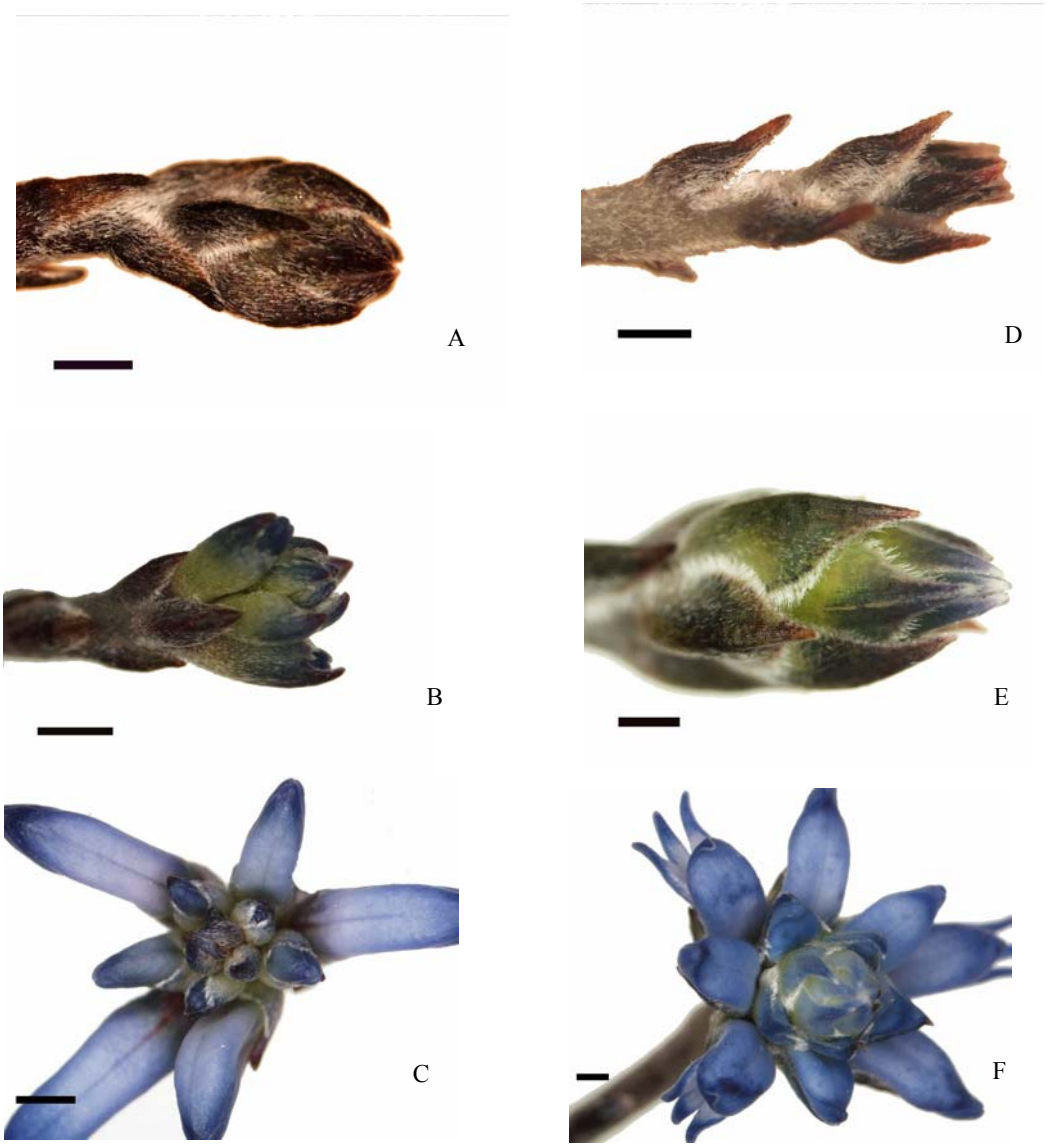

Figure 2.1 Developmental stages of *Conospermum eatoniae* (A-C) and *C. caeruleum* (D-F) flowers. A. Vegetative buds of *C. eatoniae* in May. B. Semi-floral buds of *C. eatoniae* in June. C. Floral *C. eatoniae* in July. D. Vegetative buds of *C. caeruleum* in May. E. Semi-floral buds of *C. caeruleum* in June. F. Floral *C. caeruleum* in August.

combination with BAP and 2,4-D enabled some differentiation into shoots, and callus formed in the presence of 2,4-D (Table 2.2). There was no difference in response between whole or half buds. The floral tips from *in vitro* culture swelled and expanded, then produced shoots on the media tested.

Vegetative buds from *C. caeruleum* initiated into culture produced some shoots on TDZ and BAP, and callus on TDZ and 2,4-D (Table 2.3), however fungal contamination resulted in approximately 75 % of culture treatments being discarded. This high rate of contamination was consistently a problem for initiating *C. caeruleum* explants into culture. Stems were unresponsive in culture and were eventually discarded, as were semi-floral explants from *C. amoenum* (Table 2.4).

Fruit set for *Conospermum* begins in early October; mature seed are shed from the plants in December (Figure 2.2). All immature *C. eatoniae* and *C. caeruleum* fruits collected in October and initiated into culture died (Table 2.2, 2.3). In December, mature zygotic embryos were excised from *C. caeruleum, C. eatoniae,C. amoenum, C. brownii, C. dorrienii* and *C. spectabile* seeds. One *C. dorrienii* zygotic embryo germinated with a root and shoot. Direct somatic embryos were produced on mature *C. caeruleum* zygotes cultured on MS supplemented with 0.1 μM TDZ and 0.1 μM BAP (Table 2.3), on growth regulator free MS and ½ MS for *C. spectabile* (Table 2.5). Embryogenic callus formed on mature zygotes from all species tested except *C. eatoniae* (Table 2.2) and *C. amoenum* (Table 2.4); however in all species except *C. caeruleum* embryogenic callus eventually produced shoots, became hard green callus or died after 12 - 14 weeks in culture. In *C. caeruleum* only, embryogenic cultures could be maintained for over 18 months as multiplying clumps of embryos at

globular, heart (not shown) or cotyledonary stages (Figure 2.3). No torpedo stage was observed. Contamination of initiated seeds was high, for example 85 % of *C. eatoniae*, 80 % of *C. amoenum* and 44 % of *C. caeruleum* seeds were discarded. Tables 2.3 and 2.4 show only callus growth or differentiation of explants in culture because of the high level of contamination and some death.

Table 2.2 Media on which *Conospermum eatoniae* explants showed callus growth or differentiation in culture (+). Subscripts represent concentration of growth regulators (μM). MS represents basal medium with no growth regulators.

Explant	Medium	Shoots	Callus
Vegetative buds	MS		
	MS + TDZ_1	+	
	MS + TDZ_5	+	
	MS + TDZ_{10}	+	
	MS + TDZ_1 BAP_1	+	
	MS + TDZ_1 BAP_5	+	
	MS + TDZ_5, BAP_5	+	
	MS + TDZ_{10}, BAP_5	+	+
	MS + TDZ_1 2,4-D_1		+
	MS + TDZ_5, 2,4-D_5		+
Semi floral	MS + $TDZ_{0.1}$ $BAP_{0.1}$		
	MS + TDZ_1 2,4-D_1	+	+
	MS + 2,4-D_5		+
	MS + 2,4-D_5 TDZ_1		+
	MS + 2,4-D_5 TDZ_5		+
	MS + 2,4-D_5 TDZ_{10}		+
Floral	MS + TDZ_5, 2,4-D_5		+
	MS + TDZ_5 BAP_5	+	
In vitro floral tips	MS + $TDZ_{0.5}$ $K_{0.1}$	+	
Immature zygotic embryos	MS + TDZ_1 BAP_1		
	MS + TDZ_1 BAP_5		
	MS + TDZ_1 2,4-D_1		
Mature zygotic embryos	MS		
	MS + $TDZ_{0.1}$ $BAP_{0.1}$		

Table 2.3 Media on which *Conospermum caeruleum* explants showed callus growth or differentiation (+) in culture. Subscripts represent concentration of growth regulators (μM). MS represents basal medium with no growth regulators.

Explant	Medium	Small shoots	Callus	Direct embryos	Embryogenic callus	Discard
Vegetative buds	MS					+
	MS + $TDZ_{0.5}$					+
	MS + TDZ_5					+
	MS + TDZ_1 BAP_5	+				+
	MS + TDZ_1 2,4-D_5					+
	MS + TDZ_5 BAP_5	+				+
	MS + TDZ_5 2,4-D_5		+			+
Stems	MS					+
	MS + $TDZ_{0.5}$					+
Immature zygotic embryos	MS					+
	MS + $TDZ_{0.1}$ $BAP_{0.1}$					+
Mature zygotic embryos	MS + $TDZ_{0.1}BAP_{0.1}$			+	+	+

Table 2.4 Media on which *Conospermum amoenum* explants showed callus growth or differentiation (+) in culture.

Explant	Medium	Shoots	Callus
Semi floral buds	MS + TDZ_2 2,4-D_1		
	MS + TDZ_2, 2,4-D_5		
	MS + TDZ_2 BAP_5		
Mature zygotic embryos	MS + $TDZ_{0.1}$ $BAP_{0.1}$	+	+

Table 2.5 Response of *Conospermum brownii, C. dorrienii* and *C. spectabile* mature zygotes in culture. (Week) refers to weeks after initiation into culture, (germinated) refers to embryos that germinated with a root and shoot, (direct embryogenesis) refers to embryos formed on the zygote, (embryogenic callus) is callus that was embryogenic, (callus) was hard and green. Note that although up to 50 zygotes were originally initiated, contaminated ones were excluded from this data. For each medium, 5 – 22 sterile explants were used.

		Number of zygotic embryos showing responses							
Species	**Media**	**Week**	**Germinated**	**Shoot**	**Direct embryogenesis**	**Embryogenic callus**	**Callus**	**Death**	**No response**
C. brownii	½MS	4				15			7
		8				10	3	8	1
		14				6	5	3	
C. dorrienii	½MS	6	1	5					3
		10	1	3		1		4	
		13	1	3				1	
	½ MS $TDZ_{0.1}BAP_{0.1}$	6						5	
	MS	6				4		7	
		10						4	
C. spectabile	½MS	6			2	1	4		2
		10			1		3	5	
		14				1	2	1	
	MS	6		1		2	1		7
		10		2		1	1	7	
		14		2			1	1	

2.4.2 *In vitro* leaves

Callus growth was observed on all media types. Medium containing 5 μM 2,4-D produced the most callus (Figure 2.4, 2.6) and overall had the best survival (Figure 2.5). There was no statistical difference between leaf region responses across all treatments (P >0.05), however growth regulator combinations did influence callus production overall (Figure 2.4). No organogenesis or embryogenesis was observed for any treatment. Many explants died within 6 weeks following some callus growth, such as on 10 μM TDZ, and 10 μM TDZ with 5 μM BAP (Figure 2.5, Figure 2.6D).

Figure 2.2. A. Mature *Conospermum caeruleum* fruit and embryo extracted from seed coat. Bar = 0.5 mm. B. Mature *C. eatoniae* fruit cut longitudinally. Bar = 1 mm.

Shoots growing *in vitro* reverted to the juvenile rosette form, but later produced soft inflorescence stalks. These were never observed to mature and flower *in vitro*

2.4.3 Effect of growth regulator pulsing and light

Hard green callus was observed on explants, to a greater extent when explants were cultured on 2,4-D rich medium. No organogenesis or embryogenesis was observed, and there was no difference between hormone concentrations. A large number of cultures were lost to contamination by bacteria and fungi, and to explant death over the 13 week period. More cultures remained alive when kept in the light but the high level of contamination made it impossible to show this was statistically significant (data not shown).

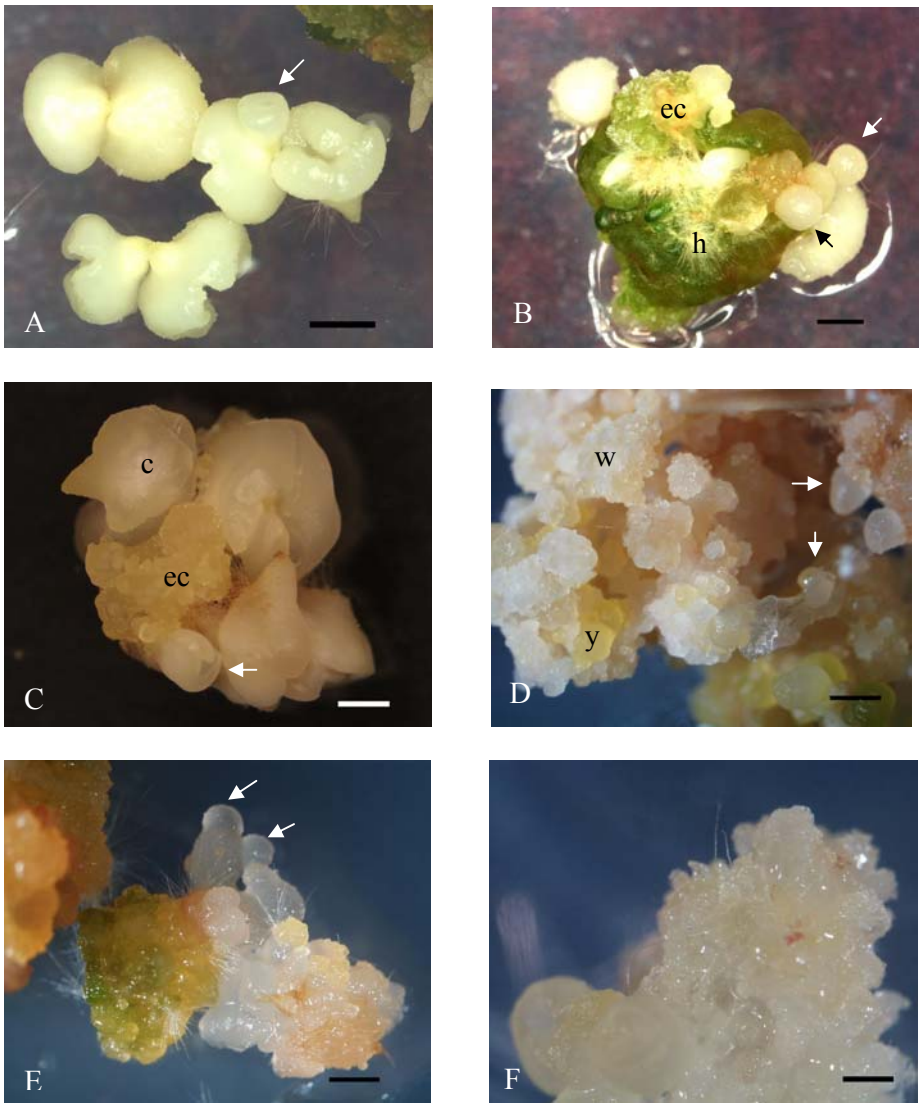

Figure 2.3 Somatic embryogenesis of *Conospermum caeruleum*. A. Mature cotyledonary embryos and a globular secondary embryo (arrow) on a root apex. B. Secondary embryogenesis on a swollen green embryo, showing globular stage embryos (arrows), embryogenic callus (ec) and root hairs (h). C. A cluster of embryos showing cotyledonary (c) and globular embryos (arrows) and embryogenic callus (ec). D. White (w) and yellow (y) embryogenic callus, with some globular embryos (arrows). E. Embryogenic callus with some early globular embryos (arrows) and root hairs, and greening of some callus. F. White embryogenic callus. Bars show 1 mm.

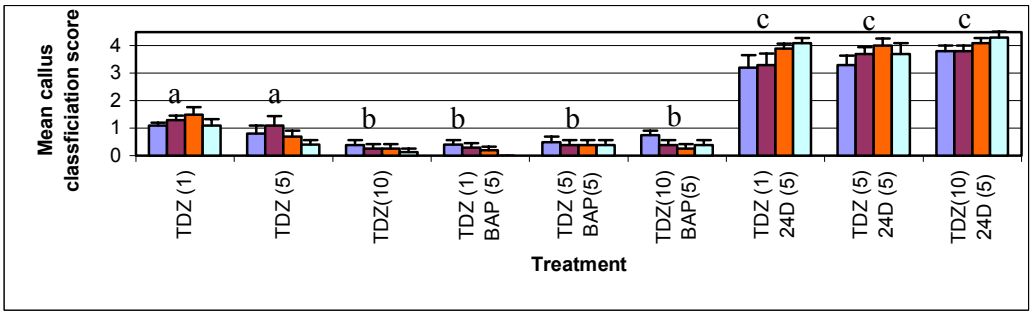

Figure 2.4 Mean callus score on *Conospermum eatoniae in vitro* leaf explants after 6 weeks in culture. Leaves were cut into 4 equal portions from tip (1) to petiole (4). Leaf segment 1. (■). Segment 2. (■). Segment 3. (■). Segment 4. (□). The scoring system for callus is described in Table 2.1. MS media was supplemented with combinations of TDZ, BAP and 2,4-D. Concentrations (µM) are shown in parentheses. There was no difference in response according to leaf position. Media with the same letters are not significantly different (P >0.05). Bars show standard error.

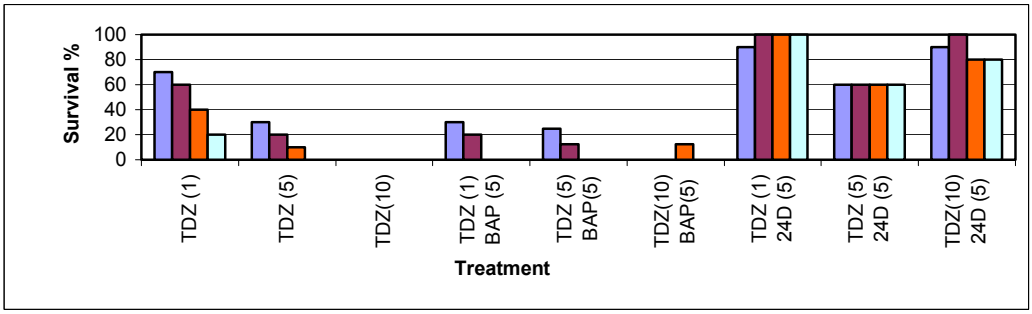

Figure 2.5 Percentage survival of *Conospermum eatoniae in vitro* leaf explants following 6 weeks of culture on MS medium with a range of growth regulators. Leaves were cut into 4 equal portions from tip (1) to petiole (4). Leaf segment 1. (■). Segment 2. (■). Segment 3. (■). Segment 4. (□).Concentrations (µM) are shown in parentheses. Media were supplemented with combinations of TDZ, BAP and 2,4-D.

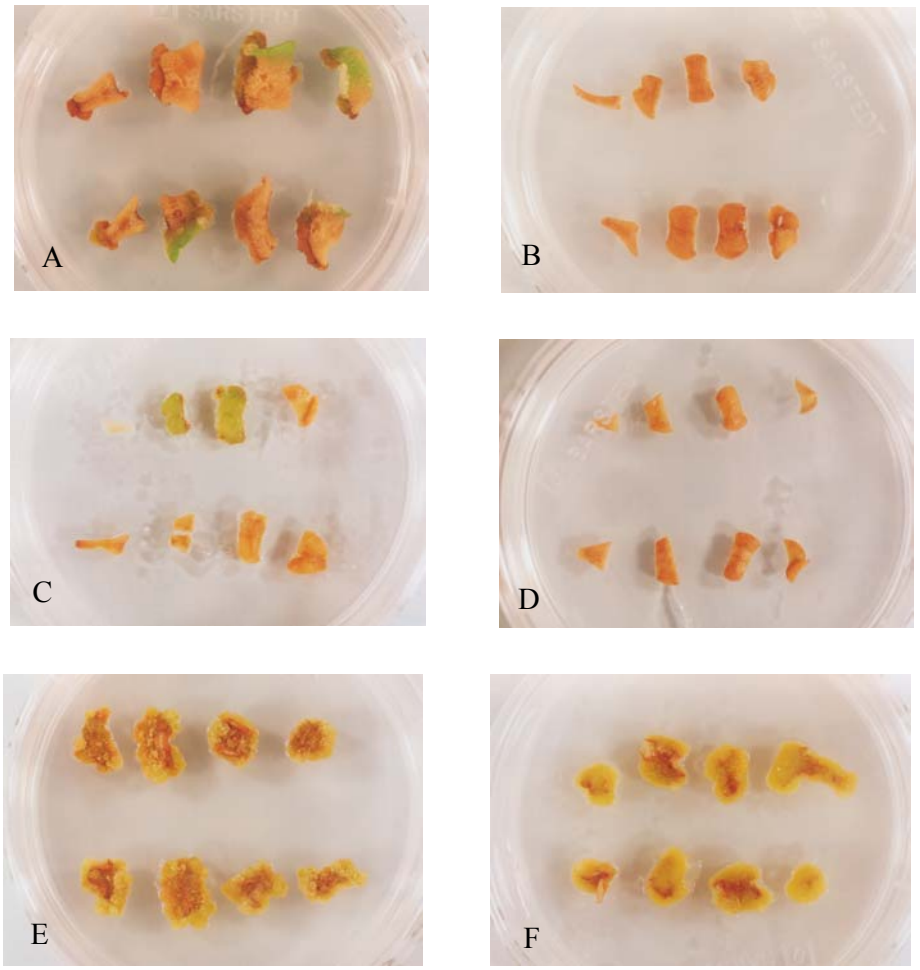

Figure 2.6 Response of *Conospermum eatoniae* leaf pieces *in* vitro: The effect of growth regulators and leaf explant position. Leaves were cut into 4 pieces tip to petiole labelled 1-4 (right to left). MS medium was supplemented with: **A**. TDZ 1 µM. **B**. TDZ 10 µM. **C**. TDZ 1 µM BAP 5 µM. **D**. TDZ 10 µM BAP 5 µM. **E**. TDZ 1 µM 2,4-D 5 µM. **F**. TDZ 10 µM 2,4-D 5 µM. Photos were taken after 6 weeks in culture. Diameter of Petri dishes is 55 mm.

2.5 DISCUSSION

On the wide range of explants and growth regulators tested for several *Conospermum* species, the only evidence for direct or indirect embryogenesis was on mature zygotic embryos.

Direct embryogenesis occurs from cells that are pre-programmed for embryogenesis. Indirect embryogenesis requires reprogramming of cells for the embryogenesis developmental pathway to be induced (Sharp *et al.* 1980). Generally, cells that are closest developmentally and physically to the zygotic embryo are more easily able to produce somatic embryos (Carman 1990). It is believed that the ability of cells to express embryogenesis is restricted to a small developmental window, which was evident for *C. caeruleum* zygotic embryos. Immature zygotic embryos collected at the beginning of November were not able to produce somatic embryos, however somatic embryos were produced on zygotic embryos collected just two weeks later. This is in contrast to some species where the immature zygotic embryo is more likely to express somatic embryogenesis (Canedo Ortiz *et al.* 2000). It was suggested by Carman (1990) that the response of a zygotic embryo in culture depends on the developmental signals the embryo was receiving at the time of the somatic embryogenesis induction signal. He concluded that immature zygotic embryos that were still in an embryogenic pathway would be more likely to produce somatic embryos, whilst more mature zygotic embryos that were entering germination pathways would be more difficult to induce embryogenesis once again. The presence of synthetic auxin disrupts developmental pathways, and may have induced embryogenesis in the mature zygotes of *C. caeruleum*.

Contamination was a substantial problem for many of the experiments, reducing replicate numbers significantly. This was particularly the case for vegetative *C. eatoniae* buds, *C. caeruleum* semi-floral buds, stems and zygotic embryos for all species. From Figure 2.1, it is obvious that the hairy surface of the buds, and tight clustering in their vegetative state made sterilisation difficult. Air bubbles trapped by the hairs and beneath the small buds protected fungi and bacteria, despite brushing during treatment with calcium hypochlorite. Increasing the sterilisation time in calcium hypochlorite did not reduce contamination (data not shown), nor did placing explants in sterilising solution in a syringe to create a vacuum to remove the air. The two-phase sterilisation of the explants has been shown to be most effective (Seaton, pers. comm.). In future experiments, the number of explants initiated into culture should allow for up to 50 % contamination. The use of mercuric chloride rather than sodium hypochlorite may reduce the level of contamination, but the mercuric compound is environmentally undesirable.

The majority of zygotic embryos, whether immature or mature, were contaminated when cultured. The seeds are approximately 3 mm in length (Figure 2.2), and also have hairs on the upper surface. The seed coat was removed from the embryo but this often resulted in some damage to the embryo. When the seed was smaller (2 mm), the seed coat was cut and a section removed to enable the embryo access to the culture medium, to reduce damage inflicted. Soaking the seeds in distilled water did help to soften the seed coat, but many of the embryos were still damaged.

Floral explants have been successful sources of somatic embryos in woody species (Li *et al.* 1998aa). For *Conospermum*, buds are available for many months of the year in

various stages, from small vegetative buds through to flower buds on fully expanded flowering inflorescences. It was hoped that a "developmental window" for induction of somatic embryogenesis would be found for one or more of these floral stages in *Conospermum*. Instead, it was noted that vegetative buds were more likely to give rise to shoots, while semi-floral buds developed callus. The majority of fully floral buds initiated into culture died, however some did produce shoots on BAP, and callus on 2,4-D. Although floral explants did not produce somatic embryos, there appears to be a pattern of *in vitro* response in relation to developmental stage of the inflorescence.

Stem and leaf explants, have also been used as explants for somatic embryogenesis in other species (Maataoui *et al.* 1990; Pedroso and Pais 1993). Many *Conospermum* species have leaves only on juvenile plants; mature plants are made up of leafless stems bearing multiple inflorescences. The production of *in vitro* shoot cultures for these species means that leaves are available year-round. No organogenesis or embryogenesis was observed for *Conospermum* leaf explants using a wide range of growth regulator treatments. Compact callus was observed on all leaf explants, beginning on the cut edges and moving to the centre. The influence of growth regulators was significant, with the presence of 2,4-D necessary for high callus production and survival of the explant. Callus colour has been associated with embryogenic capacity where green, yellow, white, brown and red *Gossypium hirsutum* L. callus were observed, but only yellow callus produced somatic embryos (Finer 1988). For *Conospermum*, callus formed on leaf explants was uniformly hard and green and showed no embryogenic response. On zygotic embryos however, white-yellow embryogenic callus formed, and for *C. caeruleum* somatic embryos

developed (Chapter 4). Callus that formed on zygotic embryos that was not embryogenic was also white, but was hard and ultimately died in culture.

The choice of growth regulator combinations and concentrations is determined by the primary explant type, and can significantly influence the *in vitro* response of explants. Somatic embryogenesis has been induced on several angiosperm dicot zygotic embryo and floral explants on a wide range of growth regulators and combinations. Of the studies surveyed by Raemakers *et al.* (1995), there was an almost even spread of auxin-supplemented media, cytokinin-supplemented media, combinations of the two, and growth regulator-free media used successfully. In contrast, vegetative explants predominantly required auxin-cytokinin supplemented media or auxin only. For *Conospermum*, somatic embryos formed on zygotic embryos on regulator free (*C. brownii, C. spectabile*) and on an auxin-cytokinin supplemented media (*C. caeruleum*) (Table 2.3, 2.5).

A wide range of auxin and cytokinin supplemented media was tested on the many explants in the experiments presented here. Thidiazuron has cytokinin-like properties, and has been found to be very effective at inducing somatic embryogenesis in woody species in recent years (Li *et al.* 1998aa; Bates *et al.* 1992; Gill and Saxena 1992; Huetteman and Preece 1993), but was unsuccessful on all types of *Conospermum* with the exception of *C. caeruleum* zygotic embryos. Auxin alone, and in combination with cytokinin was also tested, as the presence of auxin initially has also been shown to induce somatic embryogenesis in some species.

These results suggest that for *Conospermum,* somatic embryogenesis would be difficult to achieve on explant material that was not zygotic. Vegetative and floral explants produced callus and preformed buds elongated into shoots. There are two main disadvantages of using zygotic embryos as the primary explant for producing somatic embryos: the zygote is of unknown genotype, and source material is only available for a limited time each year. In a breeding program, genotypes are selected for their desirable attributes, and maintaining a homogeneous line is important for commercialisation. The difficulty overcoming contamination from field-grown explants impacted significantly on many of the experiments, and for species like *Conospermum* with hairy stems, leaves and flowers, is an important factor to consider. For these reasons, it was considered worthwhile to pursue testing *in vitro* leaves further as they are sterile, clonal, and available all year round (Chapter 3). The response of zygotic embryos in culture will also be considered further in Chapter 4.

CHAPTER 3

PLANT GROWTH REGULATORS FOR THE INDUCTION OF EMBRYOGENESIS FROM *CONOSPERMUM EATONIAE* LEAF TISSUE

3.1 ABSTRACT

Conospermum eatoniae E. Pritz. (Proteaceae) has valuable horticultural attributes but has proved difficult to propagate. A method for clonal production of plants via somatic embryogenesis may overcome these problems. A wide range of auxin/cytokinin combinations and concentrations were tested on *C. eatoniae in vitro* leaf explants to induce somatic embryos. Leaf explants were first induced to form uniform green callus on a Murashige and Skoog medium supplemented with TDZ 5 μM and 2,4-D 5 μM, before being placed on media with 42 combinations of a number of auxins, cytokinins and gibberellins. A single shoot formed on only one explant on medium with TDZ 1 μM and IBA 1 μM. The remainder died or continued to grow as callus. Friable callus formed on 2 – 44 % of explants on 18 of the growth regulator combinations, the best combination was TDZ 1 μM, 2,4-D 0.1 μM. These results indicate that callus formed from *in vitro* leaf tissue is not suitable for organogenesis or embryogenesis in this species.

3.2 INTRODUCTION

There are no generalised methods for inducing somatic embryogenesis in plants. Each species and even individual genotypes can require varied growth regulator combinations and concentrations, culture lengths and explant sources for somatic embryos to be successfully cultured (Raemakers *et al.* 1995; Pinto *et al.* 2002).

Many dicot species mainly produce somatic embryos from zygotic embryo explants and in fewer species it is possible to induce somatic embryos from vegetative explants (Raemakers *et al.* 1995). Leaves have successfully been used to produce direct somatic embryos in woody species such as *Camellia japonica* L. (Pedroso and Pais 1993) and in *Arachis hypogaea* L. (Gill and Saxena 1992). Previous studies had failed to induce direct embryogenesis from leaf tissue in *Conospermum* (Chapter 2).

