Self-incompatibility in *Eucalyptus globulus* and *E. nitens*

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Eucalyptus globulus Labill. ssp. *globulus*

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Abstract

Eucalyptus globulus and *E. nitens* are two species within the subgenus *Symphyomyrtus*, widely grown in plantations predominantly for pulpwood production. This study investigated aspects of the breeding system to determine the mechanism of self-incompatibility operating in each species, and whether cross-pollen can outcompete self-pollen following mixed pollinations in *E. globulus*.

The self-incompatibility mechanism in each species was investigated by conducting controlled self- and cross-pollinations on five mature trees. Pollen tube growth in the style and pollen tube penetration of ovules was visualised using fluorescence microscopy, and embryology was examined by bright-field microscopy on sectioned material. Some pollinated flowers were left on the trees until seed maturity, then seed counts were used to establish the self-incompatibility level of each tree.

Self-incompatibility levels of *E. nitens* trees ranged from 25.8 to 93.6 %. There was no significant difference between self- and cross-pollen treatments in the number of pollen tubes present in styles or in the percentage of ovules penetrated by pollen tubes. Fertilisation had taken place by 2 weeks after pollination with nearly every ovule showing evidence of fertilisation. Cross-pollination resulted in a greater proportion of healthy, developing ovules, at both 2 and 4 weeks after pollination, compared with self-pollination. The proportion of degenerating ovules increased from 2 to 4 weeks after pollination. Self-incompatibility in *E. nitens* appears to be a post-zygotic mechanism. Differences in ovule size from 6 weeks after pollination onwards shows potential for identifying self-incompatible trees.

Eucalyptus globulus trees had self-incompatibility levels ranging from 76 to 100 %. There was no significant difference in the number of pollen tubes in the style between self- and cross-pollen treatments although variation was present between

trees. The number of pollen tubes present was similar to the number of ovules present within flowers. At 2 weeks after pollination there was no significant difference between pollen treatments in the number of ovules penetrated by a pollen tube. By 4 weeks after pollination, there was slightly greater penetration by cross-pollen tubes. Fertilisation had taken place by 4 weeks after pollination with zygotes and free nuclear endosperm visible. There was a greater proportion of healthy, fertilised ovules in the cross- compared with the self-pollination treatment. Approximately half the ovules examined from both pollen treatments were not fertilised or were degenerating. By 6 weeks after pollination a few zygotes were starting to divide. The mechanism of self-incompatibility appears to have both late-pre and post-zygotic components. Fertilised ovules were significantly larger than non-fertilised or degenerating ovules and this difference was detectable by eye at 6 and 8 weeks after pollination. As in *E. nitens*, these differences in ovule size show potential for the identification of self-incompatible trees.

To investigate whether pollen competition occurs favouring cross- over self-pollination in *Eucalyptus globulus*, controlled pollinations using self-pollen, cross-pollen and a mixture of self- and cross-pollen were conducted on three partially self-incompatible trees. The paternity of individual seeds resulting from mixed pollination was determined by isozyme analysis. No evidence for pollen competition was found. Instead, seed paternity reflected the level of self-incompatibility of each tree as determined by separate self- and cross-pollinations. Furthermore, the number of seeds set per capsule following mixed pollination was significantly less than following cross-pollination in the two least self-compatible trees. These results suggest that both self- and cross-pollen tubes reach ovules following mixed pollination, and that a late-acting self-incompatibility mechanism operates to abort a certain proportion of self-penetrated ovules.

This research has added to our knowledge on reproductive biology strategies within *Eucalyptus* and has produced useful information for eucalypt breeders.

Declaration

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

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Leanne Marie Pound

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Chapter 1

General Introduction

1.1 Introduction

Australian trade in forest products resulted in a $2.2 billion dollar trade deficit in 1999 / 2000 with a similar value of $2 billion anticipated for 2001/2002 (Yainshet and Sledge, 2001). Expansion of Australia's plantation resources is seen as one way to reduce the trade deficit. The total area of land in Australia devoted to plantation estates was reported to be approximately 1.34 million ha (as planted to the end of September, 1999), with an expansion rate of almost 95,000 ha per annum (National Plantation Inventory, 2000).

Eucalypts have rapidly become the major component of the expanding areas of Australia's plantation estate, due to the demand for eucalypt pulp in paper production. In 1999, 85,000 ha, or 89 % of the new plantation estate, was planted with eucalypts, and the area of land planted with eucalypts is predicted to increase (National Plantation Inventory, 2000). *Eucalyptus globulus* ssp. *globulus* (hereafter referred to as *E. globulus*) and *E. nitens* are the two temperate species most widely planted in Australia, with plantation estate estimates of 200,000 and 46,000 ha respectively by 1999 (Tibbits *et al.*, 1997). *E. globulus* is planted in areas of mild climate, free of severe frost and droughts, and *E. nitens* grows well in high rainfall areas, free of drought and with frequent frosts (Eldridge *et al.*, 1993).