The aim of this experiment was to determine the growth regulators and their optimum concentrations for organogenesis and particularly embryogenesis from *C. eatoniae* leaf explants. Embryogenesis is frequently easier to induce from juvenile tissues (Raemakers *et al.* 1995) and explants from *in vitro* culture have the advantage that they do not have the stress of surface sterilisation at the time of induction into culture. *C. eatoniae* begins growth as a rosette of basal juvenile leaves before producing glabrous flowering stems with dichotomous branching (Bennett 1995). Leaves chosen for trials were growing as rosettes from plants *in vitro*. These satisfied the requirement of leaves in the juvenile stage, and not requiring further surface sterilisation before use.

3.3 MATERIALS AND METHODS

3.3.1 Plant material

Leaves were used from an *in vitro C. eatoniae* shoot culture of a single clone selected for its excellent horticultural attributes. This culture line originated from vegetative buds taken from the field and induced to produce shoots. Shoot cultures were maintained on MS medium with MS salts and vitamins, 20 gL^{-1} sucrose, 8 gL^{-1} agar and IBA 0.1 μM and BAP 0.3 μM. The pH of medium was adjusted to 7.0 before

autoclaving. Cultures were maintained at 24 ± 2°C under a 16 h photoperiod (23-37 $\mu molm^{-2}\ s^{-1}$) of cool white light and subcultured monthly.

3.3.2 Callus induction

Previous experiments had revealed that a medium with MS salts and vitamins, 20 gL^{-1} sucrose and 8 gL^{-1} agar, pH 5.8, supplemented with TDZ 5 μM and 2,4-D 5 μM produced healthy green callus from leaf explants. This medium was used to induce callus using 10 mls in 55 mm Petri dishes. Once a relatively uniform callus had been produced, various auxins, cytokinins and giberellic acid (GA_3) were tested for their potential to induce regeneration. Fully expanded leaves were cut into three to four equal portions and placed on the media abaxial side down (Figure 3.1B). After four weeks cultures were transferred to the experimental media (Table 3.1). All cultures were incubated under a 16 h photoperiod of cool white light (23 - 37 $\mu molm^{-2}\ s^{-1}$) at 24 ± 2°C.

3.3.3 Media tested for induction of differentiation from callus

Experimental media consisted of MS salts and vitamins, 20 gL^{-1} sucrose, 8 gL^{-1} agar and various auxins, cytokinins and GA_3 (Table 3.1). The media were adjusted to pH 5.8 prior to autoclaving and 10 mls poured into 55 mm Petri dishes. Four replicates of each medium were tested, with four- five callus explants per dish (Figure 3.1B).

Cultures were maintained in conditions described above and subcultured monthly for four months, before explant response to the medium was recorded. After four months, callus pieces were transferred intact to MS supplemented IBA 0.5 μM, BAP 0.5 μM medium pH 5.8 and observations were made after a further month.

3.4 RESULTS

Explants were classified as friable callus, non-friable callus, or dead (Figure 3.1C). There was a high rate of explant death for all treatments, but most explants formed compact, nonfriable callus. The highest percentage of explants that became friable was on media with 0.1 μM 2,4-D with TDZ or IBA at 1 μM. The final four-week incubation on the medium with IBA and BAP did not induce roots or shoots; there were no observable changes in the explants. With the exception of one shoot on a piece of callus on medium with TDZ 1 μM and IBA 1 μM, there was no sign of shoots, roots or embryoids.

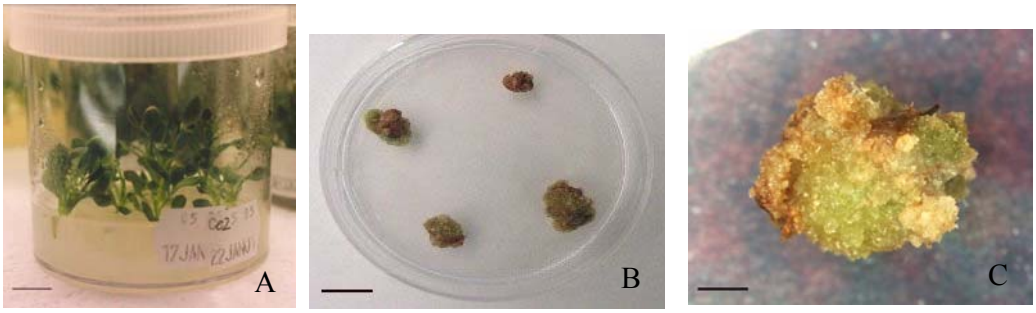

Figure 3.1 *Conospermum eatoniae in vitro.* A: shoot cultures on MS with 0.3 μM BAP and 0.1 μM IBA. Bar = 10mm. B: leaf explants forming callus on MS with 5 μM 2,4-D and 5 μM TDZ after 8 weeks. Bar = 10 mm. C: Callus formed on leaf explant on MS with 5 μM 2,4-D and 5 μM TDZ after 8 weeks. Note hard green callus and yellow friable callus. Bar = 1 mm.

Table 3.1 Percentage of *Conospermum eatoniae* explants with friable callus (F), nonfriable callus (NF) or those dead (D) for each experimental medium after four 1-monthly subcultures.
*A single shoot formed on an explant on TDZ 1 μM IBA 1 μM.

				TDZ						2,4-D						K			
Growth		0.1μM				1μM		0.1μM				1μM		0.1μM				1μM	
Regulators	μM	F	NF	D	F	NF	D	F	NF	D	F	NF	D	F	N	D	F	NF	D
	0													0	12	88			
TDZ	1							44	24	32	0	0	100	0	4	92	4	8	88
BAP	1	20	12	68	12	16	72										4	0	96
2,4-D	1	4	28	68										0	20	80	4	36	60
K	1	0	12	88	4	20	76	0	0	100	12	28	60						
NAA	1	0	8	92	14	8	78	16	28	56	2	2	92	8	12	80	0	0	100
IBA	1	24	8	68	4	12	84	32	4	64	0	16	84	12	16	72	4	6	90
GA3	1	8	8	84	8	12	80	14	8	78	12	12	76	0	0	100	4	12	84

3.5 DISCUSSION

Callus formation on leaf explants was readily inducible. Although friable callus was observed on several growth regulator combinations in up to 44 % of explants (Table 3.1), no signs of embryogenic capacity were observed. In other species such as *Gossypium hirsutum* L., friable yellow callus has been induced to produce embryos (Finer 1988). The lack of organogenesis in the *Conospermum* cultures suggests the cells were not competent at changing developmental pathways or that callus production was the dominant process.

The callus induction medium contained 2,4-D and TDZ, growth regulators that have been shown to induce embryogenesis in other species. For example, Anzidei *et al.* (2000) showed that the presence of 2,4-D produced an embryogenic secondary callus from primary non-embryogenic callus in *Foeniculum vulgare* Miller over a 12-month period. Subsequently embryos were produced at high frequency in the absence of 2,4-D. For woody species in particular, TDZ has been reported to be a potent cytokinin–like growth regulator for stimulating shoot proliferation at low

concentrations (<1 μM), and callus formation at higher concentrations (1-50 μM) (Huetteman and Preece 1993). Rugkhla and Jones (1998) also induced direct somatic embryos in *Santalum album,* on medium containing TDZ, and indirect somatic embryos on medium containing TDZ and 2,4-D. It was expected that at least roots and/or shoots would form on the *C. eatoniae* callus given the wide range of growth regulators tested. The lack of virtually any organogenesis was surprising. The shoot that did develop on media supplemented with 1 μM TDZ and 1 μM IBA did not appear vigorous, and its production was not considered indicative of an appropriate medium for shoot initiation. The *in vitro* leaves used as explants were healthy and green, but perhaps younger seedling leaves may show more response. In other species, callus has remained in culture for up to 12 months before changing to an embryogenic state, however a relatively high proportion of the *Conospermum* callus was dying at each subculture indicating that culture media or conditions were sub optimal, and it is unlikely that prolonging the period of subculture would have resulted in successful organogenesis.

It is believed the memory that cells have of previous developmental pathways can be erased with several cell divisions on media that enables dedifferentiation, particularly media with 2,4-D (Carman 1990). The effectiveness of this genetic reprogramming influences how competent the dedifferentiated cells are at changing pathways to somatic embryogenesis, for example. Vegetative leaf tissue is not developmentally close to embryogenesis in space or time, but it was thought possible that the presence of 2,4-D would enable dedifferentiation of *Conospermum* leaf tissue, and loss of "cell memory," so that a new embryogenic pathway could be induced.

In many other woody species, vegetative tissues, whether from a plant *in vivo* or *in vitro,* have proved recalcitrant for organogenesis or embryogenesis, and it is possible that the combinations of plant growth regulators in culture have been inadequate to completely reprogram the genetic material.

The other significant factor that influences embryogenic capacity is genotype. The *in vitro* leaves used in this experiment all originated from a shoot culture of a single selection of *C. eatoniae* that shows desirable horticultural attributes. The inability of selected genotypes to respond to *in vitro* culture is a widespread problem for plant breeders, however Henry *et al.* (1994) noted that plant selection should be based on agronomic importance and not on *in vitro* regeneration ability. The aim of these experiments was to develop efficient propagation methods for pre-selected lines of *Conospermum* spp., so recalcitrance attributed to genotype is a problem that cannot be avoided.

The approach taken here was to screen a single genotype with a wide range of growth regulator combinations and concentrations to identify those that may induce an embryogenic response in the explant. The alternative approach, which may prove to be more appropriate for *Conospermum*, is to screen several genotypes of horticultural value with a limited number of growth regulator combinations. This would determine how strongly the genotype of *Conospermum* influences its response in culture, the results of which would be beneficial for planning future experiments. However, the problem of contamination of explants initiated into culture must be overcome first. The results here further confirm that the zygote is the only appropriate explant for inducing somatic embryogenesis in *Conospermum* spp., as was shown in Chapter 2.

CHAPTER 4

MATURATION AND GERMINATION OF *CONOSPERMUM* SOMATIC EMBRYOS

4.1 ABSTRACT

Somatic embryogenesis was successfully induced from a zygotic embryo of *Conospermum caeruleum*, and cultures were maintained for up to 12 months. Attempts to synchronise embryo development and induce maturation pathways were unsuccessful, as cultures continued to produce secondary embryos from root, hypocotyl and cotyledonary regions of somatic embryos. Treatments with ABA, mannitol, desiccation and cold did not induce germination and conversion to plants.

4.2 INTRODUCTION

Somatic embryogenesis has been recorded for species across many genera and from a variety of plant tissues. However, conversion of somatic embryos into plants can be difficult to achieve, and is a significant problem faced by propagators. One difference between somatic and zygotic embryos is, in growth medium somatic embryos continue to grow without the period of dormancy that is usual for zygotic embryos. Many efforts have been made to simulate the conditions that result in a period of dormancy, in attempts to synchronise embryos and to obtain higher levels of germination and conversion to plants. Abscisic acid (ABA) is often used to increase conversion and maturity of somatic embryos and prevent precocious germination (Torres *et al.*, 2001). ABA accumulates during zygotic embryo maturation, and is thought to be involved in synthesis of storage proteins, desiccation tolerance and in controlling dormancy. For germination to occur, endogenous ABA levels need to be

reduced. Gibberellin (GA) stimulates the genes required for growth and utilisation of stored reserves, and is known to increase germination. Endogenous levels of GA increase as ABA levels decrease during chilling (Pearce *et al.*, 1987).

Desiccation and chilling of somatic embryos are also techniques used to improve germination efficiency, often in conjunction with each other. For some species, a cold treatment (2 - 4 °C) of 2 - 4 months increases germination (Corredoira *et al.*, 2003; Tulecke and McGranahan, 1985). Desiccation, and/or cold treatment, has improved germination in species such as *Picea glauca* (Pond *et al.*, 2002), *Juglans regia* L. (Tang *et al.*, 2000) and *Gossypium hirsutum* L.(Chaudhary *et al.*, 2003), although severe desiccation can result in injury of embryos.

The inclusion of a plasmolysing osmoticant like mannitol in the culture medium, increases the osmotic potential. It has been shown to improve shoot regeneration ability of embryogenic cultures (Emons *et al.*, 1993) and aid in synchronisation of embryo development.

Somatic embryos of *C. caeruleum* remained highly embryogenic in culture over a period of 12 months, though conversion of these embryos into plantlets rarely occurred (Chapter 2). Embryogenic cultures contained embryos of all developmental stages with the exception of the torpedo stage, from pre-embryonic calli, to mature cotyledonary embryos. Maturation and germination of embryos was uncommon on the culture maintenance medium. This chapter reports a number of experiments to achieve efficient conversion to plantlets.

4.3 MATERIALS AND METHODS

4.3.1 Somatic embryo origins

All embryogenic cultures used in these experiments originated from a single mature *C. caeruleum* zygotic embryo that became embryogenic on MS medium supplemented with 8 % agar and 2 % sucrose, 0.1 μM TDZ and 0.1 μM 2,4-D, pH 5.8. Somatic embryos were maintained in culture for up to 12 months on this and/ or half- strength MS media prior to use in some experiments.

4.3.2 Influence of growth regulators on maturation and germination of embryos

Whole, discrete embryos (late heart to cotyledonary stage) were cultured on 10 mls MS supplemented with a range of growth regulators on 55 mm Petri dishes, 4 embryos per plate, 2 replicate plates per medium. pH was adjusted to 5.8 prior to autoclaving. Embryos were assessed after 4 - 6 weeks.

4.3.3 Synchronising embryo development

Embryogenic clusters were cultured on half-strength MS medium with 8 % agar, and either 2 % sucrose, 2 % sucrose and 3 % mannitol or 3 % mannitol, and adjusted to pH 5.8 prior to autoclaving. Then, 0, 0.1, 1 or 5 μM filter-sterilised ABA was added. Three replicate 55 mm Petri dishes with 5 embryogenic clusters were cultured for each medium type. Clusters contained mostly globular embryos, with some heart and cotyledonary embryos that had secondary embryos emerging from them. Cultures were assessed after 1 month, then subcultured onto MS with 1 μM GA_3 and 1 μM BAP at pH 5.8. Cultures were scored after a further 8 weeks on this medium.

To test any effect of darkness and low temperatures on embryo development, embryogenic clusters were cultured on half-strength MS medium with 2 % sucrose, 8 % agar, pH 5.8. Three replicate 55 mm Petri dishes with 5 clusters per dish were cultured in either: darkness at 24 ± 2 °C for 1 or 2 weeks then in a 16 h light photoperiod (23-37 $\mu molm^{-2} s^{-1}$) at 24 ± 2 °C, or at 4 °C for 3 days in light then 24 ± 2 °C, or 2 weeks in light at 24 ± 2 °C. In addition, embryogenic clusters were desiccated in a Lamina Air Flow Cabinet for 3 hours before being subcultured onto half-strength basal MS medium as described above, and placed in a 16 h light photoperiod (23 - 37 $\mu molm^{-2} s^{-1}$) at 24 ± 2 °C. Cultures were assessed after 6 weeks. Embryo clusters that had not been desiccated were also cultured as a control.

A further experiment testing pH, temperature and desiccation also used half-strength MS as described above, at pH of 5.8 or 7.0. Small embryogenic clusters were desiccated for 3 hours in a Lamina Air Flow Cabinet, and placed on media of different pH at 4 °C or 24 °C, 4 clusters per 55 mm Petri dish, 2 replicate dishes. Embryos were assessed after 6 weeks.

4.4 RESULTS

4.4.1 Influence of growth regulators on maturation and germination of somatic embryos

After 4 - 6 weeks on a wide range of plant growth regulator combinations, the most common response was for somatic embryos to continue to proliferate (Table 4.1). For those with more than 50 embryos, a score of >50 was given as counting more than this number was not accurate. The highest number of embryos formed on basal MS medium, and the least on media supplemented with GA_3 (Table 4.1), although an

analysis of variance (ANOVA) showed there to be no significant difference between treatments ($F_{(12,48)} = 0.7005$, $P = 0.743$) or between embryos within treatments ($F_{(39,48)} = 0.4358$, $P = 0.9957$) Embryos were mostly cotyledonary. Media containing GA_3 however produced the most roots and root hairs, and swollen shoot apices. Embryogenic callus formed on all growth regulators used, except in the presence of low levels of NAA.

Table 4.1 Response of *Conospermum caeruleum* somatic embryos on MS media supplemented with a range of growth regulators. Table shows mean number of embryos per initial single embryo, and total number of shoots, roots, clusters with root hairs (Hairs), swollen or green shoot apices and embryogenic callus for each treatment. There were initially four embryos per plate and two replicate plates.

Media	Mean number of embryos	Shoots	Roots	Hairs	Swollen/green shoot apex	Embryogenic callus
MS	46.25					2
2,4-D 0.1μM	32.25		1			1
2,4-D 0.1μM BAP 0.1μM	34.63					1
2,4-D 1μM BAP 0.1μM	27					2
2,4-D 1μM BAP 1μM*	35.08					
NAA 0.1μM	28.75				2	
NAA 0.1μM BAP 0.1μM*	34.63					
NAA 1μM BAP 0.1μM	25	1			1	
NAA 1μM BAP 1μM	27.21					4
GA_3 0.1μM	23.5				2	2
GA_3 0.1μM BAP 0.1μM	8.38		1	4	3	1
GA_3 1μM BAP 0.1μM	19.75		1	2	4	1

* embryos showed no response on these media

4.4.2 Synchronising embryo development

Culture media designed to induce embryo dormancy, using sucrose, mannitol or ABA, were ineffective. After 1 month in culture, embryogenic clusters on sucrose and sucrose/mannitol continued to proliferate. A single root was observed on media with 5 μM ABA on sucrose and mannitol, and a single shoot was observed on media with sucrose and no growth regulators. Clusters on media with mannitol and no sucrose

were unchanged in cluster size, and many had browned. All embryos were then transferred to medium with 1 μM GA_3 and 1 μM BAP, for 8 weeks (Table 4.2). Cultures remained embryogenic, and those on mannitol regained embryogenic capacity. Roots were observed on embryos on media with sucrose and sucrose/mannitol (Table 4.2). Shoots were produced on basal MS and 5 μM ABA with sucrose, and 1 μM ABA with mannitol. Shoots and roots all formed at low frequency, but root hairs were common across all media types. Swollen cotyledons were more common on media containing sucrose and sucrose/mannitol.

Table 4.2 Response of *Conospermum caeruleum* somatic embryos on MS media containing sucrose and/or mannitol, and varying concentrations of ABA. Embryos were treated for four weeks on these media, then transferred to 1 μM GA_3 with 1 μM BAP for a further eight weeks before results were recorded. The table shows the percentage of embryo clusters that were still embryogenic, and the total number of embryo clusters with shoots, roots, root hairs, swollen shoot apices or green (non-embryogenic) callus.

Media	Embryogenic (%)	Green callus	Shoots	Roots	Root hairs	Swollen cotyledon
Sucrose 2%						
MS	100		1	5	14	7
0.1μM ABA	100				13	10
1μM ABA	100			1	16	13
5μM ABA	100		2	1	17	9
Sucrose 2%/Mannitol 3%						
MS	100				11	9
0.1μM ABA	100				9	5
1μM ABA	100			1	15	12
5μM ABA	100			1	14	7
Mannitol 3%						
MS	91.67				9	4
0.1μM ABA	100				13	4
1μM ABA	83.33	1	3		14	7
5μM ABA	100				12	3

Cultures maintained in the dark on ½ MS for 7 - 14 days showed no difference in response to those in the light, with shoots forming in clumps on the embryogenic clusters. Roots formed on two embryos cultured in the dark for 7 days, and on one

cultured for 14 days in the dark. Secondary embryos formed on these roots. (Figure 4.1). Cultures held at 4 °C for 3 days then returned to 24 °C produced a few shoots, and embryogenic callus also formed. Cultures remained embryogenic.

Desiccation of embryo clusters did not result in germination of embryos. One embryo germinated in the control treatment (Figure 4.2 A). Both the control and desiccated cultures showed multiple shoot growth from the clusters, but no root formation (Figure 4.2 B, C). Embryogenic callus formed in both treatments, and some browning was evident for both also (Figure 4.2 D, E).

Results for the experiment combining change in pH, desiccation and change in temperature were inconclusive, and did not show a particular treatment as being superior for embryo germination (Table 4.3). Cultures remained highly embryogenic across all treatments, and embryogenic callus was present in all cultures. No shoots or roots formed, but root hairs were common.

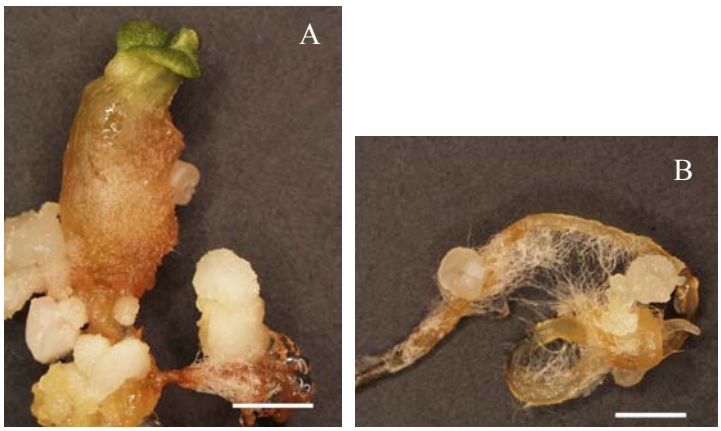

Figure 4.1 A. *Conospermum caeruleum* embryo that germinated with a cotyledon and root. Embryos formed on the root. B. Embryos on the root were at globular and cotyledonary stages. Bars show 1 mm.

Table 4.3 Influence of pH, temperature and desiccation on *Conospermum caeruleum* somatic embryo germination. Note embryogenic callus was observed on all treatments, and could be separated into four colour categories: white, yellow, deep yellow and brown. The table shows the percentage of cultures that were embryogenic and the percentage of each embryogenic callus colour. The number of embryo clusters with swollen cotyledons, root hairs and green (non-embryogenic) callus is also shown.

Treatment	Embryogenic	% Embryogenic callus				Swollen	Root	Green
	%	White	Yellow	Deep yellow	Brown	Cotyledon	Hairs	Callus
pH 5.8, 24 °C	79	50	33	0	17	4	5	0
pH 5.8, 24 °C desic	100	43	43	0	13	8	6	4
pH 5.8, 4 °C	92	23	40	13	23	1	12	5
pH 5.8, 4 °C desic	92	33	33	0	30	1	13	4
pH 7, 24 °C	100	47	37	7	10	1	11	6
pH 7, 24 °C desic	96	33	33	7	27	5	13	3
pH 7, 4 °C	79	33	43	0	23	7	11	2
pH 7, 4 °C desic	71	43	17	0	40	3	1	0

4.5 DISCUSSION

Gibberellin significantly reduced the number of secondary late heart – cotyledonary stage embryos that formed in comparison to cultures on NAA and 2,4-D, in the presence of BAP. Of the embryos that did form, more had a swollen or green hypocotyl in the presence of GA_3 than the other regulators tested, and roots and root hairs were more likely to form. Exogenous gibberellin can assist in germination of embryos by stimulating the genes required to utilise stored reserves and breaking their dormancy cycle, and can stimulate root outgrowth as in *Zea mays* L. (Emons *et al.*, 1993). Root formation for *C. caeruleum* was uncommon, but frequent presence of fine root hairs may indicate that GA could be effective in inducing further development of the radicle.

Some treatments successful in other species attempt to mimic the developmental cycle of the zygotic embryo, and include for example, a desiccation step prior to

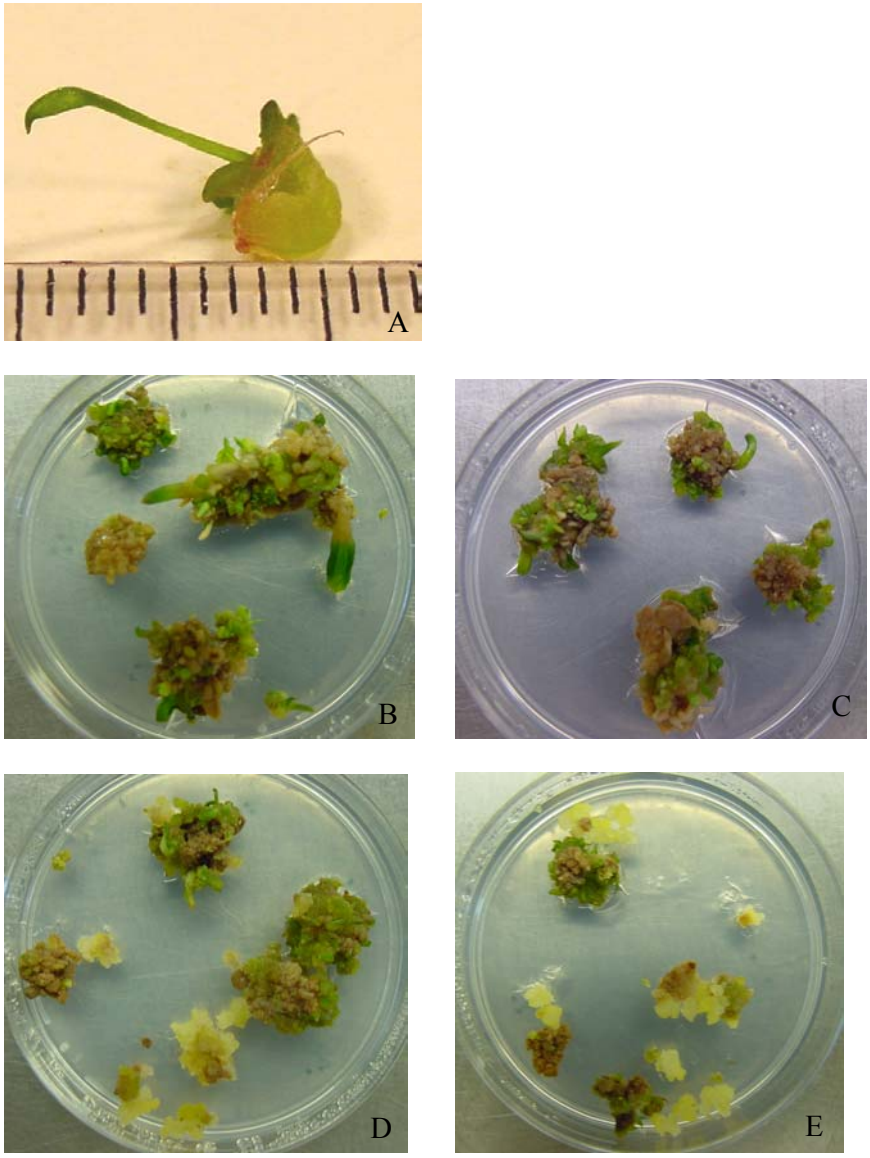

Figure 4.2 Response of *Conospermum caeruleum* embryos to desiccation. A. Germination of an embryo on control treatment of half-strength MS at 24 °C. Embryogenic callus formed on both the control (B) and desiccated plates (C). C. Shoot formation was similar for control (D) and desiccated plates (E). Plates were 55 mm diameter.

germination. Inducing hypo-hydricity in somatic embryos attempts to end embryogenic pathways and induce germination pathways.

Desiccation of *C. caeruleum* embryos did not increase germination. The presence of mannitol in the medium did reduce embryogenesis, but presence of both sucrose and mannitol did not. Subculture onto media with BAP and GA_3 with sucrose resulted in resumption of embryogenic ability for those cultures previously on mannitol only. Mannitol has been used successfully to improve embryo differentiation and maturation in species such as *Ipomoea batatas* Lam. and *Apium graveolens* (Nadel *et al.*, 2001; Torres *et al.*, 2001), but was unsuccessful in other species such as *Glycine max* (L.) Merr. (Walker and Parrott, 2001).

Abscisic acid was used successfully to improve number and synchrony of *Ipomoea batatas* Lam. embryos (Torres *et al.*, 2001). ABA induces desiccation tolerance and storage of reserves such as starch, sucrose, proteins and lipids. This is important for embryo germination, and for survival of encapsulated embryos that are first desiccated to increase conversion rates to whole plants, such as for *Saccharaum* sp. (Nieves *et al.*, 2001). For *C. caeruleum*, roots formed without addition of ABA, and on 1 - 5 μM ABA in the presence of sucrose and a combination of sucrose and mannitol, and shoots formed on 0 - 5 μM ABA with sucrose or mannitol. Overall germination was not improved.

Further experiments are required to establish a reliable system for conversion of *C. caeruleum* embryos to plants. A lack of root growth has previously been a problem in organogenic cultures (Seaton and Webb, 1997) and continues to be problematic here.

Future experiments may investigate the effect of maltose on *C. caeruleum* somatic embryos, as some reports have shown improved embryo development in woody species such as *Pinus taeda* L. (Li *et al.*, 1998b) and *Castanea sativa* Mill. (Corredoira *et al.*, 2003) and *Hevea brasiliensis* (Blanc *et al.*, 1999). How maltose improves germination is unclear, but suggestions of nutrition, benefit of slow hydrolysis or action as a signal molecule have been suggested (Blanc *et al.*, 1999).

Once embryogenesis is established in *C. caeruleum*, it appears difficult to alter developmental pathways to those of germination. This observation needs to be confirmed over a wider range of genotypes to rule out genotypic barriers to germination. Secondary embryogenesis from the cotyledon was common, and also occurred from hypocotyls and roots. This proliferation of embryos prevented normal germination of embryos. Further studies could measure storage protein accumulation after desiccation or cold treatments and manipulation of the gaseous atmosphere and spectral quality of light to see if the embryogenic pathway has been down regulated and germination pathways initiated.

CHAPTER 5

SOMACLONAL VARIATION OF EMBRYOGENIC CULTURES

5.1 ABSTRACT

Clumps of *Conospermum caeruleum s*omatic embryos derived from a single zygotic embryo underwent adventitious embryogenesis and differentiation from embryogenic callus. They were subcultured for a period of 12 months. RAPD analysis was performed on 20 shoots and 20 embryos regenerated from these cultures to assess genetic stability. Somaclonal variation was found in two shoots, both with 1.53 % variable loci. This level of variation was considered too high in a commercial setting and modification of techniques is discussed.

5.2 INTRODUCTION

Somaclonal variation describes epigenetic and genetic changes in plants that become apparent either during or after *in vitro* culture of plant cells or organs. The changes of interest to plant breeders are heritable and result from changes in the plastid or nuclear genome. The introduction of undesirable and unknown variation is problematic for horticulturists and breeders, and may occur in high frequency when adventitious regeneration techniques are used. Methods for detection of somaclonal variation have been explored for many years (Novak 1980). Scoring changes in plant morphology can be useful in some studies (Lamhamedi *et al.* 2000), but there is limited diversity and traits may be affected by environmental influences. Cytological assessment is not often used, and can be difficult in many species like *Proteaceae* where chromosomes are difficult to observe. Analysis of secondary metabolites and isozyme patterns have also been used but are limited in their sensitivity (Morell *et al.* 1995). Molecular

techniques such as Restriction Fragment Length Polymorphisms (RFLP) or Random Amplified Polymorphic DNA (RAPD) are often favoured over traditional phenotypic or cytological measurements, and generally accurately assess even small levels of variation in the genome.

The use of the PCR-based RAPD technique to detect somaclonal variation has been applied successfully to several monocot species, such as *Lolium* (Wang *et al.* 1993) and *Allium sativum* L. (Al-Zahim *et al.* 1999), and woody dicotyledonous species such as *Picea abies* (Heinze and Schmidt 1995), *Quercus suber* (Gallego *et al.* 1997) and *Melia azedarach* L. (Olmos *et al.* 2002). The technique is not always accurate, as a study by Bouman and De Klerk (2001) showed that RAPD analysis could not detect point mutations induced by a chemical mutagen, yet phenotypical assays clearly showed variation in plant appearance. However, somaclonal variation induced by tissue culture is not confined to point mutations, as chromosome breakages, DNA deletions, changes in DNA methylation and activation of transposons have all been suggested as mechanisms for inducing variation, some of which may be detected using RAPD analysis (Cassells and Curry 2001).

The likelihood of somaclonal variants emerging is influenced by the method of micropropagation. In general, direct regeneration via shoot, nodal or embryogenic culture will decrease somaclonal frequency. Adventitious shoots and indirect embryogenesis from previously non-embryogenic cells are considered at high risk of genetic change. Somaclonal variation has been reported in a number of species using a variety of original explants. *Secale cereale* L. showed a high frequency of variation in plants regenerated from immature embryos and inflorescences via a callus phase,

where 40 % of plants showed at least one variation (Linacero *et al.* 2000). Overall 8.19 % of bands were variable from the original explant. Most other recorded rates of variation are much lower than for rye, such as 16.5 % in turmeric shoots derived from leaf base callus (Salvi *et al.* 2001) and 0.35 – 1 % in garlic plants regenerated from somatic embryos derived from callus cultures (Al-Zahim *et al.* 1999).

RAPD analysis was used to detect somaclonal variation in whole embryos and shoots derived from the same original zygotic embryo.

5.3 MATERIALS AND METHODS

5.3.1 Material source

All *C. caeruleum* shoots and embryos that were used to extract DNA and test for somaclonal variation originated from a single zygotic embryo, and had been in culture for over 12 months (Chapter 4). In total 20 embryos and 20 shoots were assayed. Shoots had callus removed from the base, and all embryos were discrete cotyledonary-staged embryos that showed no secondary embryogenesis.

5.3.2 DNA extraction

DNA was extracted from embryos and shoots using a Qiagen DNeasy plant mini kit. Embryos and shoots were ground either in a 1.5 ml eppendorf tube using a hand held grinder, or in a mortar and pestle with liquid nitrogen. The instructions provided with the kit were followed, with some alterations. For those samples ground by hand in an eppendorf tube, 100 µl of the 400 µl of buffer AP1 was added to aid grinding, and the remaining buffer was added after grinding was complete. The optional centrifuge spin to remove unwanted precipitates was carried out on all shoot extractions, but not

embryo extractions. Elution of DNA from the membrane was in 50 µl of buffer AE, not 100 µl as stated in manual, since a low yield of DNA was expected. DNA yields were determined using a DyNA Quant 2000 fluorometer, by adding 2 µl DNA sample to the assay solution.

5.3.3 Random Amplification of Polymorphic DNA (RAPD)

For all forty samples, polymerase chain reactions (PCR) were carried out using 37 randomly selected UBC primers. Each 15 µl reaction mix contained: 1x PCR buffer (20mM Tris-HCl pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM primer, 0.2 unit of Taq polymerase (5 U μl^{-1}), and 3 - 5 ngμl^{-1} DNA template. The reactions were placed in a thermocycler for one cycle of 94 °C 1 min, 36 °C 30 sec, 72 °C 1 min, followed by 34 cycles of 94 °C 10 sec, 36 °C 30 sec, 72 °C 1 min.

PCR products were separated on 3 % 0.5 x TBE agarose gels subject to electrophoresis for 2.5 hrs at 150 volts, and stained in ethidium bromide before examination under UV light.

5.4 RESULTS

The quality of DNA obtained from embryos and shoots was generally high although yield was low for all samples. The amount of DNA obtained ranged from 100 ng to 900 ng and shoots generally yielded more DNA than embryos (maximum yield 900 ng compared to 300 ng) (Table 5.1).

Table 5.1 DNA yield for embryos and shoots of *Conospermum caeruleum.*

Embryo	Yield (ng)	Shoot	Yield (ng)
1	150	1	450
2	150	2	150
3	150	3	400
4	100	4	200
5	300	5	350
6	150	6	350
7	100	7	550
8	200	8	350
9	150	9	250
10	150	10	350
11	150	11	250
12	200	12	300
13	150	13	400
14	150	14	100
15	100	15	350
16	150	16	300
17	250	17	150
18	300	18	900
19	200	19	900
20	250	20	750
Mean (se)	175 (13)	Mean (se)	390 (51)

In total, 37 primers were screened on a subset of four samples, and 15 produced clear amplification products (Table 5.2). These 15 primers were then screened on all individuals. The number of bands per primer ranged from 4 to 13 with a mean of 8. Primer 287 revealed one polymorphic band of approximately 1.90 kb in shoot 10 (Figure 5.1) that was confirmed with a repeat analysis. Shoot 14 and 15 also had a polymorphic band of approximately 1.90 kb, but this was not confirmed by a repeat analysis. Primer 208 revealed a polymorphic band in shoot 5 of approximately 1.25 kb and a band of approximately 0.86 kb in shoot 5 and 10 (Figure 5.2). These polymorphisms were confirmed by repeat analysis. Shoot 16 and 17 also have the same 2 polymorphic bands but these were not confirmed in repeat reactions. Several primers showed no polymorphism in banding patterns (Figures 5.3, 5.4).

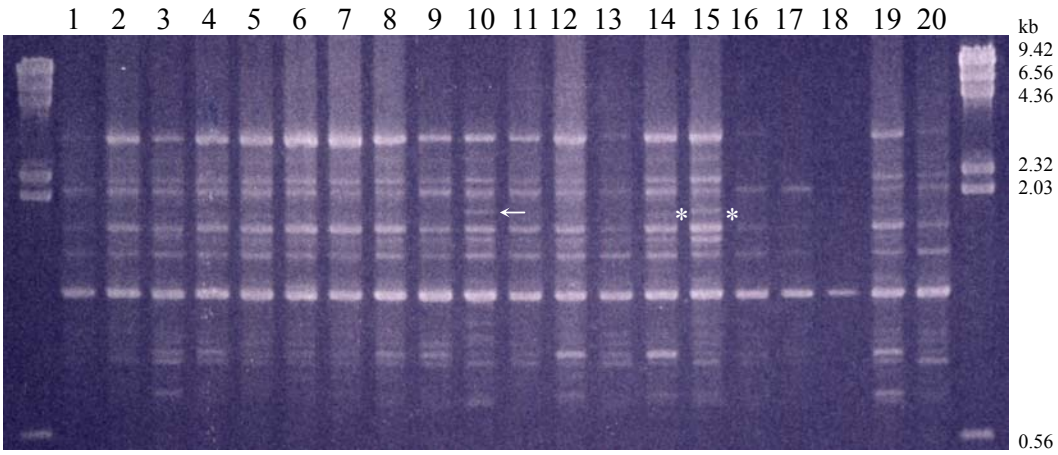

Figure 5.1 Amplification products using UBC primer 287 with template DNA from 20 *Conospermum caeruleum* shoots derived from an embryogenic culture. Shoot 10 has one polymorphic band (arrow) of approx 1.95 kb, confirmed by repeat analysis. Plantlets 14 and 15 have unconfirmed polymorphic bands (*).

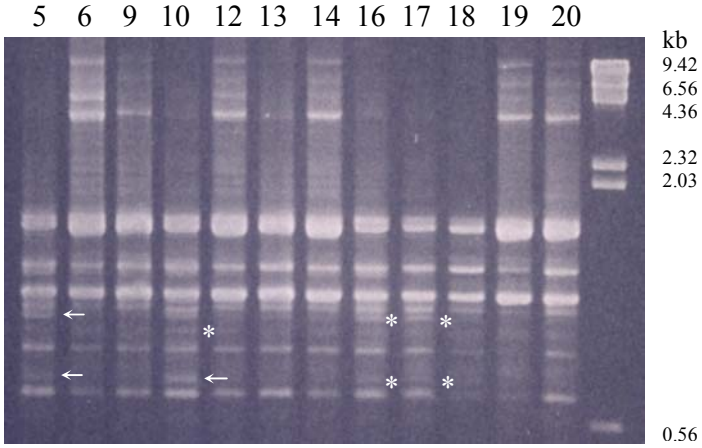

Figure 5.2 Amplification products using UBC primer 208 with template DNA from *Conospermum caeruleum* shoots derived from an embryogenic culture. Shoot 5 has 2 polymorphic bands confirmed by repeat analysis. Shoot 10 has 1 confirmed polymorphic band (arrows). Shoots 10, 16 and 17 have unconfirmed polymorphic bands (*).

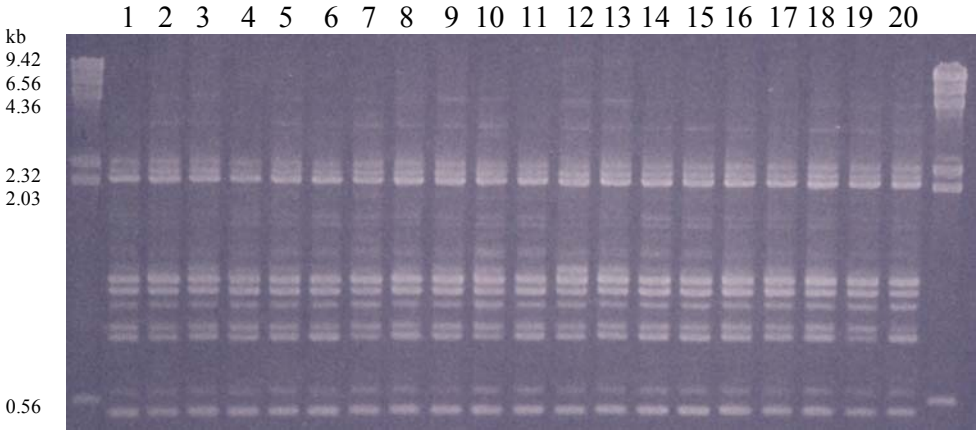

Figure 5.3 Amplification products using UBC primer 272 with template DNA from *Conospermum caeruleum* embryos. No polymorphic bands were detected.

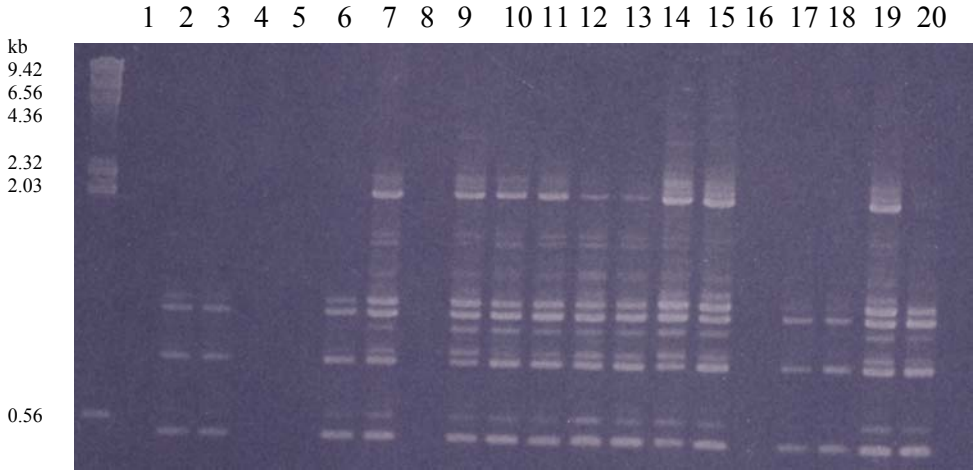

Figure 5.4 Amplification products using UBC primer 272 with template DNA from *Conospermum caeruleum* shoots derived from embryogenic culture.
No polymorphic bands were detected in the plantlets that successfully amplified.

Table 5.2 UBC primers trialed on a subset of *Conospermum caeruleum* shoots and embryos and those subsequently assayed on all individuals, and the number of loci consistently amplified.

Primer	Trialed	Assayed	No. loci
204	+	+	11
208	+	+	10
228	+	+	10
230	+	+	8
232	+	+	6
239	+	+	8
243	+	+	7
248	+	+	4
270	+	+	10
272	+	+	10
275	+	+	5
282	+	+	10
283	+	+	13
284	+	+	10
287	+	+	9
203	+		2
205	+		0
206	+		0
209	+		0
212	+		2
215	+		0
220	+		2
222	+		0
225	+		0
229	+		0
233	+		0
234	+		0
235	+		0
236	+		0
252	+		0
253	+		0
260	+		0
264	+		0
273	+		0
277	+		0
279	+		0
280	+		0

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Inconsistent amplification was a problem, for example in Figure 5.1 lanes 17, 18, and in Figure 5.4 lanes 1 – 5, and this generally occurred in primers that were screened later on or in repeat reactions. This may be related to deterioration in DNA quality Scoring of loci was only carried out for bands in the size range where consistent amplification was obtained for most individuals.

5.5 DISCUSSION

Somaclonal variation was detected in 10 % of *C. caeruleum* shoots derived from an embryogenic culture. No polymorphisms were detected in embryos derived from the same culture, resulting in 5 % of *C. caeruleum* individuals showing somaclonal variation overall. Three polymorphic bands were identified, the resulting frequency of variable loci was 1.53 % for both shoots that showed variation. The frequency of somaclonal variation could potentially be higher, but poor repeatability of some RAPD reactions meant other possible polymorphic bands were not confirmed. Poor repeatability of RAPDs can be problematic, as was found in this study, but RAPD analysis does have an advantage compared to other molecular techniques such as AFLPs, in that a small amount of DNA is required per reaction. The quantity of DNA extracted from *C. caeruleum* somatic embryos was very low, and RAPD analysis could be carried out with small amounts of DNA such as this. The small amount of template DNA available per individual meant repeat runs were often incomplete, possibly due to a decrease in DNA quality. The two individuals in which variation was confirmed had polymorphic bands that were seen in other individuals, but in the latter case the bands were not repeated in subsequent runs. Either the bands in question were not present, or the PCR reaction yielded no products, as is evident in several lanes of Figure 5.4. An approach taken by Linacero *et al.* (2000) to confirm

the presence of polymorphic bands detected using RAPDs in rye, was to clone the variable bands and use them as probes in an RFLP study to rule out error due to inconsistent amplification. This method could be considered for *Conospermum* as part of quality assurance in a breeding program, although the assay would have to be conducted on more mature plantlets since larger amounts of DNA would be required for an RFLP assay.

The individuals surveyed here were obtained from an embryogenic culture that was 12 months old and had originated from a single zygotic embryo. During that time many subcultures were performed, and somatic embryos produced more embryos, embryogenic callus, shoots and occasionally roots. The specific history of the origins of the individuals used here is not known, as subcultures were not tracked. The somaclonal variation detected may have occurred at any time during the 12 months, and been passed on through embryogenic cells to the individuals selected for this experiment. It appears possible that at some time there were at least two mutational events in separate lineages in these cultures. The presence of the mutations detected in this study in 1 or 2 out of 40 individuals suggests that the mutation occurred late in the culture period. If the other individuals with polymorphic bands were confirmed, this would indicate the mutation occurred earlier and were passed into several subculture lines. Future experiments tracking the history of each new embryo or shoot may enable more specific identification of when mutational events are most likely to occur.

The optimal method of production of plants from somatic embryos would have the fewest subcultures, and therefore the least chance of a mutational event occurring.

This would decrease the likelihood of undesirable and unknown traits being introduced, which is important for commercial production. The phenotypic effects of the somaclonal variation detected here are not known, but 5 % variability after a culture period of at least 12 months may be considered too high. To decrease the level of mutated plants, zygotic embryos could be re-initiated into culture from stored seeds periodically, possibly every six months, to enable production of somatic embryos with shorter culture times. Tracking of cultures combined with randomised screening could also be an important aspect of quality control. If randomised screening identified a mutation in a culture, then the source culture of the mutation could be more readily identified, and all cultures derived from that culture could be removed without the need to screen all cultures. Further work is required to fully determine the effects somaclonal variation may have on propagation on a commercial scale.

CHAPTER 6

BREEDING SYSTEMS

6.1 ABSTRACT

Aspects of the breeding systems of *Conospermum* were investigated, focusing on blue-flowered species. *Conospermum* have inflorescences of 5 – 10 buds that open sequentially from the basal bud upwards (acropetally). The uppermost 2 - 3 buds stayed small and closed and never opened. Many more fruits were set than seed, and both were only observed on the lower 2 – 3 flowers. Seed did not set in the absence of insect pollinators. Observations of the ultrastructure showed that basal and terminal buds appeared to be hermaphroditic, although embryo sacs were not located through transverse sectioning. Optimal stigmatic receptiveness was inconclusive, but was not at anthesis as expected. Controlled pollinations showed no incompatibility in the style between available genotypes of *C. eatoniae* at the field station, although no seed was set, so late-acting incompatibility could not be ruled out. Analysis of dominant loci using RAPDs of two plantation-grown plants and two plants from a wild population showed *C. eatoniae* to be more likely to outcross in the wild, but to be predominantly selfed when planted in clonal rows at a field station. These results would be important to consider when designing a plantation to maximise self or cross pollination between genotypes, depending on the desired outcome.

6.2 INTRODUCTION

Proteaceae are among the most widely investigated Australian native species, due to their abundance across many environments (heathland, woodland and forest), large inflorescences that provide nectar to native birds and insects, and popularity for

horticulture. Most research has focused on *Banksia* and *Grevillia* species, and there is still much to learn about their breeding systems.

Previous studies have shown that many *Proteaceae* species are self-incompatible or preferentially outcross (Chapter 1), although not for *Grevillea* where many species are self-compatible (Smith and Gross 2002). The mechanism of incompatibility varies, for example some *Banksia* species inhibit pollen tube growth in the upper style or pollen presenter (Fuss and Sedgley 1991; Sedgley *et al.* 1994), and for *Macadamia integrifolia* inhibition occurs in the lower style (Sedgley 1983).

A study by Morrison *et al.* (1994) showed that four Eastern Australian *Conospermum* species were cross-compatible at the species level to varying degrees, based on seed-set. Cross pollination was also possible between populations within these species but they were unable to self-pollinate when pollinators were excluded. No similar study on genetic barriers has been done on blue-flowered *Conospermum* endemic to Western Australia.

The extremely low fruit:flower ratios of *Proteaceae* has sparked much interest, and several studies have attempted to elucidate the evolutionary and functional reasons for this. *Conospermum* species are no exception, producing masses of inflorescences, few fruits, and even fewer seeds. Two broad hypotheses were put forward by Ayre and Whelan (1989) to explain the low fruit set in Australian *Proteaceae*; the proximate and the ultimate hypotheses. The proximate explanations for low fruit:flower ratios included: pollen limitation, genetically incompatible pollen,

resource limitations and predation. Ultimate explanations were based on past selective pressures.

Little has been reported on the stigmatic characteristics and flowering habits of *Conospermum.* It is known that the flowers are insect-pollinated. Houston (1989) identified a distinct species-group of small bees (*Hymenoptera*) (5-9 mm long) that were collected only from white-flowered *Conospermum*. These bees were observed standing on the outside of the flower and releasing the trigger mechanism with their tongues.

The parentage of seed set in a field environment is of interest to breeders and commercial growers, who often wish to maintain the genetic fidelity of their selections. In an unnatural setting such as a plantation at a field station, natural pollinators may be absent and those present may have different foraging behaviours, resulting in changes to the proportion of self or cross pollination. Methods to determine the level of outcrossing often use molecular techniques to assay dominant or codominant loci in progeny arrays. This can be carried out using allozyme analysis, or through the use of PCR-based methods such as RAPDs or AFLPs to amplify DNA. Allozyme analysis is usually carried out on germinated seed and the quantities of tissue required are quite large and enzyme activity may be low. For example allozyme analysis of *C. taxifolium* proved difficult for Mackay and Morrison (1989), who considered this was due to low enzyme activity and high levels of phenolic compounds. If polymorphism levels in a species are low, allozymes may not be sufficiently variable to detect outcrossing reliably. RAPD analysis can be carried out on extracted DNA directly from the seed, and is usually sufficiently polymorphic

to detect outcrossing with reasonable reliability. Therefore RAPDs are useful in species like *Conospermum* where seeds are difficult to germinate.

These experiments aimed to investigate the timing of anthesis and stigmatic receptiveness, and the level of fruit set of some of the blue flowering species of *Conospermum* endemic to Western Australia. In addition, the ability of *C. eatoniae* to self and cross pollinate was to be evaluated by doing controlled hand-crosses, and to compare self and cross pollination frequencies in field and wild populations using RAPD analysis to determine genetic parentage of open-set seed.

6.3 MATERIALS AND METHODS

Cultivated plants used in these experiments were located at Department of Agriculture Western Australia Medina field research station. Some *C. amoenum* was collected from a wild population located at Armidale, Western Australia, and a *C. eatoniae* wild population was sampled from Quairading, Western Australia. Other species examined were*, C. caeruleum* and *C. brownii.*

6.3.1 Bud counts and opening times

For *C. eatoniae* the numbers of flowers per inflorescence were counted for 6 - 8 stems of two different genotypes. The number of buds that opened and their timing was determined by observation every 1 - 5 days for six weeks, on 10 inflorescences from three different plants of two different genotypes.

6.3.2 Floral structure

Vegetative and whole buds from *C. eatoniae* were fixed in Carnoy's fixative and embedded in Spurr's resin (Spurr 1969). Sections were made using a microtome and stained with azur blue to observe floral structures.

Styles of flowers that were untriggered and those triggered two days prior to collection were observed under the scanning electron microscope to detect any changes that may indicate receptiveness. Styles were fixed by freeze-drying in a plastic tray and were then attached to SEM stubs using adhesive carbon disks. Stigmas were then coated with gold and viewed using the SEM.

6.3.3 Timing of stigma receptivity

Recently opened flowers of *Conospermum* spp with the style in an untriggered position and anthers intact (prior to dehiscence) were used in these experiments. Only the lower four flowers of an inflorescence were hand pollinated, as upper flowers often do not set seed naturally. For all pollinations, forceps were used to gently push down on the style to cause the trigger mechanism to be activated, and the anthers to dehisce. An insect would trigger the style by pushing on the trigger point near the anthers with its head whilst scavenging for pollen and nectar, however initial work showed that mimicking this using forceps or a dissecting needle often resulted in the stigma coming in contact with self pollen. Applying gentle pressure to the style enabled directed movement of the style downwards and protected the stigma from the dehisced pollen coming over the top. Cross or self-pollen was applied to the stigma using the flat side of a scalpel. To determine stigma receptivity, flowers were sequentially pollinated up to six days after triggering/dehiscence, and collected for

observation four days after pollination. For each day, 5 - 10 flowers were self or cross pollinated and tagged using coloured cotton tied around the base of the flower. Flowers were isolated by bagging with crispy wrap bread bags. A preliminary experiment had determined the optimum time for collection of styles after hand pollination for best observation of pollen tubes was 4 – 5 days after pollination.

The methods of Oddie and McComb (1998) were used to fix, stain and view pollen tubes in styles using fluorescence microscopy.

6.3.4 Controlled pollinations

For all pollinations, *C. eatoniae* flowers that were open and style untriggered (anthers intact) were selected. All available pollen donors were used to pollinate flowers as described previously on two plants of different genotypes; "commercial" and "51." For each cross (pollen donor x maternal genotype), 14 flowers were pollinated. Seven of the flowers were collected five days after pollen was applied, and the remaining seven flowers were left to set seed. From the flowers that were collected, the styles were removed, fixed and viewed according to Oddie and McComb (1998), using fluorescence microscopy.

6.3.5 Seed set position

For *C. amoenum,* 6 stems were collected and the position of the fruit/seed on the inflorescence was recorded.

6.3.6 Pollinator exclusion

To determine if flowers could set fruit/seed without an insect pollinator, crispy-wrap bread bags were tied around 7 - 9 stems of *C. amoenum* and *C. caeruleum* prior to the first bud opening, and left until seed set. The approximate number of buds was determined, and fruit and seed set counted.

6.3.7 Plant and seed collection for RAPD analysis

Open pollinated seed from *C. eatoniae* was collected from cultivated plants at the field research station and from the wild population near Quairading. At the field station, 11 genotypes of *C. eatoniae* were grown in clonal rows within 25 metres of each other. These genotypes were identified as "commercial" and by numbers 21, 24, 51, 53, 54, 58, 74, 80, 191, and 196. Seed was collected at maturity from "commercial" and genotype 51. Several other species of *Conospermum* were also grown in close proximity to *C. eatoniae*. The wild population was divided by a dirt road. The plants selected for this experiment were from both sides of this road and were surrounded by many plants of the same species. Mature seeds were collected from two bushes, named "A" and "B."

6.3.8 DNA extraction

DNA was extracted using a Qiagen DNeasy plant mini kit from fifteen seeds collected from genotype "commercial," 10 seeds from genotype "51" and 10 seeds from each of 2 wild plants "A" and "B." Individual seeds were ground in 1.5 ml eppendorf tubes using a hand held grinder. The seed coat from 1 seed collected from genotype "commercial" was ground and DNA extracted to confirm that maternal DNA was not being amplified in the seed samples. In addition, 100 mg of stem scrapings from the 2

wild plants, the commercial and genotype 51 plant and all potential pollen donor plants at the research station were ground in a mortar and pestle with liquid nitrogen. Scrapings were obtained by using a razor blade to remove the green outer layer from the woody stems. The instructions provided with the kit were followed, with some alterations. For those samples ground by hand in an eppendorf tube, 100 µl of the 400 µl of buffer AP1 was added to aid grinding, and the remaining buffer was added after grinding was complete. The optional centrifuge spin to remove unwanted precipitates was carried out on all stem scrapings extractions, but not seed extractions. Elution of DNA from the membrane was in 50 µl of buffer AE, not 100 µl as stated in manual, since a low yield of DNA was expected. DNA yield were determined using a DyNA Quant 2000 fluorometer, by adding 2 µl DNA sample to the assay solution.

6.3.9 Random Amplification of Polymorphic DNA (RAPD)

For all samples, a polymerase chain reaction (PCR) was carried out using randomly selected UBC primers. Each 15 µl reaction mix contained: 1x PCR buffer (20mM Tris-HCl pH 8.4, 50 mM KCl), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 mM primer, 0.2 unit Taq polymerase (5 U μl^{-1}), and 3 - 5 ngμl^{-1} DNA template. The reactions were placed in a thermocycler for one cycle of 94 °C 1 min, 36 °C 30 sec, 72 °C 1 min, followed by 34 cycles of 94 °C 10 sec, 36 °C 30 sec, 72 °C 1 min.

PCR products were separated on 3 % 0.5 x TBE agarose gel subject to electrophoresis for 2.5 hrs at 150 volts, before being stained in ethidium bromide and examined under UV light. For primers that produced scorable PCR products, reactions were repeated at least once.

6.3.10 Statistical analysis

For RAPD data, the presence or absence of bands at each locus were scored for each individual. Genetic distance between individuals was calculated using GenAlEx (Peakall and Smouse 2001). A principal co-ordinates analysis (PCA) was used to show the relationships between the mother plants, their seed progeny and the pollen donor plants from the field station, and the genetic diversity between progeny from the wild population and field station. Shannons Index (Shannon and Weaver 1949) of genetic diversity values within populations was calculated using the formula: $D = - \sum a_i \log a_i$, where a_i is allelic frequency.

6.4 RESULTS

6.4.1 Bud counts and opening times

In *C. eatoniae* over a six week period, up to 6 flowers opened sequentially on an inflorescence (Figure 6.1), from the basal flower upwards. The uppermost 2 or 3 buds stayed small and closed, and never opened. The highest mean number of flowers that opened was 5 (± 0.30) for genotype 1, and 5.9 (± 0.46) for genotype 2, both on day 33. The total mean number of flowers per inflorescence (including those that did not open) was 5.7 (± 0.27) for genotype 1 and 6.8 (± 0.21) for genotype 2, with up to 10 buds per inflorescence.

6.4.2 Floral structure

Basal flowers and terminal buds were sectioned and showed normal hermaphroditic structures. Pollen was found in both; however further sectioning is required to locate the embryo sac in the ovaries of the flowers. Sectioning of a mature *C. eatoniae* flower showed that the stylar canal was a loosely bound matrix of cells (Figure 6.2).

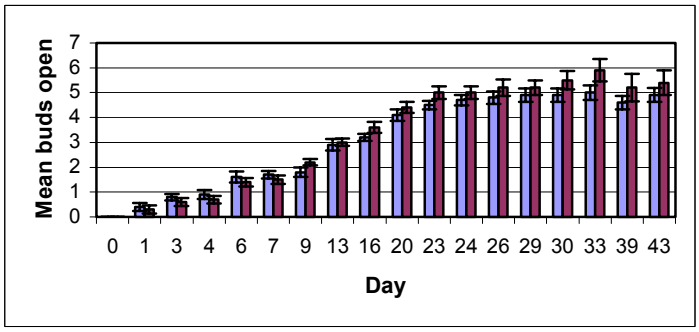

Figure 6.1 Mean number of *Conospermum eatoniae* buds that opened on 10 inflorescences of (□) genotype 1 and (■) genotype 2 over a 43 day period. Bars show standard errors.

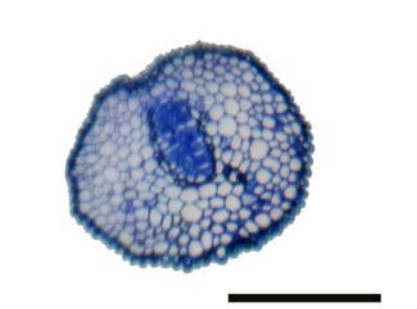

Figure 6.2 *Conospermum eatoniae* flower embedded in Spurr's resin and sectioned to show the stylar tract. Bar = 100 μm.

Observations using the SEM showed the stigmatic surface of an untriggered style was wet with a secretion, predominantly around the upper lip (Figure 6.3). The stigmatic surface of a style triggered two days earlier showed no change in secretion. Observations of the stigma *in vivo* showed no visual changes in the stigmatic secretion up to six days after anthesis.

6.4.3 Timing of stigma receptivity

The conventional technique for viewing pollen tubes using fluorescence microscopy to illuminate callose plugs was difficult for *Conospermum*, as callose plugs were rarely seen (Figure 6.4). Light microscopy revealed germinated pollen on the stigma. Pollen readily adhered to *C. brownii* stigmas for up to three days post-anther dehiscence and pollen loads generally declined after this time (Figure 6.5). Pollen did not adhere well to *C. eatoniae* at any time after dehiscence. The number of days after dehiscence that flowers were pollinated did not directly affect the number of pollen tubes observed in the style of any of the species. In all species except *C. brownii*, most pollen tubes were observed in flowers pollinated 2 - 3 days after anther dehiscence/style triggering (Figure 6.6). In *C. brownii* no tubes were observed in any flowers pollinated on days 0 - 6 after anther dehiscence. An analysis of variance (ANOVA) revealed the number of tubes observed was influenced significantly by species ($P = <0.001$). The number of days after pollination did not significantly affect the number of tubes observed, but the interaction between species and the number of days after pollination did significantly affect tube observation ($P = 0.002$). This result was consistent for the number of grains observed on the stigma; the different species were very variable in the amount of pollen that remained adhered to the stigma.

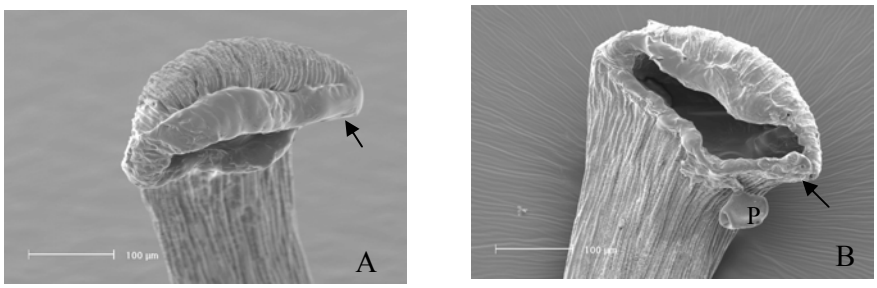

Figure 6.3 *Conospermum eatoniae* styles viewed under scanning electron microscope, showing stigmatic exudate (arrows). A. Untriggered style. B. Style 2 days after dehiscence with pollen grain attached (P).

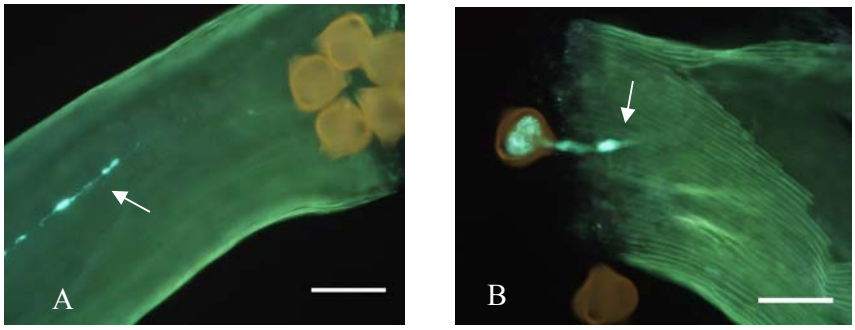

Figure 6.4 Styles of *Conospermum eatoniae* viewed under fluorescence showing callose plugs in pollen tubes (arrows). A. Pollination after anther dehiscence (Day 0). B. Pollination 2 days after anther dehiscence. Bar is 100 μm.

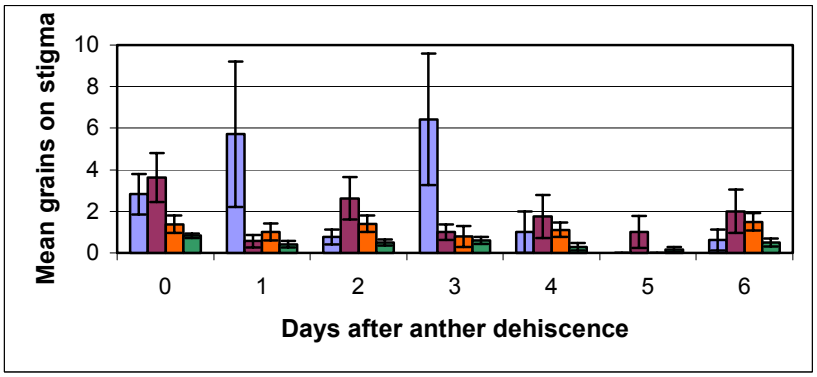

Figure 6.5 Mean number of pollen grains observed on the stigma of *Conospermum* spp. following hand pollination 0-6 days after anther dehiscence (triggering of style). (□) *Conospermum brownii.* (■) *C. caeruleum.* (■) *C. amoenum.* (■) *C. eatoniae.* Bars show standard errors. Five styles were hand pollinated for collection at each time of harvest which was four days after pollination.

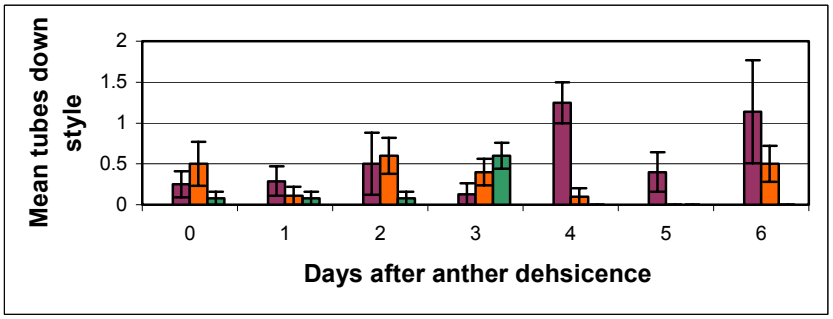

Figure 6.6 Mean number of pollen tubes observed in the style following hand pollination 0-6 days after anther dehiscence (triggering of style).
(□) *Conospermum brownii.* (■) *C. caeruleum.* (■) *C. amoenum.* (■) *C. eatoniae.* Bars show standard errors. Five styles were hand pollinated for collection at each time of harvest which was four days after pollination.

6.4.4 Controlled pollinations

Analysis of stigmas from flowers collected five days after pollination showed that *C. eatoniae* pollen from all but one of the genotypes tested can germinate, penetrate the stigma and extend down the style (Figure 6.7). Pollen loads on the stigma were low for all crosses, ranging between 0 and 6 grains, (Figure 6.8) with a mean of two grains observed on any stigma. Not all pollen grains observed on the stigma geminated for any genotype. For the commercial line, pollen from genotypes 21 and 80 were the least successful in growing down the style, but an average of one grain or less was observed on the stigma. For maternal genotype 51, pollen donors 581, 24 and 53 were the least successful at growing down the style. For the commercial line, there was a significant correlation between pollen genotype and the number and behaviour of pollen on the stigma ($P = 0.0475$), and the number of pollen tubes observed down the style ($P = 0.0383$). For maternal parent 51 there was a correlation between pollen donor genotype and pollen number and behaviour, but it was not significant ($P = 0.0738$).

All flowers left to set seed on both maternal plants used for the cross pollinations were shed, and no seed was collected from any of the crosses.

6.4.5 Seed set position

In all *Conospermum* species it was observed that only the basal 1 - 3 flowers on the inflorescence produced seeds. Detailed counts on *C. amoenum* stems showed that the mean position for fruit set was 2.61 (± 0.14) while for seeds it was 1.89 (± 0.27) (flower one was the most basal flower).

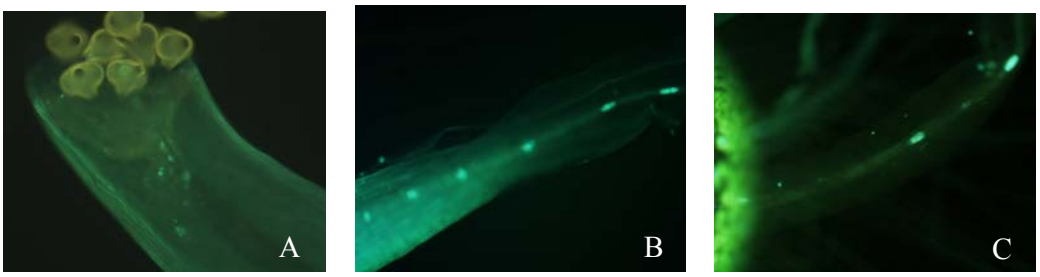

Figure 6.7 *Conospermum eatoniae* pollen tubes with callose plugs. A. *C. eatoniae* commercial genotype with self-pollen germinating on the stigma. B. *C. eatoniae* commercial genotype cross-pollinated with genotype 24, showing a pollen tube at the lower end of the style. C. *C. eatoniae* commercial genotype cross-pollinated with genotype 24, showing a pollen tube entering the ovary.

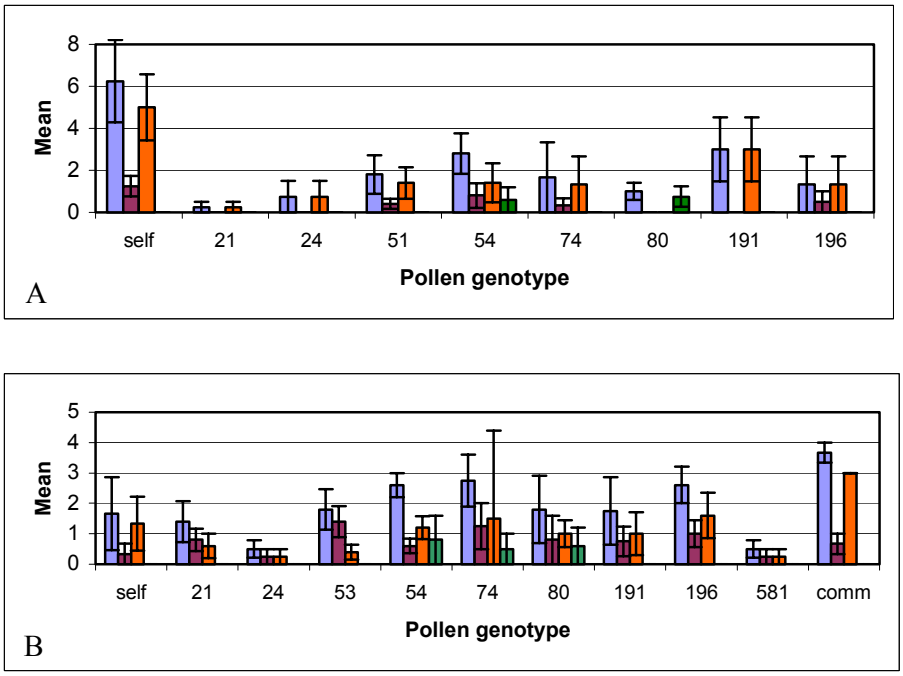

Figure 6.8 Results of controlled pollination of *Conospermum eatoniae*. (A) genotype "commercial" and (B) genotype "51" (□) Mean number of pollen grains observed on the stigma. (■) Mean number of pollen grains that had not germinated on the stigma. (■) Mean number of pollen tubes observed down the style. (■) Mean number of grains for which it was unclear whether or not they had germinated. Bars show standard errors.

6.4.6 Pollinator exclusion

When insects were excluded there was no fruit set in either *C. amoenum* or *C. caeruleum.*

6.4.7 Open pollinated seed

Open pollinated seed was collected from the "commercial" and "51" plants at the field station and from two plants in the wild population. Of the 34 UBC primers screened, seven gave consistent PCR products for seed and stem scraping samples from the "commercial" and "51" maternal plants from the field station. A total of 93 bands were scored in these two plants and 25 seeds. For the wild population, three primers gave consistent results in the two plants and their seed; a total of 50 bands were scored. DNA extracted from seeds was reliably amplified and gave clear PCR products but DNA extracted from stem scrapings was problematic, probably due to some contaminants interfering with the PCR reaction. Several primers amplified seed DNA but not stem DNA. DNA from one potential pollen donor (number 24) from the field station showed particularly inconsistent amplification and was excluded from the statistical analysis. Amplification of seed coat DNA yielded no PCR products.

A PCA was carried out on the genotypes of plants and seed from "commercial" and "51" at the field station (Figure 6.9). Potential pollen donors clustered together, with the exception of "commercial" and "51" which were quite separate. The genetic make-up of seeds from the "commercial" plant most closely resembled the mother plant, with no potential pollen donor clustering nearby (Figure 6.9). The seed collected from plant "51" were also genetically most similar to it. Shannons diversity index revealed similar genetic diversity within the progeny for "commercial" (5.228)

and “51” (5.398) but diversity in both progeny was low compared to the diversity in the potential pollen donors (10.402). PCA analysis of the four sets of progeny from the field station and wild population was carried out using the loci that gave reliable amplification in the wild population. The ordination showed that for each family the seed clustered closely together (Figure 6.10). The seed from the two field families were quite distinct from each other and from the wild families. The progeny from the two plants in the wild population were genetically similar and clustered together. Shannons index of genetic diversity showed there to be greater genetic diversity in the wild population progeny than the field station progeny (Table 6.1).

Table 6.1 Shannons Diversity Index values for seed progeny of two field station *Conospermum eatoniae* plants, “commercial” and “51”, and two plants, “A” and “B,” from a wild population. A total of 50 loci were scored.

Identity	"commercial" progeny	"51" progeny	"A" progeny	"B" progeny
Diversity index	1.676	2.284	3.191	3.267

6.5 DISCUSSION

Conospermum are different from most *Proteaceae* in that pollen is not deposited on a pollen presenter just prior to anthesis, but is released in a single dusty mass when a pollinator visits the flower and triggers the style that has been held in a state of tension. The stigma is forced down toward the insect pollinator, and the sticky upper lip of the stigma contacts the insect and pollen that it is carrying is attached.

It was expected that the stigma would be most receptive at the time of anther dehiscence/triggering of style, however the results for the timing of stigma receptivity were inconclusive. Hand pollination was the method of choice for determining the timing of stigmatic receptivity; however the number of pollen tubes in the style was

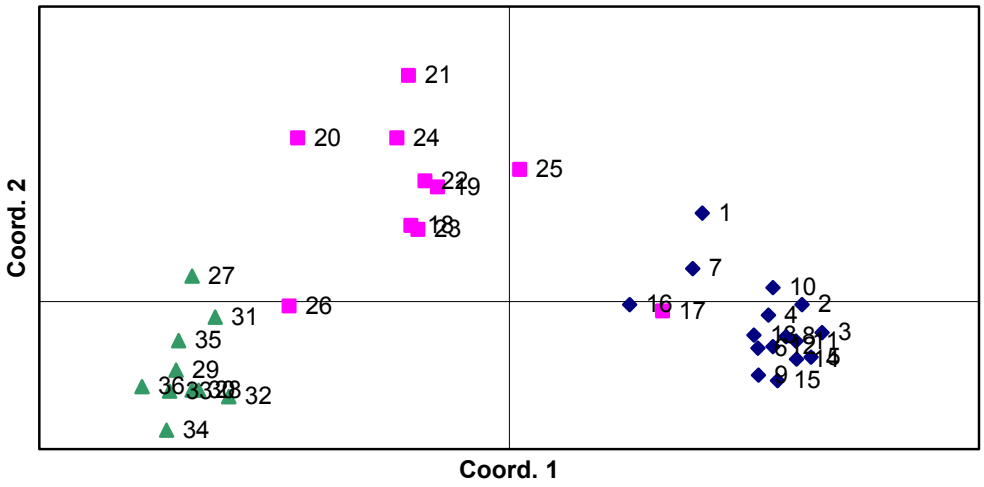

Figure 6.9 Principle Co-ordinates Analysis (PCA) of *Conospermum eatoniae* field populations "commercial" and "51." ( ) 15 seed progeny from genotype "commercial." (△) 10 seed progeny from genotype "51." (□) potential pollen donors. Individual 17 is the genotype "commercial." Individual 26 is genotype "51."

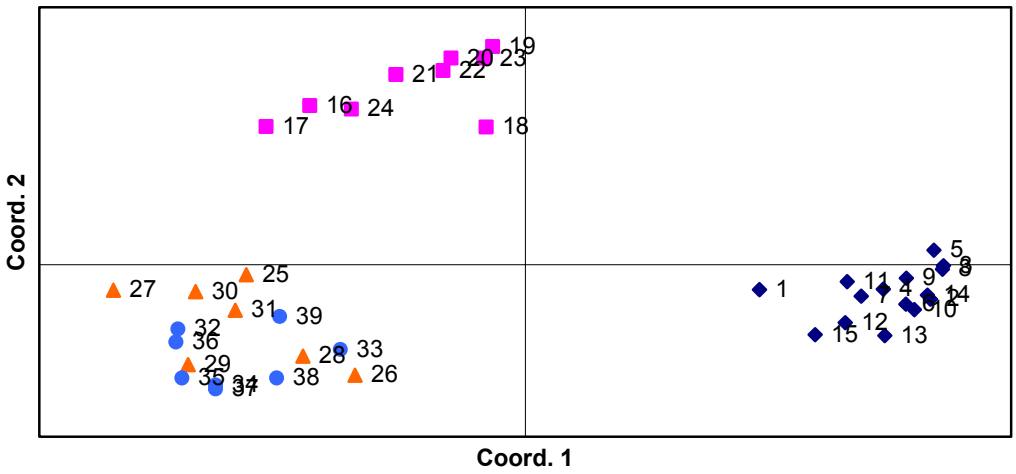

Figure 6.10 Principle Coordinates Analysis (PCA) using co-ordinates 1 and 2, of seed progeny from two field station grown plants "commercial" and "51," and two plants from a wild population "A" and "B." (◆) progeny from "commercial." (□) progeny from "51," (△) progeny from "A," (○) progeny from "B."

always low and gave no clear indication of the optimal time for stigma receptiveness. In a review by Goldingay and Carthew (1998) on methods used to determine stigma receptivity, it was concluded that hand pollinations were the most commonly used and reliable method for determining receptiveness of the stigma. Other methods included changes in stigmatic structure at the time of receptiveness. Some *Proteaceae,* such as some *Banksia* species, have dry stigmas, where grooves in the stigma open as receptiveness increases. For *Grevillea* spp., stigmatic papillae enlarge to indicate receptiveness. Some *Proteaceae* have wet stigmas, such as *Banksia coccinea* R. Br., where secretion increases as receptiveness increases (Fuss and Sedgley 1991). *Conospermum* have wet stigmas, however increases in secretion were not observed up to six days after dehiscence, nor were any grooves or other physiological changes observed. The number of pollen grains found on the stigma up to six days after dehiscence varied widely. It was assumed that approximately similar numbers of grains were deposited by hand onto each stigma. The variation in grain numbers observed could be due to a loss of adhesion over the time period, weather conditions (for example rain), or loss during the fixation process. The number of pollen grains observed did not directly correlate with the number of pollen tubes observed growing down the style. For all species, the number of pollen tubes observed was far less than the number of pollen grains, and for *C. brownii,* there were no pollen tubes observed at all (Figure 6.6). From this data, it appears that the stigma is receptive for six days from the time of anther dehiscence. Previous studies on receptivity in *Proteaceae* by Goldingay and Carthew (1998) did not include species with actively triggered styles, but revealed three broad patterns of receptivity. These were: stigma receptivity at anthesis; receptivity within 12 hours, increasing to days 2 - 4; and no receptivity until 24 hours following pollen removal (from the pollen presenter) and declining after 48

hours. *Conospermum caeruleum* and *C. eatoniae* appear to fit with the second pattern, also seen in some *Banksia* species (Vaughton and Ramsey 1991), while *C. amoenum* could fit either of the first two patterns. This is surprising as initial expectations were that the stigma would pick up pollen at the time the style was triggered. However, it is clear that later visits of insects to previously triggered flowers must also result in pollen transfer to the stigma.

The low fruit:flower ratios observed in *Conospermum* are largely unexplained but poor pollen germination, rather than pollen availability would appear to be the main reason. The uppermost buds of an inflorescence do not mature and open, and it is the lower flowers that set seed. Sections of upper flowers both terminal (unopened) and open ones, revealed normal hermaphroditic structures, although further sections to find the embryo sac are required to confirm that the inability to fertilise is not due to a lack of functional gametophytes. These flowers are blue in colour and may serve to only attract pollinators.

C. amoenum and *C. caeruleum* cannot self pollinate in the absence of insect pollinators, which is consistent with a study on Eastern Australian species *C. taxifolium, C. ericifolium, C. ellipticum* and *C. longifolium* (Morrison *et al.* 1994).

Genetic diversity in seed progeny arises through segregation of heterozygous loci and recombination of maternal genes, and capture of genes from the paternal contribution. The genetic diversity in the field station seed progeny was only half that of the potential pollen donors, indicating that the diversity present in the potential pollen donors was not captured in the seed progeny. This suggests a high level of

inbreeding in the seed progeny and the diversity present in the seed has come from segregation of heterozygous loci and recombination of maternal genes. In contrast, the level of genetic diversity captured in the seed progeny from the wild population was greater than that of the field plants and suggests a higher level of outcrossing in these seed progeny. The seed progeny of the wild plants occupied greater ordination space than the seed progeny of the field station plants, although this difference was not large. However, the seed progeny from both wild plants were genetically similar which suggests that they have captured the genetic diversity available within the population. The level of genetic diversity in the population was not determined but the genetic diversity in a population is bounded by the population itself. In contrast, the plants in the field station come from three distinct populations across the geographical range of *C. eatoniae* habitation, thereby representing the genetic diversity likely to be present. If the seed progeny from the field plants arose through outcrossing, then genetic diversity would be expected to be much greater than that of the wild plants because the diversity available in the pollen pool would be much greater.

The lack of outcrossing in the field station is unlikely to be the result of incompatibility with available pollen donors, based on the observation of pollen tubes in the style from available donors. Hand pollinations of both "commercial" and "51" flowers using all available pollen donors showed that pollen could germinate and extend down the style for many of these pollen donors. If incompatibility systems were operating, these must occur in the ovary. No seed was set from hand pollinations using either self-pollen or cross-pollen from ten different paternal genotypes, so late acting incompatibility could not be ruled out. However, the similar

diversity in the seed progeny from wild plants is not consistent with incompatibility. Inbreeding of the field station is more likely to have resulted from the physical layout of plants (with clonal plants in rows) and the foraging behaviour of the insect pollinators.

The foraging behaviour of insect pollinators was not studied at the field station or wild population sites, but this could be an area of future interest. The *C. eatoniae* plants are planted in rows at the Medina field station, with several other species of *Conospermum* also in close proximity. It is likely that insects visit successive bushes in the same row which are of the same genotype, and therefore promote self-pollination in this field station. However, as open-set seed was largely from self pollination (Figure 6.8), it is not clear why controlled self-pollinations did not result in seed set.

Conospermum eatoniae genotypes were originally selected for their flower colour, flowering time, and number of inflorescences. Genotypes 191 and 196 were selected from a northern population, the "commercial" genotype was also from the North but a separate population, and the remainder were from an eastern population near Quairading. Greater genetic diversity was expected for the potential pollen donors at the field station, as these populations were collected from three diverse areas of Western Australia, at the North and South end of the range of the species. This could indicate that *C. eatoniae* has limited genetic diversity at the species level.

For broad genetic studies on *Conospermum* populations, DNA from mature seeds may be preferable to stem scrapings. The sometimes inconsistent nature of RAPD analysis

is difficult to avoid when the amount of DNA available is limited. Many other molecular techniques that may be more reliable require larger quantities of DNA that were not available here. Germinating the seeds and growing plants may produce a greater yield of DNA, however *C. eatoniae* shows seed dormancy for up to eight years (Seaton pers. comm), and the difficulties in seed germination have not been adequately resolved yet.

The results obtained here would be important to consider when designing a commercial breeding program and field planting design. Genetic integrity of selected lines could be maintained in an open pollinated situation by positioning clonal plants close together in a field planting. Alternatively, if cross pollination is desirable to produce new genotypes with improved horticultural properties, then the maternal plant should be surrounded by the desired pollen donor. Further studies on the presence of insect pollinators and their foraging in areas used for seed production would enable a better understanding of how to control pollination, and maximise desired seed set. Further studies on seed viability and seedling vigour from self or cross pollinations are also required before seed production can be fully exploited in a plantation. *Conospermum* are essentially outcrossing species, but the results here indicate that self-pollination is possible.

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CHAPTER 7

OBSERVATIONS OF RAPID GERMINATION AND GROWTH OF MULTIPLE POLLEN TUBES IN *CONOSPERMUM*

7.1 ABSTRACT

Conospermum pollen is triporate, and is able to germinate with up to three pollen tubes both *in vivo* and *in vitro*, the first tube emerging within seconds of being placed in pollen germination medium. Multiple tubes were observed in six species of *Conospermum* and close relative *Synaphea petiolaris*. Up to 77 % of *C. incurvum*, 72 % of *C. dorrienii* and 46 % of *C. amoenum* viable pollen grains produced three tubes. The sum length of all tubes varied significantly between species and the number of tubes that emerged from the grain. All tubes elongated at a remarkable rate over the first two seconds after emergence from the grain, at up to 55 μms^{-1}. Extension rates 5 seconds after emergence declined to approx 16 μms^{-1}, and by 30 seconds to approx 3 μms^{-1}. The *in vitro* pollen tube growth rates observed here are 20-fold faster than has previously been reported for any other species.

7.2 INTRODUCTION

The rate at which pollen tubes elongate varies widely between species and also between *in vivo* and *in vitro* conditions. Some of the fastest recorded *in vivo* speeds are around 1.8 μms^{-1} (evening primrose) to 2.7 μms^{-1} (maize) (Stanley 1971; Barnabas and Fridvalszky 1984). Growth rates *in vitro* are slower, *Zea mays* at 1.14 μms^{-1} and *Ornithogalum virens* at 0.25 μms^{-1} (Heslop-Harrison and Heslop-Harrison 1984; Stepka *et al.* 2000).

The *in vitro* conditions required for pollen germination vary between species, but in general the optimum temperatures range from 20 - 30 °C, and the medium composition includes boron (Vasil 1960), calcium and a carbon source such as sucrose, lactose or raffinose (Stanley and Linskens 1974; Cheng and McComb 1992). Pollen tubes growing *in vitro* usually do not reach the length that would be required to effect fertilisation in nature. This suggests that *in vitro* optimal growth conditions are not met, and the role of the style is more than just a tract to the ovary.

When a pollen grain lands on a compatible stigma in nature, it hydrates and germinates to produce a pollen tube. The tube carries the male gamete (generative cell) down the style to the micropyle of the ovary, where fertilisation of the egg cell occurs.

Conospermum amoenum were observed to produce multiple tubes *in vitro*, and tubes elongated rapidly. These experiments aimed to quantify multiple tube emergence and their rapid growth across a range of *Conospermum* species.

7.3 MATERIALS AND METHODS

7.3.1 Pollen source

Several blue-flowered *Conospermum* species were used; *C. amoenum* Meisn. subsp. *amoenum*, *C. spectabile* E.M. Benn., *C. eatoniae* E. Pritz., *C. caeruleum* R. Br. Subsp *caeruleum, C. brownii* Meisn., and the white-flowered *C. incurvum*. All flowers with the exception of *C. spectabile* were collected from the Department of Agriculture, Western Australia research station at Medina, Western Australia. *C. spectabile* flowers were collected from the Stirling Range National Park, also in Western

Australia. Flowers from *Synaphea petiolaris* were collected from Ellis Brook, Western Australia. *Synpahea* is a genus closely related to *Conospermum* and this species was included to see if rapid pollen germination from multiple germ pores was confined to *Conospermum*. The floral structure is similar to *Conospermum*, having an activated pollination system (see Chapter 1.1.4), however the style is positioned upside down in respect to *Conospermum* (Figure 1.2, 1.4)

7.3.2 Observation of *in vitro* multiple pollen tubes.

Fertile anthers were collected from mature *Conospermum* and *Synaphea* flowers prior to dehiscence. Pollen from 2 to 3 flowers was mixed into a drop of pollen germination medium on a slide. Pollen germination medium consisted of 0.01 M H_3BO_3, 0.03 M $CaCl_2.H_2O$ in distilled water and 500 gL^{-1} sucrose. Fluoroscein diacetate (FDA) was added to the germination medium; 100 µl of a stock solution of 0.5 % FDA was added to 5 ml of germination medium (Alexander and Ganeshan 1989). At least 4 slides were examined per species.

7.3.3 Measuring *in vitro* pollen tube growth rate

Conospermum pollen was prepared in germination medium as described above, and germination was immediately recorded with a VHS video recorder under a microscope using 4x and 10x magnification. Rate of growth of tubes, length and numbers of pollen tubes were determined from the videotape using appropriate scales. The time between germination of multiple tubes was also recorded and the average length of pollen tubes was calculated. For all species, the rate of pollen tube growth (μms^{-1}) was measured at 2, 5, 10 and 30 s following tube emergence. Data were analysed with StatSoft Statistica (1999) using repeated measures analysis of variance

(MANOVA). Factors analysed were species, the number of tubes produced (1, 2 or 3) and time (the repeated measures factor). Separate analyses were performed for pollen that produced 1, 2 or 3 tubes. Greenhouse-Geisser epsilons (∈) were used to modify degrees of freedom for all main effects and interactions involving the repeated measures factor, to protect against the possibility of a violation of the sphericity assumption.

In addition, pollen was germinated on cellophane as described by Alexander and Ganeshan (1989), using the pollen germination medium described above. Pollen germination was monitored every 3 - 6 h for 24 h to observe if any pollen which did not initially germinate at a rapid speed, did so slowly over the 24 h period.

7.3.4 Observations of cytoplasm and nuclei movement

Cytoplasmic movement was observed under UV and light microscopy. To observe the vegetative and generative nuclei in *C. amoenum,* pollen from 2 to 3 flowers was placed in a drop of 1 $\mu g ml^{-1}$ DAPI (a'-6-Diamidino-2-phenylindole. 2HCl) in 0.05 M citrate-phosphate buffer (pH 4) and left for 1 - 2 h in a humid container in the dark at room temperature (Vergne *et al.* 1987). Locations of the nuclei were observed using UV microscopy. In some experiments fluorescein diacetate (FDA) was added to the germination medium. 100 µl of a stock solution of 0.5 % FDA was added to 5 ml of germination medium (Widholm 1972).

7.3.5 Observations *in vivo*

Open, untriggered *C. amoenum* and *C. eatoniae* flowers were triggered with forceps and pollinated with self and cross pollen; flowering stems were then covered with

crispy-wrap bread bags. After 5 days, flowers were removed, fixed and stained according to Oddie and McComb (1998), and styles viewed under light and fluorescent microscopy.

7.4 RESULTS

7.4.1 Observations *in vitro*

Conospermum pollen is triporate, and when cultured *in vitro* up to three pollen tubes can develop from one grain. In the first 2 s after germination, pollen tubes grew at 30 - 55 μms^{-1} and showed growth pulses. Tubes continued to grow after 30 s but at a slow rate, and the initial tube(s) continued to grow slowly as the second or third tube emerged. Grains that did not germinate immediately did not do so over a 24 hour period. The sequence of *C. amoenum* pollen tube growth *in vitro* in 500 gL^{-1} sucrose for up to 101 s is shown in Figure 7.1. The time between the emergence of subsequent tubes showed that the second pollen tube emerged 1 – 189 s after the first tube (mean 54 s); the third tube emerged 2 - 106 s (mean 43 s) after the second.

Pollen germination data for grains that produced 1, 2 and 3 tubes were analysed separately. When growth rates of the first, second and third tubes were compared, they were found to be very similar, particularly over the first 2s, and the mean growth rates were not significantly different between these species ($P = 0.806$) (Figures 7.2-7.6).

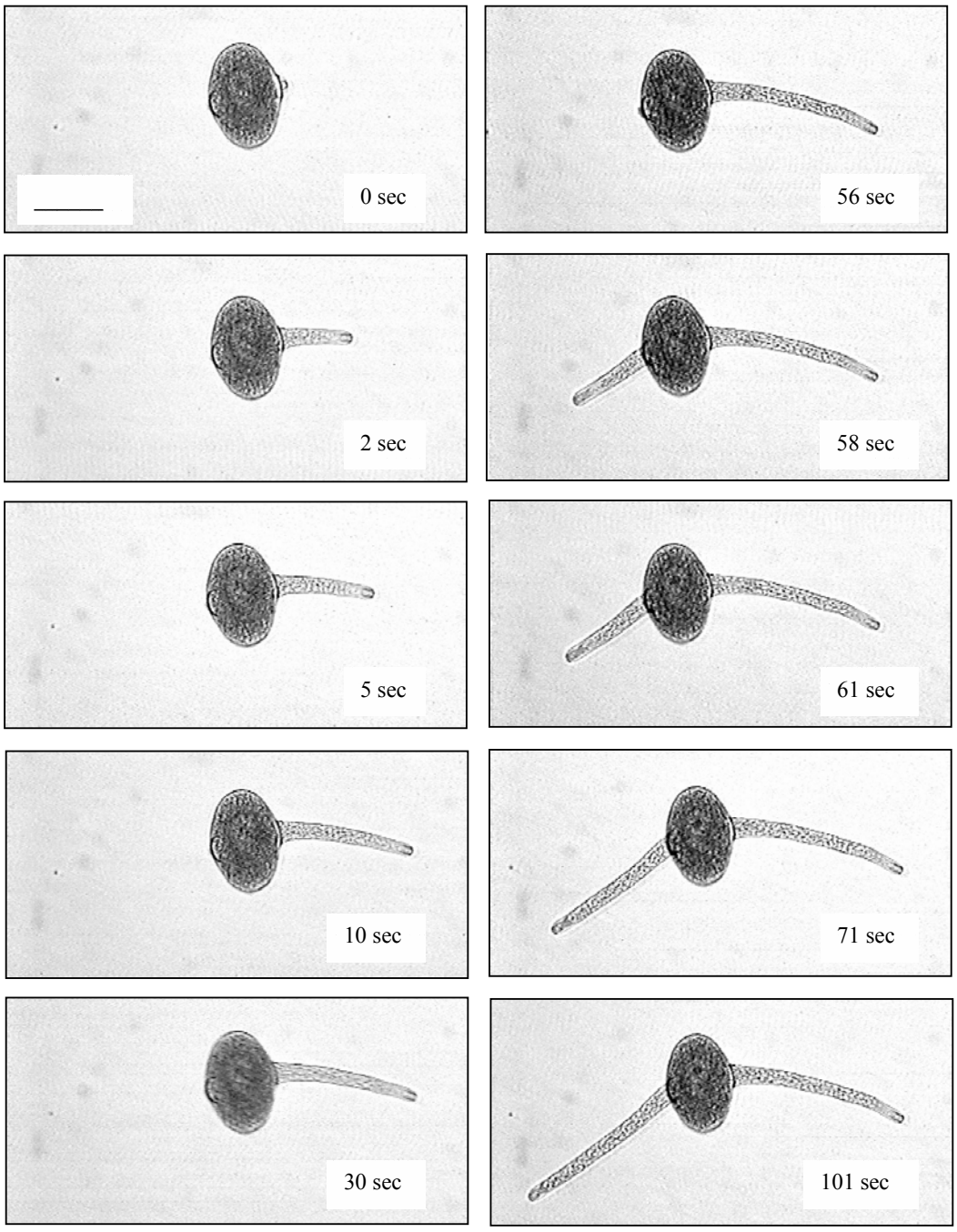

Figure 7.1 *Conospermum amoenum* pollen tube growth up to 101 seconds after initial germination in pollen germination media (0.01 M H_3BO_3, 0.03 M $CaCl_2.H_2O$ in distilled water) with 500 gL^{-1} sucrose. Pictures taken from video recording of pollen germination. Bar = 75 μm.

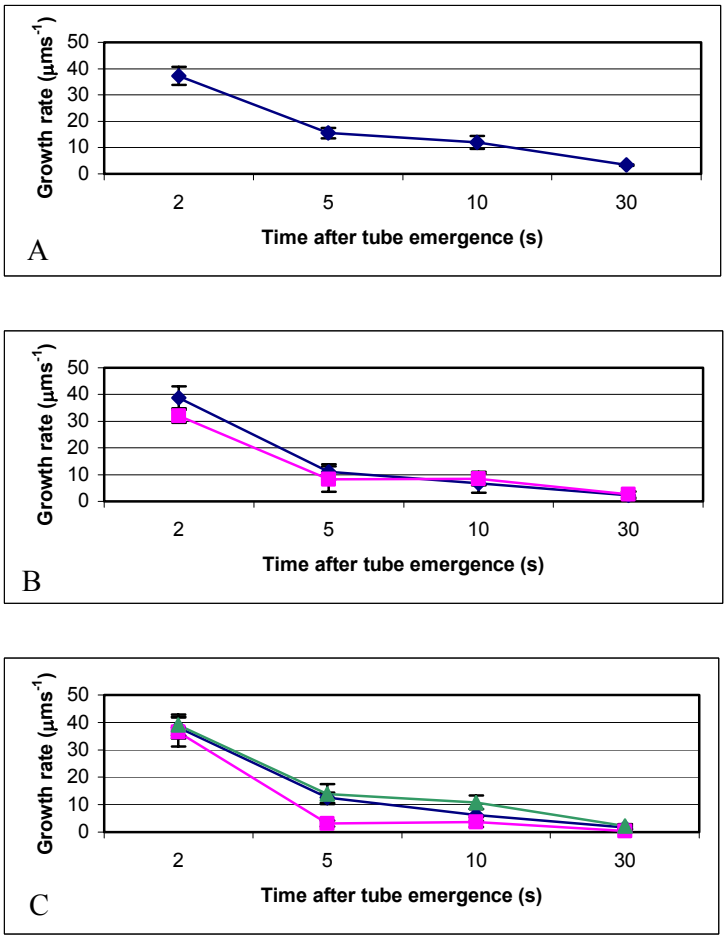

Figure 7.2 Pollen tube growth rates from *Conospermum amoenum* pollen in pollen germination medium with 500 gL^{-1} sucrose. A. Grains producing a single tube (Means from 5 grains). B. Grains producing two tubes (Means from 3 grains). C. Grains producing three tubes (Means from 4 grains). Bars represent standard errors.

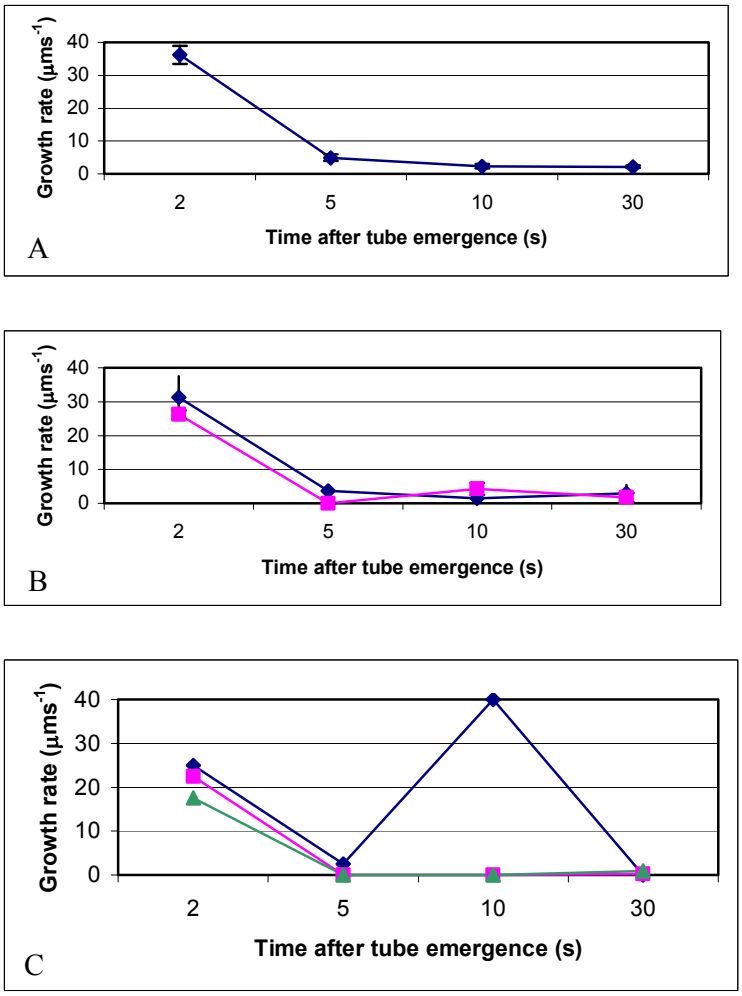

Figure 7.3 Pollen tube growth rates from *Conospermum brownii* pollen in pollen germination medium with 500 gL^{-1} sucrose. A. Grains producing a single tube (Means from 9 grains). B. Grains producing two tubes (Means from 2 grains). C. Grains producing three tubes (1 grain). Bars show standard errors.

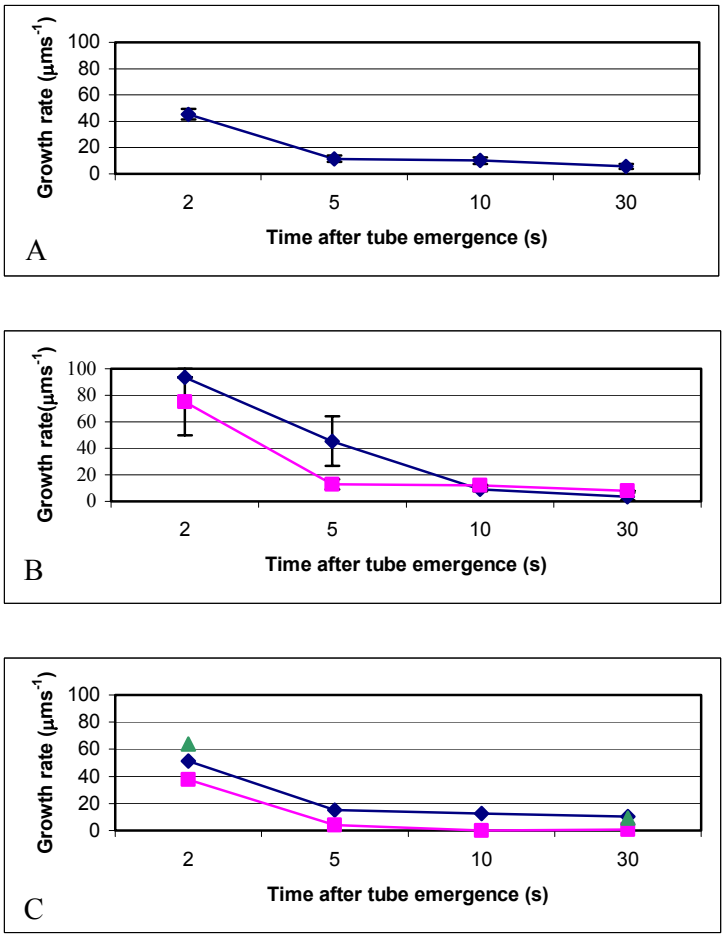

Figure 7.4 Pollen tube growth rates from *Conospermum eatoniae* pollen in pollen germination medium with 500 gL^{-1} sucrose. A. Grains producing a single tube (Means from 4 grains). B. Grains producing two tubes (Means from 2 grains). C. Grains producing three tubes (1 grain). Bars represent standard error.

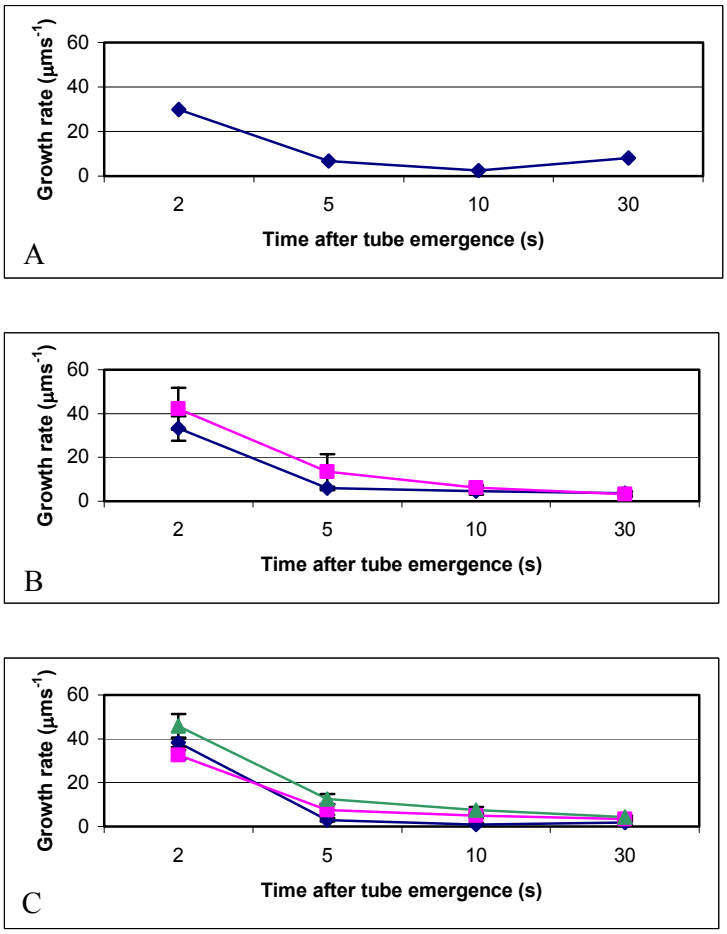

Figure 7.5 Pollen tube growth rates from *Conospermum spectabile* pollen in pollen germination medium with 500 gL^{-1} sucrose. A. Grains producing a single tube (1 grain). B. Grains producing two tubes (Means from 7 grains). C. Grains producing three tubes (Means from 8 grains). Bars represent standard error.

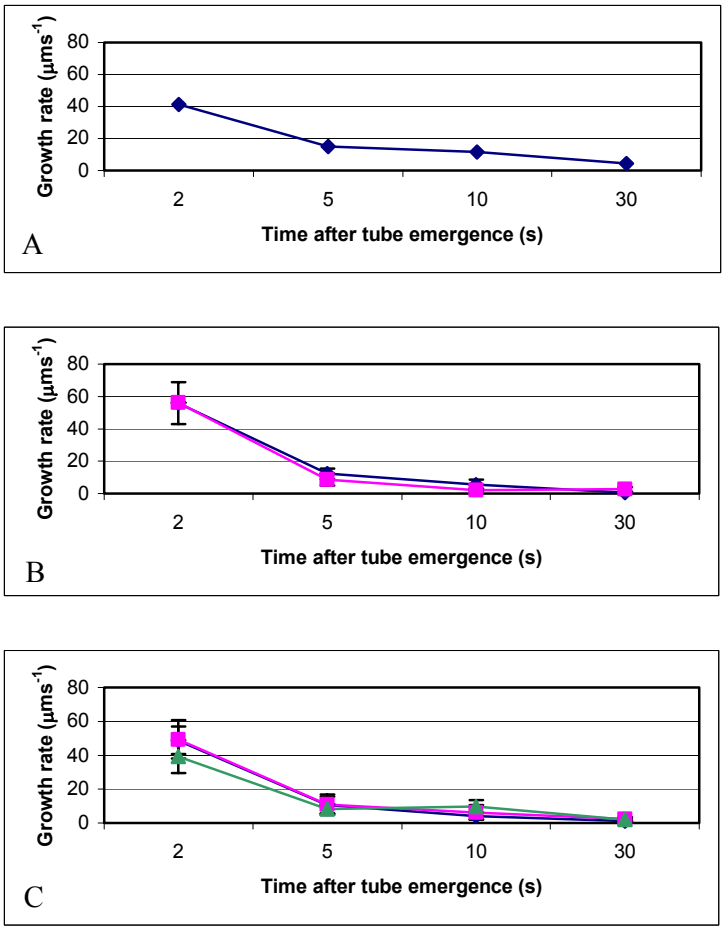

Figure 7.6 Pollen tube growth rates from *Conospermum incurvum* pollen in pollen germination medium containing 500 gL^{-1} sucrose. A. Grains producing a single tube (1 grain). B. Grains producing two tubes (Means from 3 grains). C. Grains producing three tubes (Means from 4 grains). Bars represent standard errors.

Species significantly influenced whether one tube ($P < 0.001$), two tubes ($P < 0.001$) or three tubes ($P < 0.001$) emerged from a pollen grain, which is evident in Figure 7.7. Most pollen from *C. amoenum* and *C. dorrienii* and *C. incurvum* germinated with three tubes, pollen from the rest of the species tested mostly produced a single tube. The number of viable grains that did not germinate was high for *C. eatoniae, C. caeruleum* and *C. incurvum* (45 %, 64 % and 83 % respectively) and 48 % of *C. eatoniae* grains burst without germinating. Inviability of pollen was high for *C. caeruleum* (48 %), *C. brownii* (20 %) and *C. incurvum* (29 %).

The total length of all pollen tubes produced from a grain was calculated for grains that produced 1, 2 and 3 tubes in 500 gL^{-1} sucrose (Figure 7.8). Sum lengths varied significantly between species ($P < 0.001$) and the number of tubes produced significantly increased sum tube length ($P <0.001$) (Figure 7.9).

7.4.2 Observation of cytoplasm and nuclei movement

Pollen tubes had a distinct clear tip region (Figure 7.10). Staining with FDA and illuminating using fluorescence clearly showed the cytoplasm flowing from the pollen grain into each pollen tube. A reversal of the flow could be seen into and out of the pollen grain. This was also visible on the video footage. DAPI staining revealed that the pollen had the normal number of nuclei and that the vegetative and generative nuclei moved into one of the tubes (Figure 7.11).

7.4.3 Observations *in vivo*

Styles of *C. amoenum* and *C. eatoniae* viewed under fluorescent microscopy revealed pollen tubes extending down the style towards the ovary. Pollen germinated from

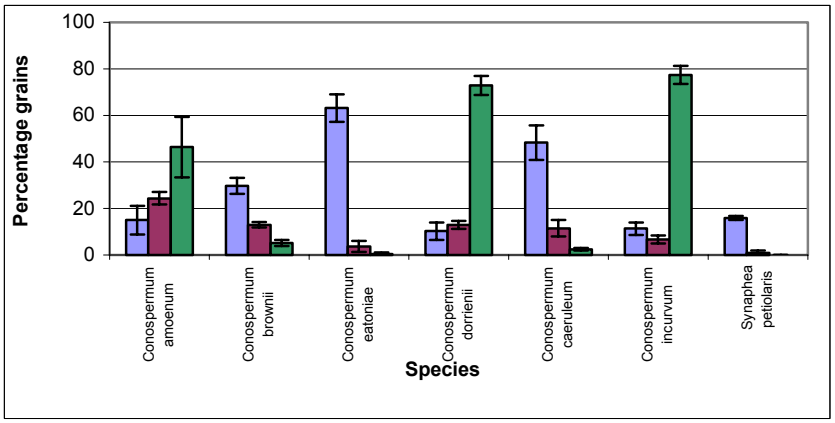

Figure 7.7 Percentage of grains that produced one (▭), two (▭) or three tubes (▭) in pollen germination medium (0.01 M H_3BO_3, 0.03 M $CaCl_2.H_2O$ in distilled water) with 500 gL^{-1} sucrose after three to five minutes, for several species of *Conospermum* and *Synaphea petiolaris.*

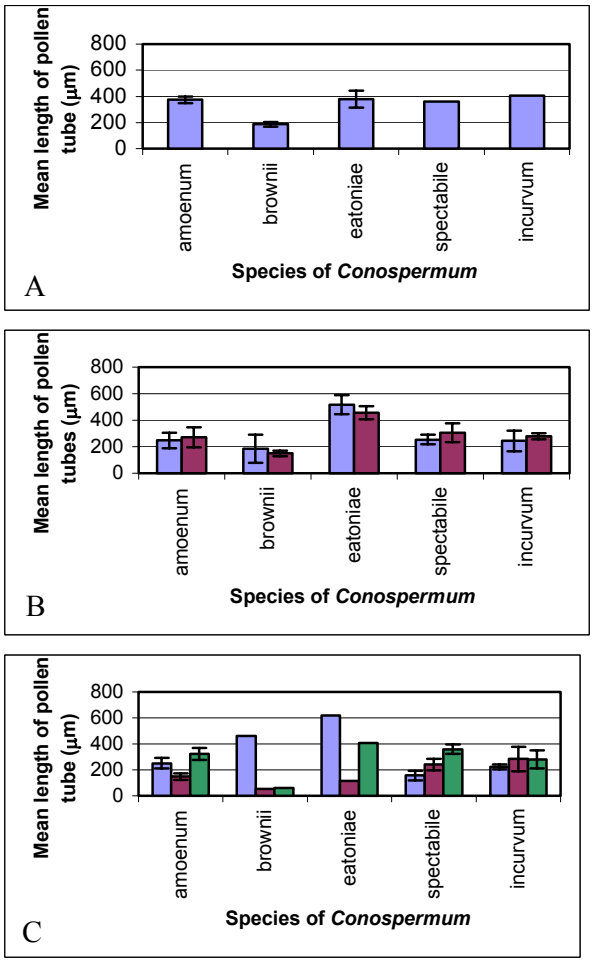

Figure 7.8 Mean length of pollen tubes (µm) in species of *Conospermum* grown *in vitro.* Pollen tube 1 (■), pollen tube 2 (■) and pollen tube 3 (■). A. Grains producing a single tube. B. Grains producing 2 tubes. C. Grains producing 3 tubes. Bars show standard errors. Where there are no standard error bars, only one grain was measured.

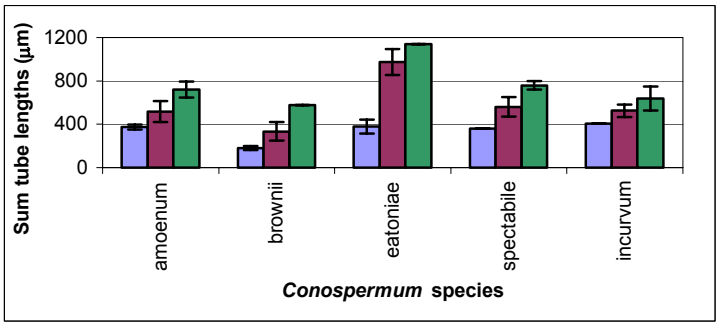

Figure 7.9 Mean total length of all pollen tubes emerging from *Conospermum* pollen grains. (▭) Grains producing a single tube; (▭) Grains producing two tubes; (▭) Grains producing three tubes. Bars show standard errors. Where there are no bars, only one grain was measured.

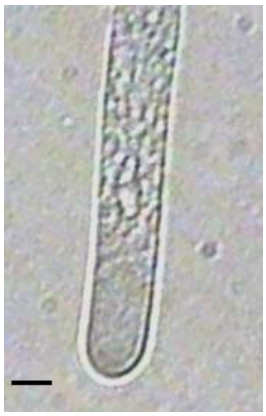

Figure 7.10 Clear tip region of *Conospermum amoenum* pollen tube taken from a video recording of pollen germinating *in vitro*, in pollen germination medium (0.01 M H_3BO_3, 0.03 M $CaCl_2.H_2O$ in distilled water) and 500gL^{-1} sucrose. Bar = 5 μm.

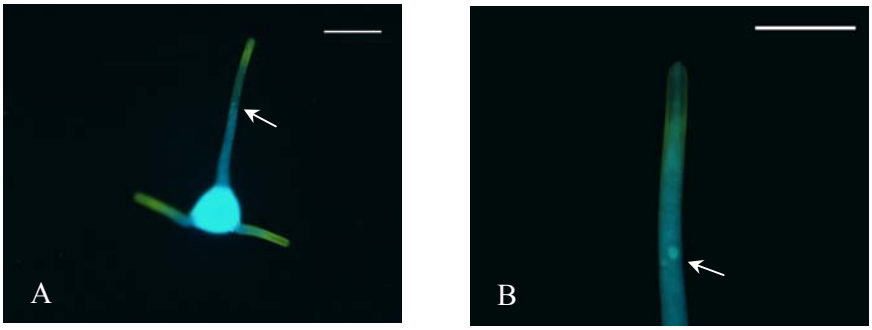

Figure 7.11 A. Germinated pollen of *Conospermum spectabile* stained with DAPI in 300 gL^{-1} sucrose. Arrow indicates white fluorescing nuclei. Bar = 100 μm. B. Pollen tube of *C. spectabile* stained with DAPI in 300 gL^{-1}. Arrow indicates white fluorescing nuclei. Bar = 50 μm.

multiple pores on the stigma in both self and cross-pollination, though only one pollen tube from a grain appeared to enter the style. These multiple tubes could only be seen under light microscopy. For some grains, fluorescence of callose plugs in the tube extending down the style were observed, but never in the second or third tubes to emerge. Many grains showed no fluorescence at all, suggesting that no callose plugs were formed at this stage.

7.5 DISCUSSION

When placed in *in vitro* culture solution with appropriate sucrose content, pollen of most species rehydrates then germinates from a single pore over a period of 2 - 24 hours. The pollen tube extends at a rate typically less than 1 μms^{-1} and stops growing at variable lengths. This type of "normal" germination was never observed in *Conospermum*. The speed of germination and the rate of pollen tube growth at up to 55 μms^{-1} greatly exceeds previously reported rates *in vitro* or *in vivo* (Heslop-Harrison and Heslop-Harrison 1984; Stepka *et al.* 2000). *Conospermum* pollen grains from several species were able to produce up to three pollen tubes that displayed similar rates of growth, and achieved similar lengths.

It was hypothesised that the initial fast growth of the pollen tube could be attributed to the pre-synthesis of cell wall material in the pollen grain prior to germination. The rate of initial pollen tube growth is so fast it would be difficult for the Golgi and other organelles which normally produce cell wall precursors to manufacture, transport and deposit these components. Ultrastructural observations in Chapter 9 support these suggestions.

The total length of pollen tube produced from 2 or 3 pores is greater than from a single pore (Figure 7.3). This suggests that metabolites in the cytoplasm of the pollen grain are more fully transported and utilised when there are 2 or 3 pollen tubes rather than 1 tube, or that resources available to the grain limits the number of tubes to emerge. *In vitro*, multiple tubes were more common than single tubes for *C. amoenum, C. brownii* and *C. spectabile.*

A biological function for multiple tubes has not been identified; however observations of multiple pollen tube emergence on the stigma suggests that these results are not an artifact of *in vitro* culture. Cytoplasmic streaming into and out of all tubes that emerged, and presence of both the generative cell and vegetative nuclei, suggest that these pollen can function normally to deliver the male gamete to the ovary.

The longest pollen tube lengths observed *in vitro* would not be sufficient to enable fertilisation *in vivo*; how these pollen tubes extend to the micropyl *in vivo* remains unknown. Observations 30 s after tube emergence *in vitro* showed a remarkable drop in extension rate, as evident in Figures 7.2 - 7.6, and tubes monitored over 24 hours showed no further growth after the first few minutes. Pollen tubes with callose plugs were observed at the ovary end of the style five – six days after pollination (Chapter 6), so as it extends down the style, the pollen tube must seal off the cytoplasm at the growing tip.

Chapter 8 discusses further experiments that aimed to determine the effects that changes in osmoticum had on growth rates, and the influence of calcium channel blockers.

CHAPTER 8

EFFECT OF SUCROSE AND CALCIUM CHANNEL BLOCKERS ON RAPID GROWTH OF MULTIPLE TUBES IN *CONOSPERMUM*

8.1 ABSTRACT

Conospermum pollen germinates with up to three pollen tubes within seconds of being placed in pollen germination medium, at rates of up to 55 μms^{-1}. Changing the sucrose content of the germination medium influenced percentage germination and the number of tubes to emerge, but did not significantly affect tube extension rates. This was also true for a closely related species, *Synaphea petiolaris.* The addition of calcium channel blockers did not alter pulsatory tube growth or affect the rate of growth. These results further support the hypothesis that pollen tube cell wall components are manufactured prior to germination ready for rapid hydration and germination of the pollen grain.

8. 2 INTRODUCTION

The pollen of *Conospermum* germinates within seconds of being placed in pollen germination medium, and tubes can emerge from each of its three pores at speeds up to 55 μms^{-1} (Chapter 7). This rapid *in vitro* growth is unparalleled in other species, and the consistent emergence of multiple tubes from a single grain is rare (Sniezko 1997). The osmoticum of a pollen germination medium can influence the hydration and germination of pollen. Generally, a hypotonic solution will cause bursting of pollen grains, and media with high sucrose will inhibit hydration and germination

(Heslop-Harrison and Heslop-Harrison 1992). High sucrose levels can also result in leaching of metabolites from the pollen tube into the media (Taylor and Hepler 1997).

The role of calcium in pollen tube growth has been extensively studied (Geitmann and Cresti 1998; Holdaway-Clarke *et al.* 1997; Pierson *et al.* 1994). Intracellular calcium has a steep tip-focused gradient, calcium ions entering the tube at the extreme apex where growth occurs. Ion channels, possibly stretch activated (Pierson *et al.* 1996), regulate the uptake of calcium at this point. Pulsatory growth of pollen tubes was observed in lily by Pierson *et al.* (1995), and was later shown to be linked to oscillations in $[Ca^{2+}]$ (Holdaway-Clarke *et al.* 1997). Other studies using inorganic calcium channel blockers (Geitmann and Cresti 1998), reported that calcium channel blockers prevented pulsatory growth but not tube extension.

The experiments presented here aimed to determine the effects of change in osmoticum and presence of calcium channel blockers on pollen tube growth rate in *Conospermum*.

8.3 MATERIALS AND METHODS

8.3.1 Pollen source

Several blue-flowered *Conospermum* species were used; *C. amoenum* Meisn. subsp. *amoenum*, *C. spectabile* E.M. Benn., *C. eatoniae* E. Pritz., *C. caeruleum* R. Br. subsp *caeruleum, C. brownii* Meisn., and the white flowered *C. incurvum*. All flowers with the exception of *C. spectabile* and some *C. amoenum* were collected from the Department of Agriculture, Western Australia research station at Medina, Western Australia. *C. spectabile* flowers were collected from the Stirling Range National

Park, also in Western Australia. Additional *C. amoenum* flowers were collected from a wild population in Armidale, WA. Flowers from close relative, *Synaphea petiolaris,* were collected from Ellis Brook, Western Australia.

8.3.2 Effect of sucrose on pollen germination and tube growth

Fertile anthers were removed from mature *Conospermum* flowers that were open, but styles were in an untriggered position (see Chapter 1). Pollen from two to three flowers was mixed into a drop of pollen germination medium on a slide. Pollen germination medium consisted of 0.01 M H_3BO_3, 0.03 M $CaCl_2.H_2O$ in distilled water with 300 - 800 gL^{-1} sucrose. At least 4 slides were examined per species. Germination was immediately recorded with a VHS video recorder using 4x and 10x magnification. The rate of pollen tube growth (μms^{-1}) was measured at 2, 5, 10 and 30 s following tube emergence from the pollen grain, from the videotape using appropriate scales. Data were analysed using repeated measures analysis of variance. Factors analysed were species, the number of tubes produced (1, 2 or 3) and time (the repeated measures factor). Separate analyses were performed for pollen that produced 1, 2 or 3 tubes. Greenhouse-Geisser epsilons ($\in$) were used to modify degrees of freedom for all main effects and interactions involving the repeated measures factor, to protect against the possibility of a violation of the sphericity assumption.

8.3.3 Effect of calcium channel blockers

Pollen from *C. amoenum* was germinated in pollen germination medium (see above) with 500 gL^{-1} sucrose, and Aluminium chloride ($AlCl_3$) or Lanthanum chloride ($LaCl_3$) at concentrations ranging from 0 μM to 300 μM. Stock solutions of the channel blockers were prepared in distilled water and stored at 4 °C. Germination

was recorded immediately using a VHS video recorder, and growth rate of pollen tubes determined at 2, 5, 10 and 30 seconds following tube emergence. In addition, tube length was measured in each video frame (24 frames per second) for the first 5 seconds for at least 4 grains per treatment. Data were analysed using repeated measures ANOVA as described previously.

8.4 RESULTS

8.4.1 Effect of sucrose

Pollen germination *in vitro* was observed in media with sucrose concentrations ranging from 100 gL^{-1} to 1000 gL^{-1}. For *C. amoenum g*ermination percentages varied significantly across sucrose concentrations, as shown by ANOVA ($F_{(5,18)}$ = 32.83, P <0.001) with optimum germination at 300 gL^{-1} (Figure 8.1). Pollen was also observed to germinate in water at a rapid rate (not measured); pollen tubes emerged and elongated rapidly, then burst, with cytoplasm streaming out of the burst tip.

Figure 8.2 shows the response of pollen of *C. amoenum, C. brownii, C. eatoniae, C. dorrienii, C. caeruleum, C. incurvum* and *S. petiolaris* in sucrose concentrations from 100 – 800 gL^{-1}. Percentage germination varied significantly across the seven species (P < 0.0001), with *C. brownii* and *C. caeruleum* showing particularly poor germination, regardless of sucrose concentration. An analysis of variance showed that sucrose (P < 0.0001) was a significant factor influencing germination.

The number of tubes to emerge were also significantly influenced by sucrose and species (Figure 8.2), with the exception of grains producing two tubes where the

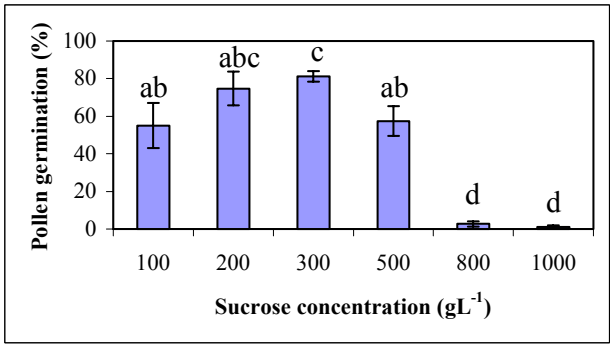

Figure 8.1 Germination of *Conospermum amoenum* pollen 15-30 minutes after being placed in pollen germination medium containing different sucrose concentrations. Bars indicate standard errors. Columns with the same letter are not significantly different ($P > 0.05$).

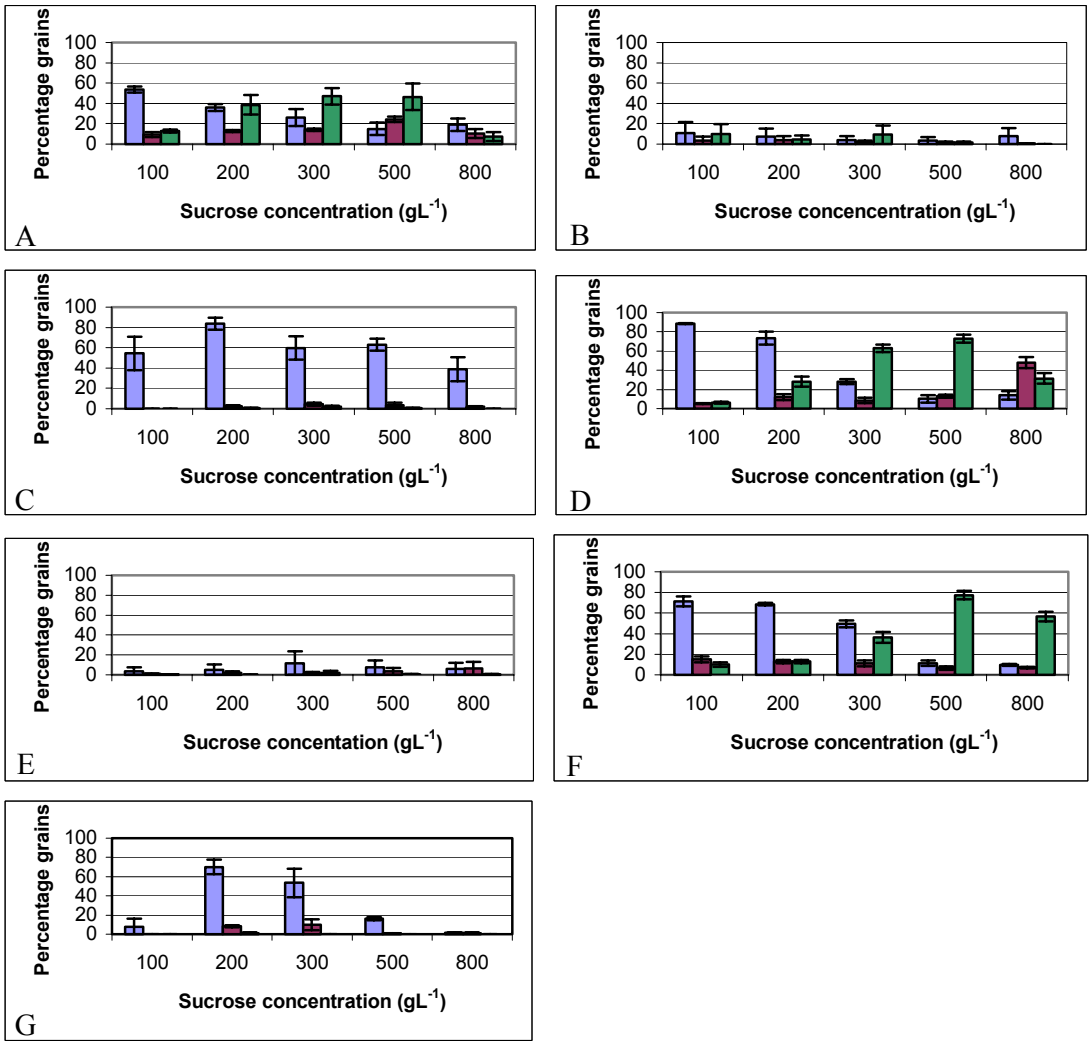

Figure 8.2 Percentage of pollen grains that produced one (□), two (■) or three (■) tubes in pollen germination medium (0.01 M H_3BO_3, 0.03 M $CaCl_2.H_2O$ in distilled water) containing 100 – 800 gL^{-1} sucrose. A. *Conospermum amoenum.* B. *C. brownii.* C. *C. eatoniae.* D. *C. dorrienii.* E. *C. caeruleum.* F. *C. incurvum.* G. *Synaphea petiolaris.*

effect of sucrose was not significant (P = 0.1283). Multiple tubes were common for *C. amoenum, C. dorrienii* and *C. incurvum,* whereas a single tube was most frequent across all sucrose concentrations for *C. eatoniae* (Figure 8.2). Rate of tube growth was not measured for species other than *C. amoenum*, however observations showed very similar patterns of growth and rapid elongation of all tubes that emerged from the other species, including *S. petiolaris.*

With the exception of *C. incurvum* and *S. petiolaris*, the largest percentage of grains that did not germinate was in 800 gL^{-1} sucrose (Figure 8.3). For *C. incurvum*, 100 gL^{-1} sucrose prevented the most grains from germinating (53 %), and for *S. petiolaris* the number of grains that did not germinate was almost identical for 100 gL^{-1} and 800 gL^{-1}. For *C. dorrienii* pollen, up to 95 % germinated across the range of sucrose tested.

Low sucrose (100 gL^{-1}) caused pollen grains to burst in 24 % of *C. eatoniae* and 19 % of *C. caeruleum* (Figure 8.4). For *C. incurvum,* sucrose of 500 – 800 gL^{-1} caused 19 – 28 % of grains to burst. *C. amoenum, C. brownii, C. dorrienii* and *S. petiolaris* were unaffected by change in sucrose concentration with only 0 – 5 % of grains bursting.

An analysis was made of the growth rates of the first, second and third tubes to emerge from *C. amoenum* pollen in different sucrose concentrations, but it was not possible to obtain a complete set of data. Although multiple tubes emerged from pollen grains across a range of sucrose from 100-1000 gL^{-1} (Figure 8.5), in media with less than 300 gL^{-1} sucrose, tubes burst within a minute. Too few grains produced

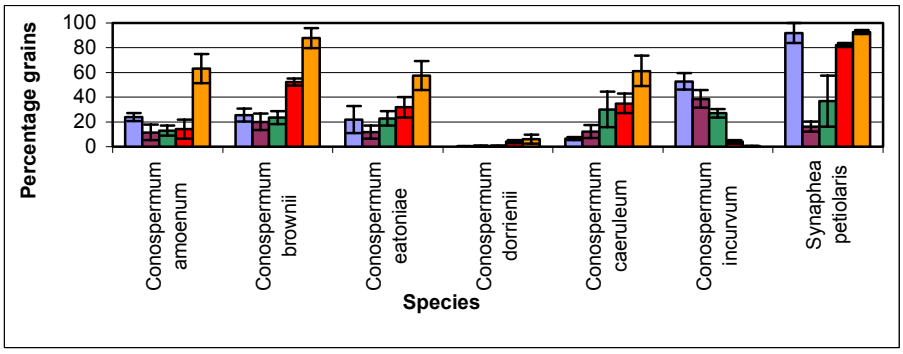

Figure 8.3 Ungerminated pollen grains for several *Conospermum* species and *Synaphea petiolaris* in pollen germination media with: 100 gL^{-1} sucrose (), 200 gL^{-1} sucrose (), 300 gL^{-1} sucrose (), 500 gL^{-1} sucrose () and 800 gL^{-1} sucrose ().

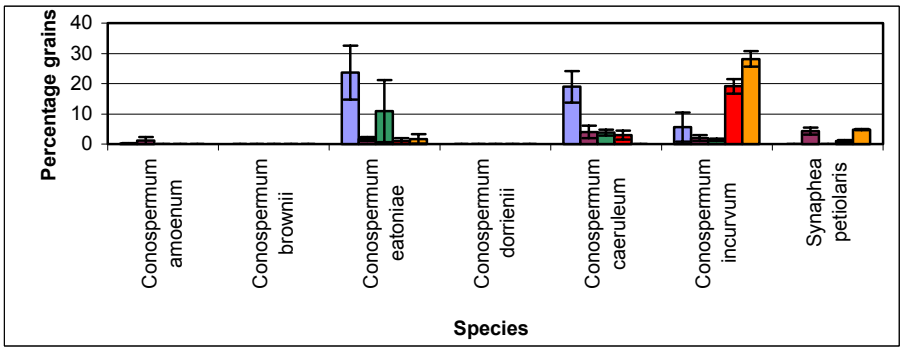

Figure 8.4 Pollen grains that burst for several *Conospermum* species and *Synaphea petiolaris* in pollen germination media with: () 100 gL^{-1} sucrose, () 200 gL^{-1} sucrose, () 300 gL^{-1} sucrose, () 500 gL^{-1} sucrose and () 800 gL^{-1} sucrose.

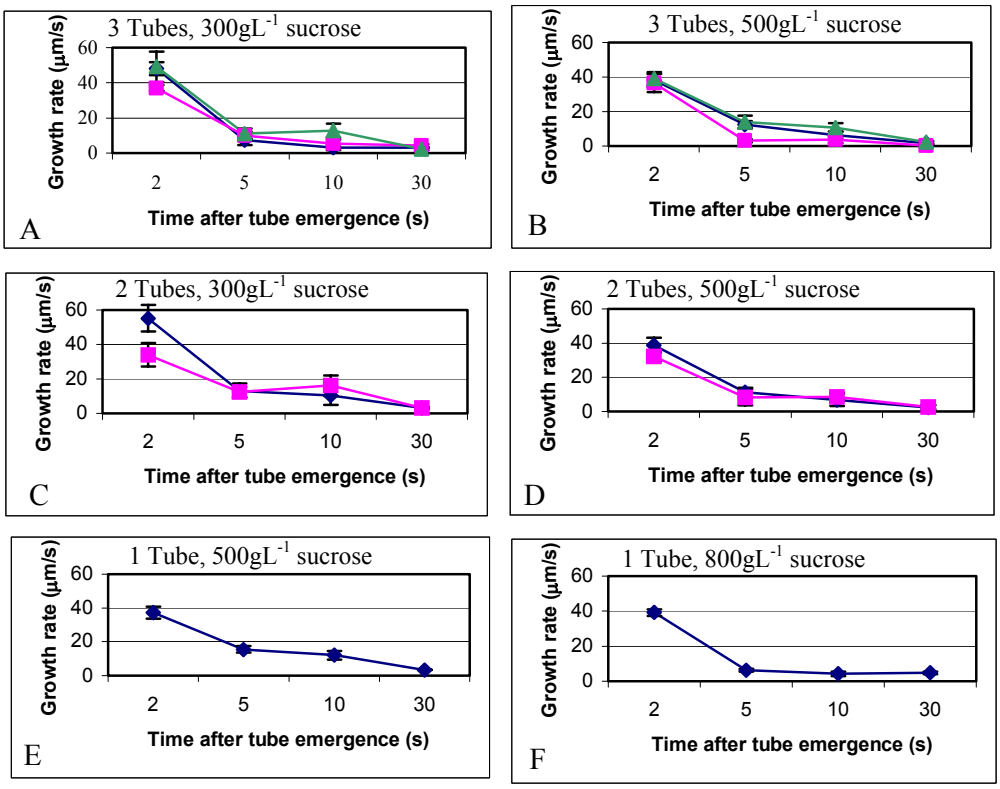

Figure 8.5 Effect of sucrose on *Conospermum amoenum* pollen tube growth rate. A: Grains that produced three tubes in 300gL^{-1} sucrose. B. Grains that produced three tubes in 500 gL^{-1} sucrose. C. Grains that produced two tubes in 300 gL^{-1} sucrose. D. Grains that produced two tubes in 500 gL^{-1} sucrose. E. Grains that produced a single tube in 500 gL^{-1} sucrose. F. Grains that produced a single tube in 800 gL^{-1} sucrose. Pollen tube 1(◆); pollen tube 2 (■); pollen tube 3 (▲). Bars show standard error.

a single tube in 300 gL^{-1} sucrose to enable collection of sufficient data at this concentration. At 800 gL^{-1} sucrose, multiple tubes were rarely produced. A multiple analysis of variance showed that multiple tubes usually grow at similar rates even in different sucrose concentrations, but that there was a significant decrease in growth (P <0.001) over the 30 s.

8.4.2 Effect of calcium channel blockers

Concentrations of 80 μM to 150 μM of $AlCl_3$ and $LaCl_3$ induced no obvious change in pollen tube growth from the control (Figure 8.6), and no significant difference between concentrations of either channel blocker. A closer investigation of the first 5 seconds of pollen tube growth by measuring growth per video frame (24 frames/second) revealed no change to the irregular pulsing growth of the tubes. (Figure 8.7, Table 8.1). Pollen germination solution with 300 μM of both $AlCl_3$ and $LaCl_3$ resulted in the bursting of *C. amoenum* pollen grains.

Table 8.1 Mean number of pulses in 30 s and mean time between pulses of pollen tube growth in the presence and absence of calcium channel blockers.

Treatment	Number of pulses	Time between pulses (s)
Control	14.75	0.26
100 μM $AlCl_3$	15.75	0.26
100 μM $LaCl_3$	13	0.24
P value	0.294	0.636

8.5 DISCUSSION

The emergence of multiple pollen tubes at rates of up to 55 μms^{-1} from a single pollen grain has not previously been observed in any species. In these experiments multiple

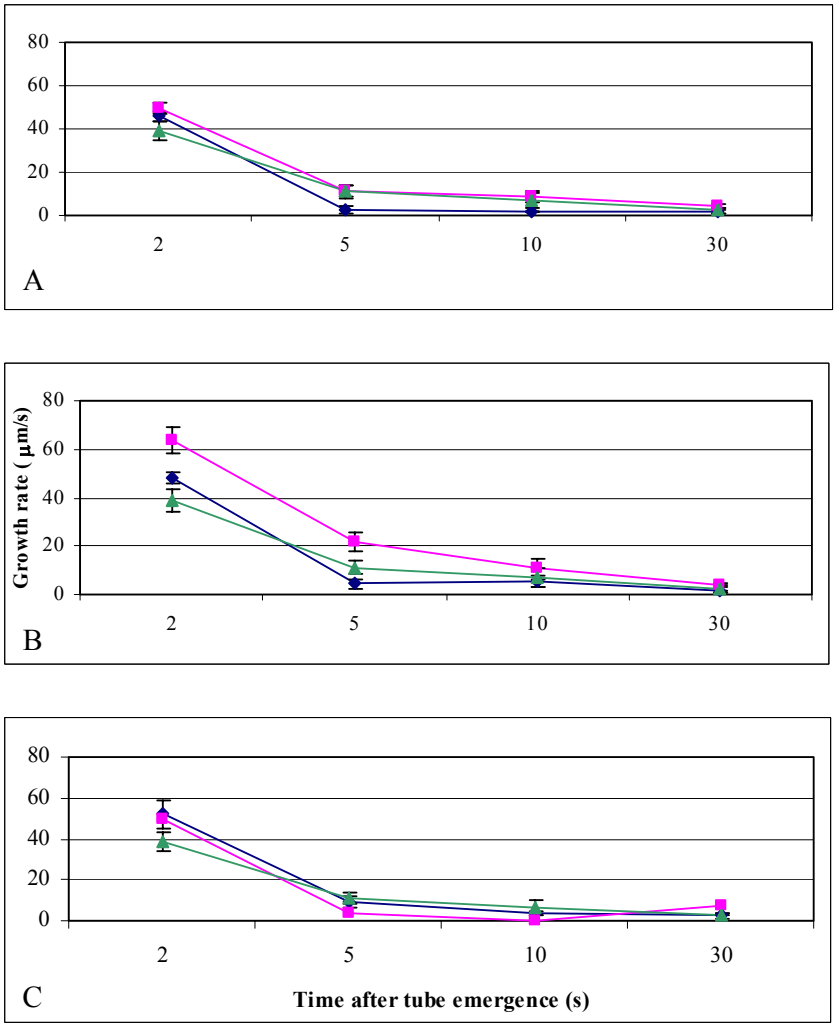

Figure 8.6 Effect of calcium channel inhibitors on *Conospermum amoenum* pollen tube growth in pollen germination medium with 500gL^{-1} sucrose, in grains producing two tubes. Growth of first tube to emerge is shown. A. Channel blockers at 80µM. B. Channel blockers at 100µM. C. Channel blockers at 150µM. Bars show standard error. Aluminium chloride (◆); Lanthanum chloride (■); Control (absence of blockers) (▲).

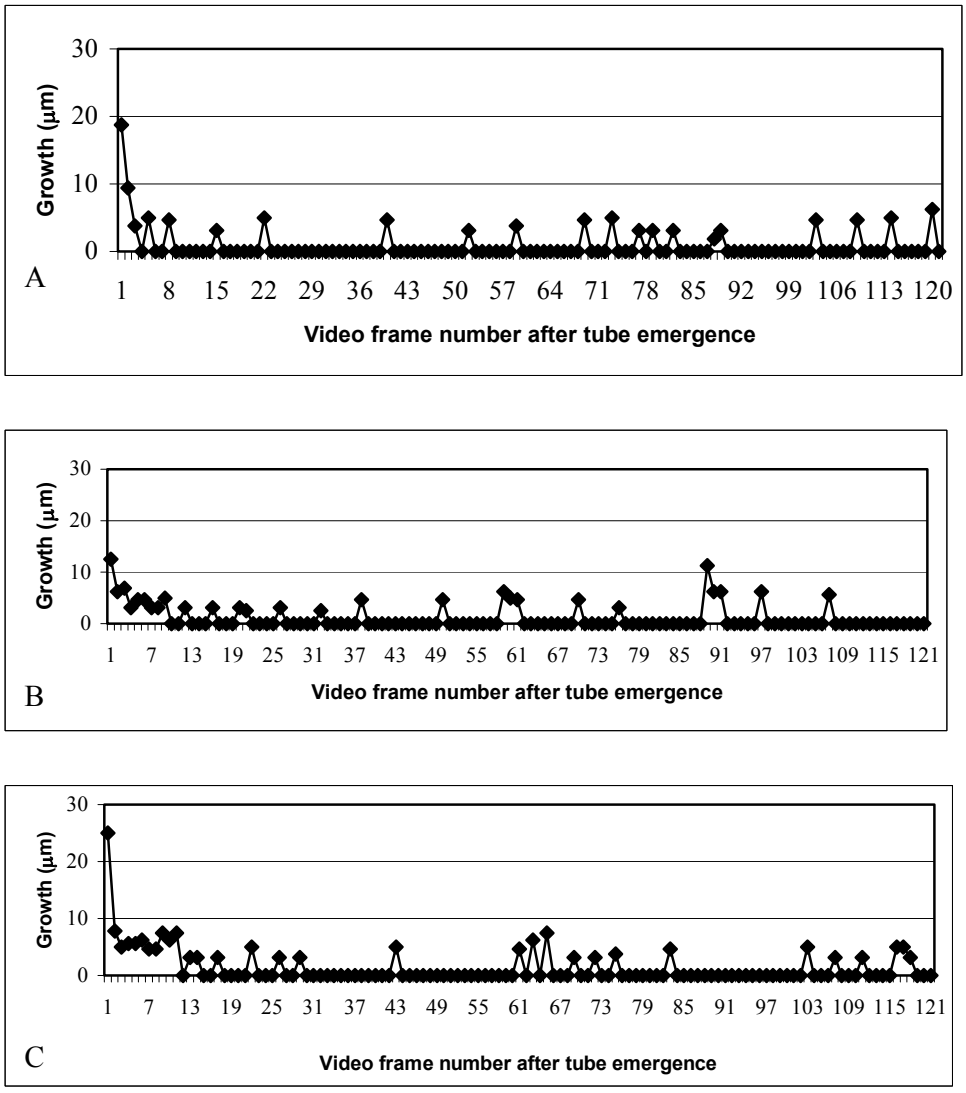

Figure 8.7 An example of the pulsing growth of a *Conospermum amoenum* pollen tube in the presence of: A. no calcium channel blockers. B. 100 µM Aluminium chloride, C. 100 µM Lanthanum chloride, in pollen germination medium containing 500 gL^{-1} sucrose. There were 24 frames per second.

tube emergence was shown to occur in six species of *Conospermum* and a related species, *Synaphea petiolaris*. Change in osmotic pressure of the pollen germination media usually affects germination response, and has been used to manipulate pollen tube growth and to examine pectin ring formation in the cell wall (Li *et al.* 1996). For *Conospermum*, pollen could germinate in 100 – 800 gL^{-1} sucrose, and growth rates were not significantly affected by increasing turgor. The number of pollen grains that germinated was significantly affected by sucrose concentration and species, but for some species, for example *C. brownii* and *C. caeruleum*, germination was poor across all sucrose concentrations. The emergence of multiple tubes was observed in all species, but more consistently in *C. amoenum, C. dorrienii* and *C. incurvum.* The initial growth of *Conospermum* pollen tubes appear unaffected by increasing turgor pressure, which further supports the hypothesis that tube wall components are pre-manufactured ready for germination when the pollen grain hydrates. The ability of the grain to hydrate under such high osmotic pressure needs closer examination to fully understand the influence of sucrose on germination; the molecular make-up of the wet stigmatic surface may give some idea on the dynamics of hydration for this genus.

To gain some understanding of how *Conospermum* tubes grow, attempts were made to slow tube growth during the initial rapid extension (first 5 seconds after tube emergence) using calcium channel blockers. Calcium is known to play an active and important role in pollen tube growth, and compounds that block the channels allowing entry of calcium into the tube have been effective at preventing pulsatory growth. In the case of *Conospermum,* growth was not slowed by either of two blockers, aluminum chloride or lanthanum chloride, and spurts were observed to last an average

of 0.24-0.26 seconds in the presence and absence of the calcium channel blockers. Other studies using inorganic calcium channel blockers took measurements every 15 seconds for up to one hour (Geitmann and Cresti 1998), and reported that calcium channel blockers prevented pulsatory growth but not tube extension. In *Conospermum* the growth rate is 20 –fold faster, and the majority of pollen tube growth occurs in the first 30 seconds, and so much more frequent recordings were required. The pulses observed are likely to be transitory rather than oscillatory (Feijo *et al.* 2001), and it is questionable whether the channel blocking compounds had time to be effective on *C. amoenum*, given the extremely fast germination rate. Further investigation is required into the role calcium plays for *Conospermum* tube growth, and whether or not it is involved at all during the initial rapid growth period.

CHAPTER 9

ULTRASTRUCTURE OF *CONOSPERMUM* POLLEN TUBES

9.1 ABSTRACT

Although *Conospermum* pollen germinates *in vitro* up to 20-fold faster than rates reported for other species, the ultrastructure revealed structural similarities to species that grow at more normal rates. The pollen grain wall was bilayered, with a smooth dense exine and an intine that showed intrusions into the plasmamembrane. The cytoplasm was dense with golgi-vesicles packed with material utilised for inner wall production during tube growth. Golgi-apparatus producing secretory vesicles were observed, and mitochondria were found throughout the tube. The pollen tube did not show the apical zonation that is typical of many species; the tip contained small secretory vesicles, mitochondria and golgi-vesicles. The tube wall was bilayered; both layers were fibrous and loosely packed. The results suggest that *Conospermum* pollen tubes extend by transport of cell wall components to the tip as described for other species.

9.2 INTRODUCTION

The growth of *Conospermum* pollen tubes *in vitro* is exceptionally fast, up to 55 μms^{-1}. It is likely that this remarkable growth is an artefact of *in vitro* culture, however the tubes are able to extend without bursting and appear normal under light microscopy. The ultrastructure of the wall and cytoplasm was investigated to determine whether or not the fast growth results from speeding up the normal processes of pollen tube growth or is a result of an abnormal, different process.

When a pollen grain germinates, the intine of the pollen grain ruptures and the tube emerges with a pectocellulosic wall that is continuous with the intine. As the tube grows, the wall in the apical zone is composed of pectin deposited by secretory vesicles. This is de-esterified and cross-linked by Ca^{2+} to form a rigid framework. Further back from the tip in the sub-apical zone, a second wall layer is formed inside the fibrillar pectin layer. It is primarily callose and cellulose (Ferguson *et al.* 1998).

Pollen tubes typically have distinct zones in their cytoplasm (Franklin-Tong 1999; Heslop-Harrison 1987). The extreme tip has a "clear" zone that is generally free from larger organelles, and is the site of pectin cell wall material transport and deposition by secretory vesicles (p-particles), enabling growth of the tube (Cresti *et al.* 1985; Steer and Steer 1989). There are also coated pits in this region of the cytoplasm which are thought to absorb excess membrane material via endocytosis (Steer 1988).

In the subapical zone, the cytoplasm is rich in mitochondria, golgi-vesicles, endoplasmic reticulum (ER), lipid droplets and dictyosomes (Derksen *et al.* 2002; Lancelle and Hepler 1992), and is metabolically very active. The numerous golgi-vesicles are filled with callose-cellulose wall material which is released when the vesicles merge with the plasmamembrane.

The central or nuclear zone carries the generative cell and vegetative nucleus surrounded by actin filament bundles that are carried along in the cytoplasm. Also present are more mitochondria, other large organelles, lipid droplets and ER (Cresti *et al.* 1980; Pierson *et al.* 1990) generally arranged longitudinally along the main tube

axis. Behind this zone, the tube becomes vacuolated and callose plugs are deposited to maintain the concentration of the cytoplasm in the growing region of the tube.

9.3 MATERIALS AND METHODS

Pollen from *Conospermum amoenum* was collected from recently opened flowers prior to anther dehiscence. Pollen from five flowers was collected in a 1.5 ml eppendorf tube.

Fixation and sectioning of pollen and pollen tubes followed the method of Ferguson *et al.* (1998) with some modifications. Some pollen was fixed immediately in 1 ml Karnovskys fixative (4 % paraformaldehude, 0.1 % guleraldehyde in 50 mM Na-phosphate buffer at pH 7.2) without the addition of 500 gL^{-1} sucrose. Additional samples were germinated in 1 ml of pollen germination solution (500 gL^{-1} sucrose, 1 mgL^{-1} H_3BO_3 and 3 mgL^{-1} $CaCl_2$) for 4 - 5 minutes, then germination solution was drained and germinated pollen fixed in Karnovsky's fixative (with or without the addition of 500 gL^{-1} sucrose). Samples were kept at room temperature for up to 3 hours, then overnight at 4 °C. Tubes were centrifuged at 4000 x g for 3 minutes, and the supernatant carefully removed. Pellets were washed twice in Na-phosphate buffer by resuspension and centrifugation at 4000 x g for 3 minutes. Pollen was then treated in 1 % OsO_4 in half-strength Na-phosphate buffer for 1 hour and washed in distilled water three times following centrifugation at 4000 x g for 2 minutes. Pellets were resuspended in 10 ml of 10 % agar dissolved in distilled water. To resuspend the pellet, 1.5mls was added to each eppendorf tube, which was then added to the remaining 8.5 mls in a 10 cm Petri dish and swirled to give an even distribution of pollen and pollen with tubes. Agar was allowed to set at room temperature. Petri

dishes were examined under a dissecting microscope to locate pollen or germinated pollen, and these were excised with a scalpel. Excised agar sections were dehydrated through a series of 30 – 100 % acetone before embedding in Spurrs resin (Spurr 1969) using a series of infiltration steps of 10 – 100 % resin for 2 hours each, and a final infiltration overnight in 100 % resin.

9.4 RESULTS

Germinated pollen of *Conospermum* had two distinct layers, a bilayered exine, and a thick intine. The ectexine and endexine appeared smooth, dense and homogeneous, and were of identical electron densities. A vacuolated area separated the two, the ectexine being discontinuous in places. The intine was 4.8 -11.2 μm thick, and up to 22 μm at the pores. The inner surface of the intine was not smooth but had large intrusions into the cytoplasm (Figure 9.1)

The cytoplasmic contents of the vegetative cell appeared similar to other species with some starch storage evident, but also very high numbers of golgi-vesicles distributed throughout the pollen grain and tube (Figure 9.2). Mitochondria, endoplasmic reticulum, and lipids were also present. Dictyosomes were present but uncommon. Figure 9.3 from a region midway along the tube shows a dictyosome with 5 cisternae, and 2 secretory vesicles present on the *cis*-face, and many mitochondria. Sections taken from the point of emergence from the grain to approximately the subapical region showed similar cytoplasmic contents, no zonation was evident (Figure 9.4). Callose plugs were not deposited, nor was the tube vacuolated near the grain in tubes up to 300 μm long. Sections taken near the tip showed abundant smaller secretory

vesicles of 55 nm diameter, golgi-vesicles fusing with each other, and many mitochondria (Figure 9.5)

The abundant golgi-vesicles appear to be carrying the inner callose/cellulose cell wall material, and can be seen in various states of fusion with each other, and in contact with the pollen tube membrane (Figure 9.2). They range in size from approximately 0.17 μm to 0.42 μm. Large vacuoles were also observed that appear to be filled with cell wall material and were up to 3.6 μm in diameter.

The wall of the pollen tube was bilayered, and appeared continuous with the intine of the grain. The outer layer consisted of a loosely bound matrix of fibrous, textured material. The inner layer was of even more dispersed fibrils, but was made up of material similar to the outer wall but less dense (Figure 9.5, 9.6). The wall was wrinkled in both longitudinal and transverse sections (Figure 9.6). This wrinkling is likely to be an artefact of fixation.

The addition of sucrose to the fixative medium greatly improved the integrity of the pollen tube wall and cytoplasmic contents. Fixation of pollen tubes without an osmoticant revealed a disorganised cytoplasm, and a disrupted plasmamembrane and cell wall (Figure 9.7).

Figure 9.1. *Conospermum amoenum* pollen grain wall showing smooth homogenous ectexine (EC) and endexine (EN), and intine (I) with intrusions into the cytoplasm. Bar = 1 μm.

Figure 9.2. An abundance of golgi-vesicles in the pollen tube in various states of fusion with each other (A) and the plasmamembrane (B). Bar = 1 μm.

Figure 9.3. Cytoplasm of pollen tube showing a dictyosome with secretory vesicles attached (D), many mitochondria (arrowheads) and a large starch grain (S). Bar = 1 μm.

Figure 9.4. Germinating *Conospermum amoenum* pollen grain. Note the tube wall (T) appears continuous with the pollen grain intine (I). Bar = 10 μm.

Figure 9.5. Pollen tube near the tip. Secretory vesicles are numerous (arrows) as are mitochondria (arrowheads). Larger golgi-vesicles are also present. Bar = 0.5 μm

Figure 9.6. Transverse section of a pollen tube showing a wrinkled pectic outer layer of the cell wall (O) and loosely bound fibrils of the inner callose/cellulosic tube wall (I). Bar = 1 μm.

Figure 9.7. Pollen tube fixed with Karnovsky's fixative in the absence of sucrose. Cytoplasm is highly disorganised and the cell wall disrupted (W). Bar = 1 μm.

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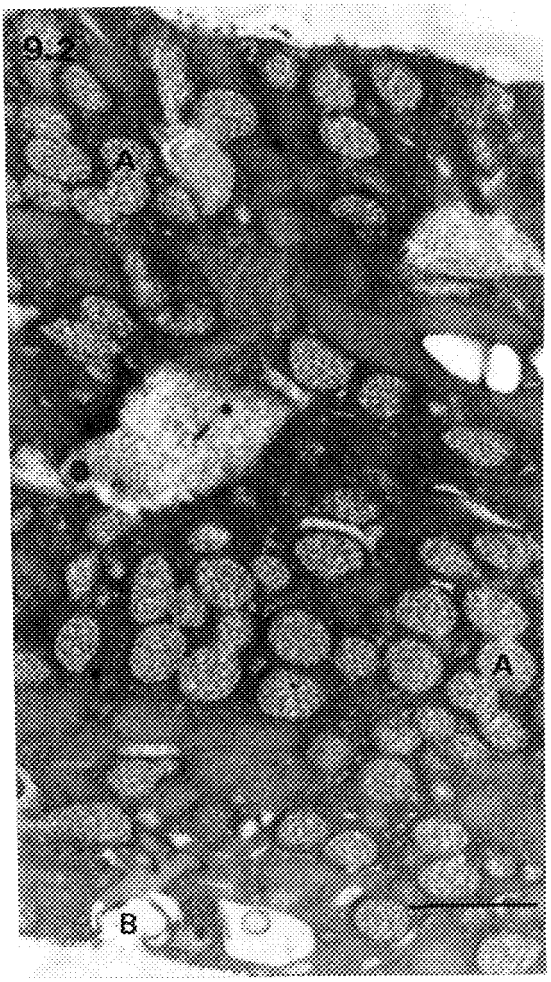

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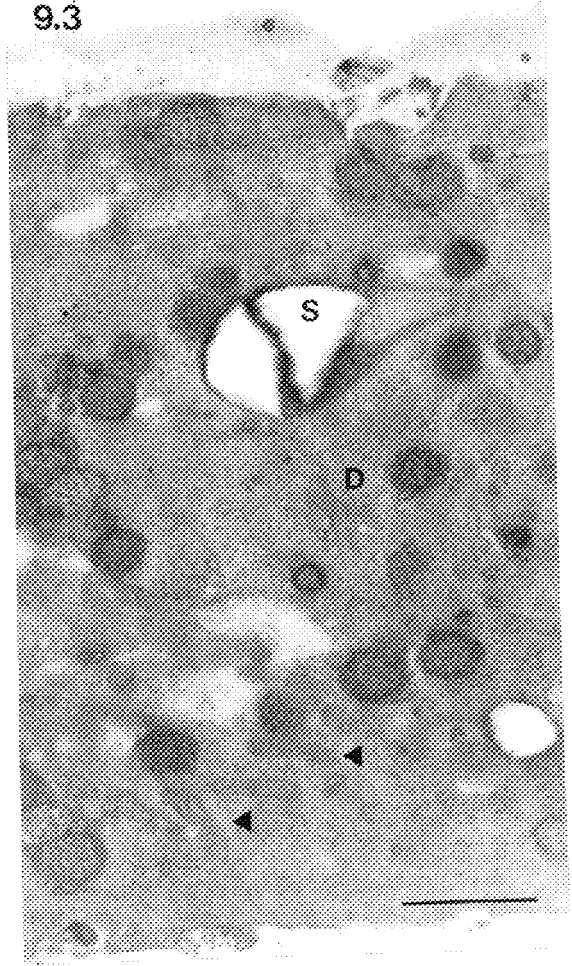

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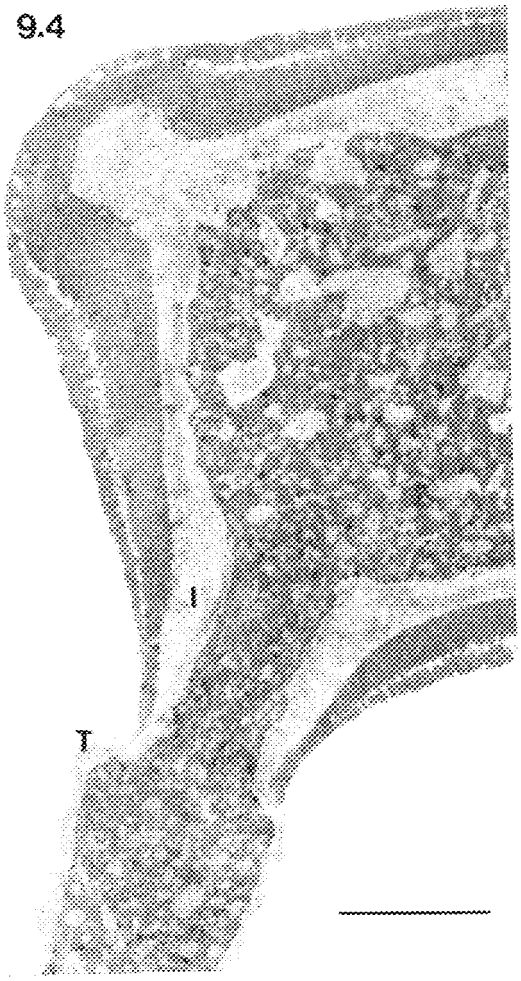

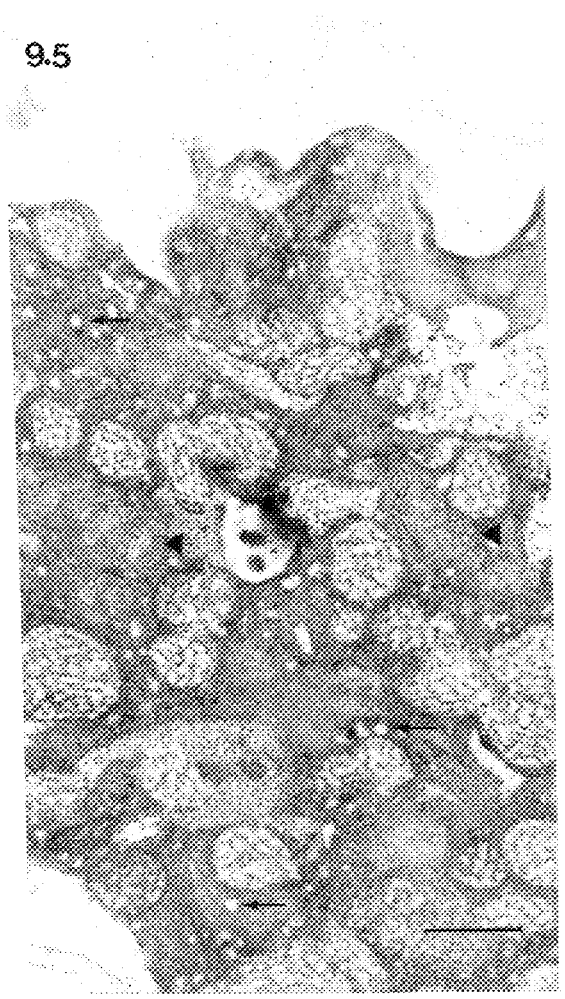
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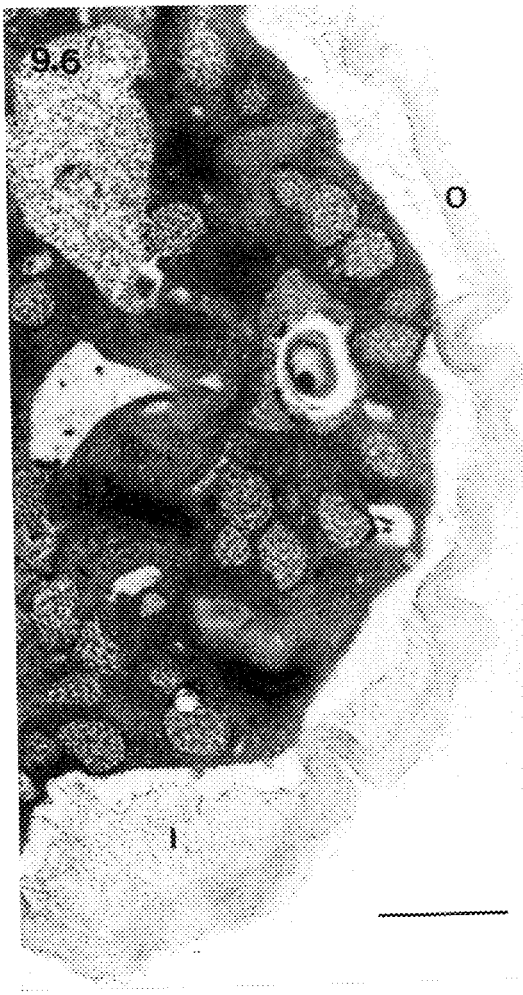
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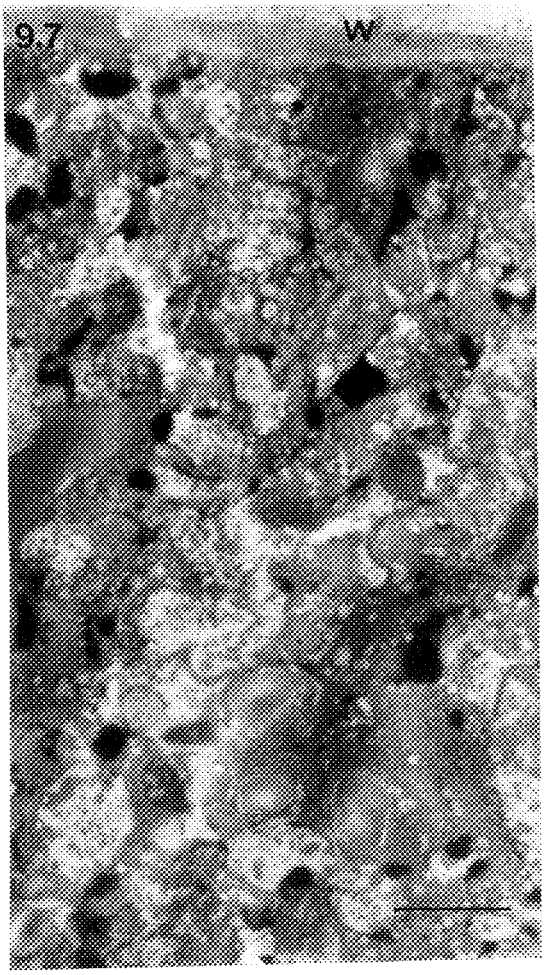
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9.5 DISCUSSION

Although the speed of germination and elongation of *Conospermum* pollen tubes is unparalleled in other species, tube ultrastructure indicated a normal discrete cell wall and plasma membrane, and cytoplasmic contents similar to those reported in species such as *Lilium* (Lancelle and Hepler 1992), *Nicotiana* (Cresti *et al.* 1985) and *Lycopersicum* (Cresti *et al.* 1980). The lack of distinct zones in the cytoplasm however, is consistent with grasses such as *Secale cereale* L. cv Rheidol and *Pennisetum typhoideum* Rich. (Heslop-Harrison and Heslop-Harrison 1982), as is the lack of dictyosomes observed.

It appears mature *Conospermum* pollen grains have a large quantity of cell wall material stored in golgi and secretory vesicles prior to germination. The small secretory vesicles seen near the tube tip must be rapidly carried to the tip of the tube to release their contents by exocytosis when the grain germinates, to allow the extension of the pollen tube at rates of up to 55 μms^{-1}. The transport and deposition of this wall material must occur at remarkable speed, as a more "normal" growth rate of pollen tubes is up to 3 μms^{-1} (Stanley 1971). The secretory vesicles (or p-particles) found near the tip are of similar size to other species, for example, the average diameter of p-particles at the tip for *Nicotiana tabacum* is 195 nm (Derksen *et al.* 1995) and for *Arabidopsis thaliana*, 85 nm (Derksen *et al.* 2002). Heslop-Harrison and Heslop-Harrison (1982) however, noted that for grasses, p-particles may occupy up to 30 % of the grain volume prior to germination, stored to enable rapid initial growth. These particles are distributed throughout the growing tube during this early phase resulting in a lack of apical zonation seen in other species. This also appears true for *Conospermum.*

The abundant golgi-vesicles appeared packed with inner cell wall material that is deposited when the vesicles fuse with the plasmamembrane, a process that must also occur rapidly once the tube emerges. The density of golgi-vesicles is much higher than in species such as *Tradescantia* but similar to that seen in *Impatiens* (Cresti *et al.* 1992). The content of these vesicles is thought to be wall material due to similar electron densities.

Pollen tubes were examined from the point of emergence from the pollen grain, to close to the tube tip. The generative cell and vegetative nuclei were not observed, nor were any callose plugs. Other studies on *Conospermum* have revealed that callose plugs are not readily observed and that the generative cell and vegetative nuclei are small (Chapter 4). A vacuolated region was not found for *Conospermum,* which is in contrast to observations from other species. It may be related to the fact that when multiple tubes arise from the second and/or third germ pore of *Conospermum* pollen, they grow at similar initial rates to the first tube to emerge (Chapter 7).

The optimal method for fixation of pollen tubes is freeze-fixation and substitution, which minimises cellular disruption and enables a clearer view of the cytoplasm and cell wall structures. This was not available for these experiments; however future comparisons between chemical and freeze-fixation would be beneficial in determining the precise make up of the pollen tube wall, and the identification of microtubules and actin filaments. This may provide greater insight into how *Conospermum* pollen can elongate so rapidly. A comparison between chemical fixation, and rapid freeze-fixation and freeze-substitution (RF-FS) in *Lilium longiflorum* by Lancelle and Hepler

(1992) showed much higher detail could be achieved using RF-FS. This method enabled identification of organised "slow" and "fast" lanes in the cytoplasm, microtubule and microfilament bundles and their arrangement and many other improvements over the knowledge gained from chemical fixation. Lancelle *et al.* (1986) concluded that rapid freeze-fixation is superior for preservation of cells that exhibit motility, such as cytoplasmic streaming in pollen tubes.

The ultrastructure of pollen tubes fixed with 500 gL^{-1} sucrose in the fixative indicated that the fast elongation of pollen tubes involves exceptionally fast wall deposition but these tubes show structural similarities to tubes of other species that grow at slower, more normal extension rates.

CHAPTER 10

GENERAL DISCUSSION

10.1 Problems in propagation of *Conospermum*

The desire to propagate blue-flowered *Conospermum* species to satisfy demand from the cut-flower industry and to help in the conservation of these increasingly rare species was the impetus behind this study. It has been shown here that *Conospermum* has some novel aspects of reproduction.

The selections of *Conospermum* suitable for horticulture, particularly the blue-flowered species, had been shown to be difficult to propagate conventionally or through micro-propagation. Somatic embryogenesis was considered a highly desirable alternative method of clonal production, having the potential to produce many hundreds of clonal plants from a single original explant. In addition, the presence of a root and shoot apex on a somatic embryo would reduce the problematic root initiation process and decrease production times.

10.2 Responses *in vitro*

A broad survey of explant tissue types was carried out for *C. eatoniae* and *C. caeruleum* (Chapter 2) in an attempt to identify possible sources of cells that could be induced into embryogenic pathways. The development of *Conospermum* flowers in plants takes place on leafless stems for many of the species, over a period of months, beginning with vegetative buds that remain dormant from February, until June when they begin developing into immature, then mature floral buds. Floral explants were tested at various stages of development for their ability to produce somatic embryos,

but all were unsuitable as explants, producing shoots, callus or dying in culture. Callus formed on leaves from *in vitro* shoot cultures in the presence of 2,4-D and BAP, but showed virtually no differentiation in culture, despite tests with a wide array of growth regulator combinations and concentrations (Chapter 3). In addition, immature zygotes were consistently unresponsive in culture and died. Direct embryogenesis and/or embryogenic callus were observed from mature zygotes of the species *C. caeruleum, C. spectabile, C. dorrienii* and *C. brownii*. Embryogenesis was able to be maintained in culture only for *C. caeruleum.* The failure to obtain somatic embryos from vegetative tissues means that the technique has limited value for commercial production. However, recent improvements on induction of *in vitro* root formation on microcuttings of *C. eatoniae* by increasing medium aeration (Newell *et al.* 2003) could be explored for rooting of somatic embryos.

The results from this survey indicated that developmental pathways in *Conospermum* are well defined and are not easily altered *in vitro.* A similar problem was encountered in attempts to induce maturation and germination of somatic embryos (Chapter 4). Once the developmental pathway for embryogenesis was turned on in *C. caeruleum*, it was difficult to switch to one of germination. An array of techniques used successfully in other species to synchronise embryo development and induce germination were unsuccessful here. These included desiccation, chilling, and treatments with mannitol and ABA. Clearly the maturation and germination of somatic embryos is essential for the technique to have practical application.

Somatic embryo production in *C. caeruleum* occurred through direct, indirect and secondary embryogenesis. Analysis of the genetic stability of the embryos after 12

months in culture showed that 5 % somaclonal variation was evident. Although it is not certain that the genetic changes would result in obvious morphological or physiological mutants, this level of somaclonal variation is unacceptable in a commercial setting. The production of clonal plants of known genetic and morphological make-up is essential for consistent production of high quality flowers. If possible, secondary embryogenesis and the number of subcultures that embryogenic calli undergo should be minimalised, perhaps by initiating new zygotic embryos from stored seed every six months. It is not known when mutations occurred to produce the somaclonal variants observed, but a future study tracking subcultures and the genetic fidelity of the new embryos would give a more precise timeline for when fresh cultures should be initiated.

10.3 Future *in vitro* work

Further experiments on inducing embryogenesis, and stimulation of conversion of somatic embryos to plantlets in *Conospermum* should include a wide range of initial genotypes, as it was only possible to screen one or a limited number of genotypes in these experiments. Genotypic barriers to certain developmental pathways have been reported in other species, and may be a reason for the lack of success reported here. This study has laid the foundations for future work, by screening a small number of genotypes with a large number of methods and techniques.

10.4 Floral biology of *Conospermum*

Controlled pollinations of *C. eatoniae* showed pollen tubes extending down the style for both self and cross pollen in two genotypes, however no seed set. Thus *C. eatoniae* appears self-compatible but post-zygotic incompatibility could not be ruled

out. The loss of flowers has two possible explanations; the method of hand pollination may have damaged floral parts causing the flower to be shed, or the very low natural flower/fruit ratio meant that statistically the loss was to be expected from the natural level of flower shedding.

A previous study on *Conospermum* species from Eastern Australia showed that they were unable to self-fertilise in the absence of insect pollinators, and that some cross-incompatibility existed between populations of *C. longifolium*, but not *C. taxifolium* *C. ellipticum* or *C. ericifolium* (Morrison *et al.* 1994). It was not stated if these species were self-compatible, but results from Chapter 6 indicate that *C. eatoniae* is self-compatible.

DNA analysis of open-set seed progeny from two *C. eatoniae* genotypes growing as rows of clonal selections in a field station showed that plants primarily self-pollinate. This is in contrast to progeny from two plants from a wild population, where pollen from several donors successfully fertilised flowers to set seed (Chapter 6). The high level of inbreeding at the field station was likely due to the foraging behaviour of insect pollinators along the clonal rows, rather than incompatibility. The genetic diversity in the wild population was captured in the seed progeny, but was limited by the diversity in the population itself. It appears that *C. eatoniae* has limited genetic diversity at the species level. In a commercial setting, self-pollinated seed are reliably obtained by planting in clonal rows, whereas cross-pollinated seed would be maximised by surrounding the maternal plant with the desired paternal plants.

10.5 Pollen biology

In several *Conospermum* species, up to three pollen tubes emerged from the triporate pollen, and grew at a rate that was over 20 times faster than previously recorded in any species. Rapid germination with a single tube were reported in Poaceae where growth rates of up to 2 μms^{-1} have been observed just minutes after hydration, but *Conospermum* extended at up to 55 μms^{-1} for up to 2 seconds.

Various sucrose concentrations and calcium channel blockers were added to the pollen germination medium to try to stop or slow tube growth. Usually, low osmotic pressure causes pollen to burst, and high osmotic pressure prevents hydration and subsequent germination (Heslop-Harrison and Heslop-Harrison 1992). *Conospermum* was able to germinate in sucrose ranging from 100 – 800 gL^{-1}. Although the rate of tube extension was unaffected by a change in sucrose concentration, percentage germination and the number of tubes that emerged were influenced significantly (Chapter 8).

Calcium has a variety of essential roles in plants, and in pollen germination. It is well established that there is a tip-focused calcium gradient in the pollen tube, calcium ions entering through a variety of channels in this region, some thought to be stretch activated (Pierson *et al.* 1996). Pollen tubes in some species extend in pulses, which were shown to accompany oscillations in calcium ions (Holdaway-Clarke *et al.* 1997). By using calcium channel blockers, pulsing tube growth was inhibited in lily, causing tubes to extend at a constant rate. Closer observation of *Conospermum* tube growth showed pulsing growth; however these growth spurts lasted a fraction of a second, and are unlikely to be related to calcium influx. This conclusion was further

supported by the absence of a change in tube growth with the addition of calcium channel blockers to the *in vitro* growth medium (Chapter 8).

Conospermum shows greater ultrastructural similarity to Poaceae pollen than to other species reported in the literature. Few golgi-apparatus were identified, and the cytoplasm appeared packed with vesicles filled with material of similar electron density to the cell wall. Ultrastructural observations of grass pollen showed that golgi-apparatus are active during the last stages of pollen development in the anther prior to desiccation, and produce large amounts of vesicles filled with polysaccharides ready for tube extension via the tip. As a consequence, the extending tube actually has very few golgi in the cytoplasm (Heslop-Harrison and Heslop-Harrison 1982).

In species such as lily distinct zones in the cytoplasm exist, that are not observed in Poaceae or *Conospermum* (Pierson *et al.* 1990, Franklin-Tong 1999). In lily, the extreme tip region is free of large organelles and is the site of cell wall deposition. In *C. amoenum,* the apical end of the tube contained larger vesicles filled with cell wall material, smaller secretory vesicles and mitochondria. The subapical zone in lily is rich in mitochondria, golgi-vesicles, endoplasmic reticulum, lipids and golgi-apparatus. This region is very metabolically active, where numerous golgi-apparatus in the cytoplasm produce cell wall material as the tube grows. Few golgi were identified in *C. amoenum*, and no zonation was evident. The generative cell and vegetative nucleus were not located in *C. amoenum.* In lily, the central zone carries the generative cell and vegetative nucleus surrounded by actin filament bundles. The region behind this becomes vacuolated as callose plugs are deposited, maintaining a high concentration of cytoplasm at the growing tip.

The optimal method of fixation of tubes, freeze fixation and substitution, was unavailable for this study, however the addition of sucrose to the chemical fixative greatly improved the integrity of the cytoplasm and cell wall. Future studies would ideally use freeze-fixation, as several studies have shown superior preservation of the cytoplasmic contents. For example, microtubules were not observed in *C. amoenum*, but have clearly been seen in other species (Cai *et al.* 1997; Cai *et al.* 2000).

10.6 Possible biological significance of unusual pollen behaviour

A biological reason for producing multiple tubes was not elucidated, but may involve adhesion to the stigma or prevention of further pollination once an initial pollen load has been deposited. The stigma is cup-shaped and the exudate covers the stigmatic opening and the upper lip. Pollen adheres easily to this surface, and becomes very difficult to observe *in vivo* when surrounded by the exudate. *Conospermum* pollen is large relative to the stigma size, and so few grains can adhere at one time. Counts were made of between 10 - 20 grains on the stigma, although one to six grains was more common. The emergence of multiple tubes would reduce the possibility of late pollen reaching the exudate and becoming attached.

The energy required to produce three tubes is significant, the majority of pollen in other species produces only a single tube. Observations were made of *Conospermum* pollen that had germinated on the stigma with one tube down the style and others in the air. It may be possible that there is a mechanism by which the generative cell and vegetative nucleus selectively travel down the cytoplasm of the tube in the style rather

than the aerial tubes. The nuclei are small and difficult to see with DAPI staining, but this is an important question that remains to be answered.

Although an area of controversy, it is accepted that *Proteaceae* are a family quite unique and isolated in evolutionary terms, and date back to the mid-Cretaceous period in the Mesozoic era (Hopper *et al.* 1996; Johnson 1998). They existed in Gondwana (Southern Hemisphere), and adapted to nutrient-poor soils and later, drought and fire. The similarity of the behaviour of *Conospermum* pollen to grasses is unexplained, but the rapid emergence of multiple tubes was also observed in *Synaphea*, the closest relative to *Conospermum.* Closer examination of pollen from other subtribes in the Conospermeae tribe, which include Cenarrheninae, Dilobeiinae, Stirlingiinae and Petrophilinae (Bennett 1995) would be valuable.

10.7 Future of *Conospermum* species

Proteaceae species are difficult to work with *in vitro* and have intricate and variable floral structures. This is also true for *Conospermum.* However, the potential benefits to the cut-flower industry, and to the conservation of the species, means that further effort is needed to extend and improve on the results reported here. The remnant populations that remain in Western Australia continue to be threatened by urban sprawl, farming and diseases such *Phytothora cinnamomi*, and commercial propagation may be the only means by which they will survive.

Figure 10.1: *Conospermum amoenum* pollen grain wall showing smooth homogenous ectexine (EC) and endexine (EN), and intine (I) with intrusions into the cytoplasm. Bar = μm.

Figure 10.2: An abundance of golgi-vesicles (arrows) in the pollen tube in various states of fusion with each other and the plasmamembrane. Bar = μm.

Figure 10.3: Cytoplasm of pollen tube showing a dictyosome with secretory vesicles attached (D), many mitochondria (arrows) and a large starch grain. Bar = μm.

Figure 10.4: Germinating *Conospermum amoenum* pollen grain. Note the tube wall appears continuous with the pollen grain intine. Bar = μm.

Figure 10.5: Pollen tube near the tip. Secretory vesicles are numerous (arrows) as are mitochondria (arrow heads). Larger golgi-vesicles are also present. Bar = μm

Figure 10.6: Transverse section of a pollen tube showing a wrinkled cell wall and loosely bound fibrils of the inner tube wall (arrow). Bar = μm.

Figure 10.7: Pollen tube fixed with Karnovsky's fixative in the absence of sucrose. Cytoplasm is highly disorganised and the cell wall disrupted. Bar = μm.

CHAPTER 11

REFERENCES

(2003) *Florabase* - *www.florabase.calm.wa.gov.au.* Department of Conservation and Land Management, Western Australia.,

Alexander M P and Ganeshan S (1989) An improved cellophane method for *in vitro* germination of recalcitrant pollen. *Stain Technology*. 644:225-257.

Al-Zahim M, Ford-Lloyd B and Newbury H (1999) Detection of somaclonal variation in garlic (*Allium sativum* L.) using RAPD and cytological analysis. *Plant Cell Reports*. 18:473-477.

Anzidei M, Bennici A, Schiff S, Tani C and Mori B (2000) Organogenesis and somatic embryogenesis in *Foeniculum vulgare*: histological observations of developing embryogenic callus. *Plant Cell, Tissue and Organ Culture*. 61:69-79.

Ayre D J and Whelan R J (1989) Factors controlling fruit set in hermaphroditic plants: studies with the Australian Proteaceae. *Trends in Ecology and Evolution*. 4:267-272.

Barnabas B and Fridvalszky L (1984) Adhesion and germination of differently treated maize pollen grains on the stigma. *Acta Botanica Hungary*. 30:329-332.

Bates S, Preece J E, Navarrete N E, Van Sambeek J W and Gaffney G R (1992) Thidiazuron stimulates shoot organogenesis and somatic embryogenesis in white ash (*Fraxinus americana* L.). *Plant Cell, Tissue and Organ Culture*. 31:21-29.

Bennett E M (1995) *Conospermum, Flora of Australia.* 16.

Blanc G, Michaux-Ferriere N, Teisson C, Lardet L and Carron M P (1999) Effects of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasiliensis*. *Plan Cell, Tissue and Organ Culture*. 59:103-112.

Bouman H and De Klerk G (2001) Measurement of the extent of somaclonal variation in begonia plants regenerated under various conditions. Comparison of three assays. *Theoretical and Applied Genetics*. 102:111-117.

Brewbaker J L and Kwack B H (1963) The essential role of calcium ion in pollen germination and pollen tube growth. *American Journal of Botany*. 50:859-865.

Briskin D and Hanson J B (1992) How does the plasma membrane K^+-ATPase pump protons. *Journal of Experimental Botany*. 43:269-289.

Cai G, Moscatelli A and Cresti M (1997) Cytoskeletal organization and pollen tube growth. *Trends in Plant Science*. 2:86-91.

Cai G, Romagnoli S and Cresti M (2001) Microtubule motor proteins and the organization of the pollen tube cytoplasm. *Sexual Plant Reproduction*. 14:27-34.

Canedo Ortiz B O, Reyes M E O and Balch E P M (2000) Somatic embryogenesis and plant regeneration in *Acacia farnesiana* and *A. schaffneri*. *In Vitro Cell Developmental Biology*. 36:268-272.

Carman J G (1990) Invited Review: Embryogenic cells in plant tissue cultures: occurrence and behaviour. *In Vitro Cell Developmental Biology*. 26:746-753.

Carolin R (1961) Pollination of the Proteaceae. *The Australian Museum Magazine*.

Cassells A and Curry R (2001) Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. *Plant Cell, Tissue and Organ Culture*. 64:145-157.

Chaudhary B, Kumar S, Prasad K V S K, Oinam G S, Burma P K and Pental D (2003) Slow desiccation leads to high-frequency shoot recovery from transformed somatic embryos of cotton (*Gossypium hirsutum* L. cv. Coker 310FR). *Plant Cell Reports*. 21:955-960.

Cheng C and McComb J A (1992) *In vitro* germination of wheat pollen on raffinose medium. *New Phytologist*. 120:459-462.

Cheung A Y, Wang H and Wu H-M (1995) A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. *Cell*. 82:383-393.

Chibbar R N, Shyluk J, Georges F, Mallard C S and Constabel F (1988) Esterase isozymes as markers of somatic embryogenesis in cultured carrot cells. *Plant Physiology*. 133:367-370.

Choi Y, Yang D and Yoon E (1999) Rapid propagation of *Eleutherococcus senticosus* via direct somatic embryogenesis from explants of seedlings. *Plant Cell, Tissue and Organ Culture*. 58:93-97.

Coppens I and Gillis E (1987) Isoenzyme electrofocusing as a biochemical marker system of embryogenesis and organogenesis in callus tissues of *Hordeum vulgare* L. *Journal of Plant Physiology*. 127:153-158.

Corredoira E, Ballester A and Vietez A (2003) Proliferation, maturation and germination of *Castanea sativa* Mill. somatic embryos originated from leaf explants. *Annals of Botany*. 92:129-136.

Cresti M, Blackmore S and Van Went J L (1992) *Atlas of Sexual Reproduction in Flowering Plants.* Springer-Verlag, Germany.

Cresti M, Ciampolini F, Mulcahy D L M and Mulcahy G (1985) Ultrastructure of *Nicotiana alata* pollen, its germination and early tube formation. *American Journal of Botany*. 72:719-727.

Cresti M, Ciampolini F and Sarfatti G (1980) Ultrastructural investigations on *Lycopersicum peruvianum* pollen activation and pollen tube organisation after self- and cross- pollination. *Planta*. 150:211-217.

Davis G L (1966) *Systematic Embryology of the Angiosperms*. John Wiley and Sons, Inc, USA.

de Vries S C, Booij H, Meyerink P, Huisman G, Wilde H D, Thomas T L and Van Kammen A (1988) Acquisition of embryogenic potential in carrot cell-suspension cultures. *Planta*. 176:196-204.

Del Casino C, Li Y Q, Moscatelli A, Scali M, Tiezzi A and Cresti M (1993) Distribution of microtubules during the growth of tobacco pollen tubes. *Biology of the Cell*. 79:125-132.

Delmer D P and Stone B A (1988) *Biosynthesis of plant cell walls. The Biochemistry of Plants.* 14. Academic Press,

Derksen J, Knuiman B, J. H, Guyon A, Bonhomme S and Pierson E S (2002) Growth and cellular organisation of *Arabidopsis* pollen tubes *in vitro*. *Sexual Plant Reproduction*. 15:133-139.

Derksen J, Rutten T, Lichtscheidl I K, de Win A H N, Pierson E S and Rongen G (1995) Quantitative analysis of the distribution of organelles in tobacco pollen tubes: implications for exocytosis and endocytosis. *Protoplasma*. 188:267-276.

Dodeman V L, Ducreux G and Kreis M (1997) Zygotic embryogenesis versus somatic embryogenesis. *Journal of Experimental Botany*. 48:1493-1509.

Elkonin L A and Pakhomaova N V (2000) Influence of nitrogen and phosphorus on induction embryogenic callus of sorghum. *Plant Cell, Tissue and Organ Culture*. 61:115-123.

Emons A M C and Kieft H (1991) Histological comparison of single somatic embryos of maize from suspension culture with somatic embryos attached to callus cells. *Plant Cell Reports*. 10:485-488.

Emons A M C, Samallo-Droppers A and van der Toorn C (1993) The influence of sucrose, mannitol, L-proline, abscisic acid and gibberellic acid on the maturation of somatic embryos of *Zea mays* L. from suspension cultures. *Journal of Plant Physiology*. 142:597-604.

Esau K (1977) *Anatomy of Seed Plants.* 2. John Wiley & Sons, Inc, USA.

Ettienne H, Lartaud M, Carron M P and Michaux-Ferriere N (1997) Use of calcium to optimize long-term proliferation of embryogenic calluses and plant regeneration in *Hevea brasiliensis* (Mull. Arg.). *Journal of Experimental Botany*. 48:129-137.

Feijo J A, Sainhas J, Holdaway-Clarke T L, Cordeiro M S, Kunkel J G and Hepler P K (2001) Cellular oscillations and the regulation of growth: the pollen tube paradigm. *BioEssays*. 23:86-94.

Ferguson C, Teeri T T, Siika-aho M, Read S M and Bacic A (1998) Location of cellulose and callose in pollen tubes and grains of *Nicotiana tabacum*. *Planta*. 206:452-460.

Filonova L H, Bozhkov P V and von Arnold S (2000) Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. *Journal of Experimental Botany*. 51:249-264.

Finer J J (1988) Plant regeneration from somatic embryogenic suspension cultures of cotton (*Gossypium hirsutum* L.). *Plant Cell Reports*. 7:399-402.

Franklin-Tong V E (1999) Signaling and the modulation of pollen tube growth. *The Plant Cell*. 11:727-738.

Fuss A M and Sedgley M (1991) Pollen tube growth and seed set of *Banksia coccinea* R.Br. (Proteaceae). *Annals of Botany*. 68:377-384.

Gallego F J, Martinez I, Celestino C and Toribio M (1997) Testing somaclonal variation using RAPDs in *Quercus suber* L. somatic embryos. *International Journal of Plant Science*. 158:563-567.

Geitmann A and Cresti M (1998) Ca^{2+} channels control the rapid expansions in pulsating growth of *Petunia hybrida* pollen tubes. *Journal of Plant Physiology*. 152:439-447.

Gill R and Saxena P K (1992) Direct somatic embryogenesis and regeneration of plants from seedling explants of peanut (*Arachis hypogaea*): promotive role of thidiazuron. *Canadian Journal of Botany*. 70:1186-1192.

Goldberg R B, Barker S J and Perez-Grau L (1989) Regulation of gene expression during plant embryogenesis. *Cell*. 56:149-160.

Goldingay R L and Carthew S M (1998) Breeding and Mating Systems of Australian Proteaceae. *Australian Journal of Botany*. 46:421-437.

Hasegawa Y, Nakamura S, Kakizoe S, Sato M and Nakamura N (1998) Immunocytochemical and chemical analyses of golgi vesicles isolated from the germinated pollen of *Camellia japonica*. *Journal of Plant Research*. 111:421-429.

Heinze B and Schmidt J (1995) Monitoring genetic fidelity vs. somaclonal variation in Norway spruce (*Picea abies*) somatic embryogenesis by RAPD analysis. *Euphytica*. 85:341-345.

Henry Y, Vain P and De Buyser J (1994) Genetic analysis of *in vitro* plant tissue culture responses and regeneration capacities. *Euphytica*. 79:45-58.

Heslop-Harrison J (1987) Pollen germination and pollen-tube growth. *International Review of Cytology*. 107:1-78.

Heslop-Harrison J and Heslop-Harrison Y (1982) The growth of the grass pollen tube: 1. Characteristics of the polysaccharide particles ("P-particles") associated with apical growth. *Protoplasma*. 112:71-80.

Heslop-Harrison J and Heslop-Harrison Y (1984) The disposition of gamete and vegetative-cell nuclei in the extending pollen tubes of a grass species, *Alopecurus pratensis* L. *Acta Botanica Neerlandica*. 33:131-134.

Heslop-Harrison J and Heslop-Harrison Y (1992) Germination of monocolpate angiosperm pollen: effects of inhibitory factors and the Ca^{2+}-channel blocker, nifedipine. *Annals of Botany*. 69:395-403.

Ho W J and Vasil I K (1983) Somatic embryogenesis in sugarcane (*Saccharum officinarum* L.) I. The morphology and physiology of callus formation and the ontogeny of somatic embryos. *Protoplasma*. 118:169-180.

Holdaway-Clarke T L, Feijo J A, Hackett G R, Kunkel J G and Hepler P K (1997) Pollen tube growth and the intracellular cytosolic calcium gradient oscillate in phase while extracellular calcium influx is delayed. *The Plant Cell*. 9:1999-2010.

Houston T F (1989) *Leioproctus* bees associated with Western Australian smoke bushes (*Conospermum* spp.) and their adaptions for foraging and concealment (Hymenoptera: Colletidae: Paracolletini). *Records of the Western Australian Museum*. 14:275-292.

Huetteman C, A. and Preece J, E. (1993) Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture*. 33:105-119.

Hulskamp M, Schneitz K and Pruitt R (1995) Genetic evidence for a long-range activity that directs pollen tube guidance in *Arabidopsis. Plant Cell*. 7:57-64.

Ikeda S, Nasrallah J B, Dixit R, Preiss S and Nasrallah M E (1997) An aquaporin-like gene required for the *Brassica* self-incompatibility response. *Science*. 276:1564-1566.

Joersbo M, Andersen J, M., Okkels F, T. and Rajagopal R (1989) Isoperoxidases as markers of somatic embryogenesis in carrot cell suspension cultures. *Physiologia Plantarum*. 76:10-16.

Klimaszewska K, Bernier-Cardou M, Cyr D R and Sutton B C S (2000) Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. *In Vitro Cell Developmental Biology- Plant*. 36:279-286.

Krauss S (1994) Preferential outcrossing in the complex species *Persoonia mollis* R. Br. (Proteaceae). *Oecologia*. 97:256-264.

Kreuger M and Van Holst G-J (1993) Arabinogalactan proteins are essential in somatic embryogenesis of *Daucus carota* L. *Planta*. 189:243-248.

Lamhamedi M, S., Chanberland H, Bernier P, Y. and Tremblay F, M. (2000) Clonal variation in morphology, growth, physiology, anatomy and ultrastructure of container-grown white spruce somatic plants. *Tree Physiology*. 20:869-880.

Lancelle S A, Callaham D A and Hepler P K (1986) A method for rapid freeze fixation of plant cells. *Protoplasma*. 131:153-165.

Lancelle S A and Hepler P K (1992) Ultrastructure of freeze-substituted pollen tubes of *Lilium longiflorum*. *Acta Botanica Hungary*. 167:215-230.

Lelu M A, Bastien C, Klimaszewska K, Ward C and Charest P J (1994) An improved method for somatic plantlet production in hybrid larch (*Larix* x *leptoeuropaea*) Part 1. Somatic embryo maturation. *Plant Cell, Tissue and Organ Culture*. 36:107-115.

Li X Y, Feng H, Huang H, Murphy K B and Gbur E E J (1998b) Polyethylene glycol and maltose enhance somatic embryo maturation in loblolly pine (*Pinus taeda* L.). *in Vitro Cell Developmental Biology - Plant*. 34:22-26.

Li Y-Q, Zhang H-Q, Pierson E S, Huang F-Y, Linskens H F, Hepler P K and Cresti M (1996) Enforced growth-rate fluctuation causes pectin ring formation in the cell wall of *Lilium longiflorum* pollen tubes. *Planta*. 200:41-49.

Li Z, Traore A, Maximova S and Guiltinan M J (1998a) Somatic embryogenesis and plant regeneration from floral explants of cacao (*Theobroma cacao* L.) using thidiazuron. *In Vitro Cell Developmental Biology-Plant*. 34:293-299.

Linacero R, Freitas Alves E and Vazquez A (2000) Hot spots of DNA instability revealed through the study of somaclonal variation in rye. *Theoretical and Applied Genetics*. 100:506-511.

Lord E (2001) Adhesion molecules in lily pollination. *Sexual Plant Reproduction*. 14:57-62.

Lotan T, Ohto M, Yee K M, West M A L, Lo R, Kwong W, Yamagishi K, Fisher R L, Goldberg R B and Harada J J (1998) *Arabidopsis LEAF COTYLEDON1* is sufficient to induce embryo development in vegetative cells. *Cell*. 93:1195-1205.

Lush W M (1999) Whither chemotropism and pollen tube guidance. *Trends in Plant Science*. 4:413-418.

Lush W M, Spurck T and Joosten R (2000) Pollen tube guidance by the pistil of a Solonaceous plant. *Annals of Botany*. 85:39-47.

Luu D-T, Marty-Mazars D, Trick M, Dumas C and Heizmann P (1999) Pollen-stigma adhesion in *Brassica* spp. involves SLG and SLR1 glycoproteins. *Plant Cell*. 11:251-262.

Maataoui M E, Espagnac H and Michaux-Ferriere N (1990) Histology of callogenesis and somatic embryogenesis induced in stem fragments of Cork Oak (*Quercus suber*) cultured *in vitro*. *Annals of Botany*. 66:183-190.

Mackay D and Morrison D A (1989) Multivariate morphometric and allozymic analysis of the *Conospermum taxifolium* (*Proteaceae*) species complex. *Plant System Evolution*. 166:141-158.

Malho R and Trewavas A (1996) Localized apical increases of cytosolic free calcium control pollen tube orientation. *The Plant Cell*. 8:1935-1945.

McCabe P F, Valentine T A, Forsberg S and Pennell R, I. (1997) Soluble signals from cells identified at the cell wall establish a developmental pathway in carrot. *The Plant Cell*. 9:2225-2241.

Montoro P, Etienne H and Carron M P (1995) Effect of calcium on callus friability and somatic embryogenesis in *Hevea brasiliensis* Mull Arg - Relations with callus mineral nutrition, nitrogen metabolism and water parameters. *Journal of Experimental Botany*. 46:255-261.

Morell M, Peakall R, Appels R, Preston L and Lloyd H (1995) DNA profiling techniques for plant variety identification. *Australian Journal of Experimental Agriculture*. 35:807-819.

Morrison D, M. M, Bankoff P and Quirico P (1994) Reproductive isolation mechanisms among four closely-related species of *Conospermum* (Proteaceae). *Botannical Journal of the Linnean Society*. 116:13-31.

Murthy B N S and Saxena P K (1998) Somatic embryogenesis and plant regeneration of neem (*Azadirachta indica* A. Juss.). *Plant Cell Reports*. 17:469-475.

Nadel B L, Altman A and Ziv M (2001) Regulation of somatic embryogenesis in celery cell suspension. 1. Promoting effects of mannitol on somatic embryo development. *Plant Cell, Tissue and Organ Culture*. 18:181-189.

Nasrallah J B, Stein J, Kandasamy M and Nasrallah M (1994) Signaling the arrest of pollen tube development in self-incompatible plants. *Science*. 266:1505-1508.

Neuman M, C., Preece J, E., Van Sambeek J W and Gaffney G, R. (1993) Somatic embryogenesis and callus production from cotyledon explants of Eastern black walnut. *Plant Cell, Tissue and Organ Culture*. 32:9-18.

Newell C, Growns D and McComb J (2003) The influence of medium aeration on *in vitro* rooting of Australian plant microcuttings. *Plant Cell Tissue Organ Culture*. 75:131-142.

Nieves N, Martinez M E, Castillo R, Blanco M A and Gonzales-Olmedo J L (2001) Effect of abscisic acid and jasmonic acid on partial desiccation of encapsulated somatic embryos of sugarcane. *Plant Cell, Tissue and Organ Culture*. 65:15-21.

Novak F (1980) Phenotype and cytological status of plants regenerated from callus cultures of *Allium sativum* L.Z. *Pflanzenzeucht*. 84:250.

Oddie R L A and McComb J A (1998) Stigma receptivity in *Eucalyptus camaldulensis* Dehnh. *Silvae Genetica*. 47:2-3.

Olmos S, Lavia G, Di Renzo M, Mroginski L and Echenique V (2002) Genetic analysis of variation in micropropagated plants of *Melia azedarach* L. *In Vitro Cell Developmental Biology- Plant*. 38:617-622.

Peakall R and Smouse P (2001) *GenAlEx V5: Genetic Analysis in Excel. Population genetic software for teaching and research.* 2003. Australian National University, Canberra http://www.anu.edu.au/BoZo/GenAlEx/.

Pearce D, Pharis R, Rajasekaran K and Mullins M (1987) Effects of chilling and ABA on (^{3}H) gibberellin A_4 metabolism in somatic embryos of grape (*Vitis vinifera* L. x *V. rupestris* Scheele). *Plant Physiology*. 80:381-385.

Pedroso M C and Pais M S (1993) Direct embryo formation in leaves of *Camellia japonica* L. *Plant Cell Reports*. 12:639-643.

Pedroso M C, Primikirios N, Roubelakis-Angelakis K A and Pais M S (1997) Free and conjugated polyamines in embryogenic and non-embryogenic leaf regions of camellia leaves before and during direct somatic embryogensis. *Physiologia Plantarum*. 101:213-219.

Perry F and Trueman S J (1999) Cutting propagation of Victorian smokebush *Conospermum mitchellii*. *S. Afr. J. Botany*. 65:243-244.

Pierson E S, Li Y Q, Zhan H Q, Willemse M T M, Linskens H F and Cresti M (1995) Pulsatory growth of pollen tubes: investigation of a possible relationship with the periodic distribution of cell wall components. *Acta Botanica Neerlandica*. 44:121-128.

Pierson E S, Licktscheidl I K and Derksen J (1990) Structure and behaviour of organelles in living pollen tubes of *Lilium longiflorum*. *Journal of Experimental Botany*. 41:1461-1468.

Pierson E S, Miller D D, Callaham D A, Shipley A M, Rivers B A, Cresti M and Hepler P K (1994) Pollen tube growth is coupled to the extracellular calcium ion flux and the intracellular calcium gradient: Effect of BAPTA-type buffers and hypertonic media. *The Plant Cell*. 6:1815-1828.

Pierson E S, Miller D D, Callaham D A, Van Aken J, Hackett G R and Hepler P K (1996) Tip-localized calcium entry fluctuates during pollen tube growth. *Developmental Biology*. 174:160-173.

Pinto G, Santos C, Neves L and Araujo C (2002) Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. *Plant Cell Reports*. 21:208-213.

Pond S E, Von Aderkas P and Bonga J M (2002) Improving tolerance of somatic embryos of *Picea glauca* to flash desiccation with a cold treatment (desiccation after cold acclimation). *In Vitro Cell Developmental Biology- Plant*. 38:344-341.

Pound L M, Wallwork M A B, Potts B M and Sedgley M (2002) Early ovule development following self- and cross-pollinations in *Eucalyptus globulus* Labill. ssp. *globulus*. *Annals of Botany*. 89:613-620.

Raemakers C J J M, Jacobsen E and Visser R G F (1995) Secondary somatic embryogenesis and application in plant breeding. *Euphytica*. 81:93-107.

Ravishankar Rai V and McComb J (2002) Direct somatic embryogenesis from mature embryos of sandalwood. *Plant Cell, Tissue and Organ Culture*. 69:65-70.

Reinert J (1968) *Factors of embryo formation in plant tissues cultivated in vitro. Les cultures des tissus de plantes.* Coll. Nat. CNRS, Paris.

Reinert J, Tazawa M and Semenoff S (1967) Nitrogen compounds as factors of embryogenesis *in vitro*. *Nature*. 216:1215-1216.

Roberts D R (1991) Abscisic acid and mannitol promote early development, maturation and storage protein accumulation in somatic embryos of interior spruce. *Physiologia Plantarum*. 83:247-254.

Rugkhla A and Jones M G K (1998) Somatic embryogenesis and plantlet formation in *Santalum album* and *S. spicatum*. *Journal of Experimental Botany*. 49:563-571.

Sainsbury R M (1991) *A Field Guide to Smokebushes and Honeysuckles (Conospermum and Lambertia)*. University of Western Australia Press, Nedlands.

Salvi N D, George L and Eapen S (2001) Plant regeneration from leaf base callus of turmeric and random amplified polymorphic DNA analysis of regenerated plants. *Plant Cell, Tissue and Organ Culture*. 66:113-119.

Samoylov V M, Tucker D M and Parrott W A (1998) Soybean [glycine max (L). merrill] embryogenic cultures- The role of sucrose and total nitrogen content on proliferation. *In Vitro Cellular and Developmental Biology - Plant*. 34:8-13.

Schmidt E D L, de Jong A J and de Vries S C (1994) Signal molecules involved in plant embryogenesis. *Plant Molecular Biology*. 26:1305 - 1313.

Seaton K A (1996) Conospermum. The search for "new" wildflowers. *Australian Horticulture*. 4:47-48.

Seaton K A (2001) *Cutflower production of blue smokebush.* RIRDC final report,

Seaton K A (2002) Taming smokebush. *Floriculture News*. 5.

Seaton K A and Webb M G (1996) The ecology and development of *Conospermum*. *Acta Horticulturae*. 453:107-110.

Seaton K A and Webb M G (1997) *Conospermum*: A cultivated cutflower. *Proceedings 26th International Plant Propagators Society*. 13-15.

Sedgley M (1983) Pollen tube growth in *Macadamia*. *Scientia Horticulturae*. 18:333-341.

Sedgley M, Sierp M and Maguire T (1994) Interspecific hybridisation involving *Banksia prionotes* Lind. and *Banksia menziesii* R. Br. (Proteaceae). *International Journal of Plant Sciences*. 155:755-762.

Shannon C and Weaver W (1949) *The mathematical theory of communication.* University of Illinois Press, Urbana.

Sharp W R, Sondahl M R, Calda L S and Maraffa S B (1980) The Physiology of In Vitro Asexual Embryogenesis. *Horticultural Reviews*. 2:268-310.

Smith J A and Gross C L (2002) The pollination ecology of *Grevillea beadleana* McGillivray, an endangered shrub from Northern New South Wales, Australia. *Annals of Botany*. 89:97-108.

Sniezko R (1997) Postulated interaction between branching pollen tubes and ovules in *Oenthora hookeri* de Vries (Onagraceae). *Journal of Plant Research*. 110:411-416.

Spurr A (1969) A low viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructural Research*. 26:31.

Stanley R G (1971) *Pollen Chemistry and Tube Growth. Pollen: Development and Physiology.* Butterworths, London.

Stanley R G and Linskens H F (1974) *Pollen: Biology, Biochemistry Management.* Springer-Verlag, Heidelberg.

StatSoft I (1999) *STATISTICA for Windows (Computer Program Manual).* Tulsa USA.

Steer M W (1988) Plasma membrane turnover in plant cells. *Journal of Experimental Botany*. 39:987-996.

Steer M W and Steer J M (1989) Pollen tube tip growth. *New Phytologist*. 111:323-358.

Stepka M, Ciampolini F, Charzynska M and Cresti M (2000) Localization of pectins in the pollen tube wall of *Ornithogalum virens* L. Does the pattern of pectin distribution depend on the growth rate of the pollen tube? *Planta*. 210:630-635.

Steward F C, Mapes M O and Smith J (1958) Growth and organized development of cultured cells I, II, III. *American Journal of Botany*. 45:693-713.

Tan S K and Kamada H (2000) Initial identification of a phosphoprotein that appears to be involved in the induction of somatic embryogenesis in carrot. *Plant Cell Reports*. 19:739-747.

Tang H, Ren Z and Krczal G (2000) Improvement of English walnut somatic embryo germination and conversion by desiccation treatments and plantlet development by lower medium salts. *In Vitro Cell Developmental Biology- Plant*. 36:47-50.

Taylor L P and Hepler P K (1997) Pollen germination and tube growth. *Annual Review of Plant Physiology and Plant Molecular Biology*. 48:461-491.

Taylor R L (1967) The foliar embryos of *Malaxis paludosa. Canadian Journal of Botany*. 45:1553-1556.

Tchorbadjieva M and Odjakova M K (1991) An acidic esterase as a biochemical marker for somatic embryogenesis in orchardgrass (*Dactylis glomerata* L.) suspension cultures. *Plant Cell Reports*. 20:28-33.

Toonen M A J, Hendriks T, Schmidt E D L, Verhoeven H A, Kammen A and de Vries S C (1994) Description of somatic-embryo-forming single cells in carrot suspension cultures employing video cell tracking. *Planta*. 194:565-572.

Torres A C, Mfe'e Ze N and Cantliffe D J (2001) Abscisic acid and osmotic induction of synchronous somatic embryo development of sweet potato. *In Vitro Cell Developmental Biology-Plant*. 37:262-267.

Tulecke W and McGranahan G H (1985) Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. *Plant Science*. 40:57-63.

van Hengel A J, Tadesse Z, Immerzeel P, Schols H, Van Kammen A and de Vries S C (2001) N-acetylglucosamine and glucosamine-containing arabinogalactan proteins control somatic embryogenesis. *Plant Physiology*. 125:1880-1890.

van Hengel A J, van Kammen A and de Vries S C (2002) A relationship between seed development, Arabinogalactan-proteins (AGPs) and the AGP mediated promotion of somatic embryogenesis. *Physiologia Plantarum*. 114:637-644.

Vasil I K (1960) Pollen germination in some Gramineae: *Pennisetum typhoideum*. *Nature*. 187:1134-1135.

Vaughton G and Ramsey M (1991) Floral biology and inefficient pollen removal in *Banksia spinulosa* var. *neoanglica* (Proteaceae). *Australian Journal of Botany*. 39:167-177.

Vergne P, Delvallee I and Dumas C (1987) Rapid assessment of microspore and pollen development stage in wheat and maize using DAPI and membrane permeabilization. *Stain Technology*. 62:299-304.

Walker D R and Parrott W A (2001) Effect of polyethylene glycol and sugar alcohols on soybean somatic embryo germination and conversion. *Plant Cell, Tissue and Organ Culture*. 64:55-62.

Wang Z, Nagel J, Potrykus I and Spangenberg G (1993) Plants from cell suspension-derived protoplasts of *Lolium* species. *Plant Science*. 94:179-193.

Wheeler M J, Franklin-Tong V E and Franklin F C H (2001) The molecular and genetic basis of pollen-pistil interactions. *New Phytologist*. 151:565-584.

Widholm E G (1972) Fluorescein diacetate for estimating viability of cells. *Stain Technology*. 47:189.

Wilhelmi L K and Preuss D (1996) Self-sterility in *Arabidopsis* due to defective pollen tube guidance. *Science*. 274:1535.

Wilhelmi L K and Preuss D (1997) Blazing new trails. Pollen tube guidance in flowering plants. *Plant Physiology*. 113:307-312.

Williams E G and Maheswaran G (1986) Review Article: Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group. *Annals of Botany*. 57:443-462.

Wolters-Arts M, Lush W M and Mariani C (1998) Lipids are required for directional pollen tube growth. *Nature*. 392:818-821.

Woodward B and Puonti-Kaerlas J (2001) Somatic embryogenesis from floral tissue of cassava (*Manihot esculenta* Crantz). *Euphytica*. 120:1-6.

Wu H-M, Wang H and Cheung A Y (1995) A floral transmitting tissue specific glycoprotein attracts pollen tubes and stimulates their growth. *Cell*. 82:383-393.

Zhang S, Wong L, Meng L and Lemaux P G (2002) Similarity of expression patterns of *knotted1* and *ZmLEC1* during somatic and zygotic embryogenesis in maize (*Zea mays* L.). *Planta*. 215:191-194.

Zinkl G M and Preuss D (2000) Dissecting *Arabidopsis* pollen-stigma interactions reveals novel mechanisms that confer mating specificity. *Annals of Botany*. 85 (A):15-21.

Zuo J, Niu Q, Frugis G and Chua N (2002) The WUSCHEL gene promotes vegetative-to-embryonic transition in *Arabidopsis. The Plant Journal*. 30:349-359.