With eucalypts now cultivated in many countries, breeding programmes play an important role in increasing the competitiveness of Australian plantations (Tibbits *et al.*, 1997). Eucalypt seed orchards are now valued as a means of producing genetically improved material for plantations (Tibbits *et al.*, 1997). Furthermore, as

clonal propagation of temperate eucalypts is both difficult and in Australia unlikely to be cost effective as a deployment strategy (Borralho, 1997; Moncur and Boland, 2000), it is foreseeable that the use of seed orchards will continue.

Seed orchards contain trees that are considered to be genetically superior and likely to produce large quantities of good quality, open pollinated seed. It is important for industry to ensure that seed orchards are optimised for maximum genetic quality and yield. However, research has shown that seed orchards of *E. globulus* and *E. nitens* have rates of self-fertilisation of 23 and 25 % respectively (Moncur *et al.*, 1995). These values are of concern, as selfed eucalypt progeny suffer from inbreeding depression. This means that selfed seed are less viable, selfed seedlings show occasional abnormalities and as young trees, selfed eucalypts show inbreeding depression for height and diameter at breast height (Hardner and Tibbits, 1998; Tibbits, 1988). Reports have shown that the trunk volume growth of selfed *E. globulus* is 48 % less than outcrossed progeny (Hardner and Potts, 1995) and that of selfed *E. nitens* is 40 % less than outcrossed progeny (Hardner and Tibbits, 1998). Such a problem highlights the need for an understanding of the reproductive biology of these species, so that the production of selfed seed may be minimised, thereby increasing the volume of wood harvested per hectare of land.

Eucalypts are known to have a mixed mating system, setting selfed as well as outcrossed seed following open-pollination, however they are considered to be preferentially outcrossing (Eldridge *et al.*, 1993; Griffin *et al.*, 1987). The rate of outcrossing within a species can be extremely variable, with individuals ranging from self-compatible to fully self-incompatible (Ellis and Sedgley, 1992; Potts and Savva, 1988). Of the 700 eucalypt species recognised (Brooker, 2000), only a handful have been studied to identify the self-incompatibility mechanism. Whilst self-incompatibility mechanisms have traditionally been found to operate in styles, where pollen tube growth is halted (de Nettancourt, 2001), the eucalypts studied have been found instead to have varying late-acting self-incompatibility mechanisms. A

late pre-zygotic barrier has been reported in *E. woodwardii* (Sedgley, 1989; Sedgley and Smith, 1989) and both late pre- and post-zygotic barriers have been found in *E. spathulata* and *E. platypus* (Sedgley and Granger, 1996). Post-zygotic self-incompatibility has been reported in *E. regnans* (Sedgley *et al.*, 1989), *E. cladocalyx* and *E. leptophylla* (Ellis and Sedgley, 1992). The nature of the self-incompatibility mechanisms operating in *E. globulus* and *E. nitens* is yet to be determined.

If seed orchard trees are to be successfully screened for self-incompatibility, not only does the mechanism of self-incompatibility need to be identified, but industry needs to develop an easy and quick screening method. Currently this is a slow process, involving controlled self- and cross-pollinations, followed by harvests of mature seed (about 12 months after pollination in *E. globulus*), and comparisons made between the number of seeds set. An understanding of the self-incompatibility mechanism is essential for the development of a quick screening method.

Even where individual eucalypt trees may be identified as self-fertile, they may still yield mostly outcrossed seed when pollinated by a mixture of self- and outcross-pollen. Studies have shown that when pollen is deposited on stigmas in excess of that required for complete seed set, different pollens can compete for ovules, thereby affecting seed paternity (Snow, 1986; Winsor *et al.*, 2000). Such an environment may be created in open pollinated seed orchards, again highlighting the need to better understand the breeding systems of plantation grown eucalypts.

1.2 Project aims

The aim of this research is to increase our understanding of the mating systems operating in *E. globulus* and *E. nitens* with a view to improving seed orchard management practises to minimize selfed seed production.

Specifically, this project aims to:

- determine the mechanism of self-incompatibility in *E. globulus* and *E. nitens*

- determine the effect mixed (self and cross) pollination has on seed paternity in partly self-compatible *E. globulus* trees
- use information gained on mating systems in *E. globulus* and *E. nitens* to propose a screening method for self-incompatibility in each species.

Chapter 2

Literature Review

2.1 Taxonomy

Eucalyptus (Myrtaceae), which contains over 700 species (Brooker, 2000), is a genus in which all species except four naturally occur within Australia. The exceptions are *Eucalyptus deglupta*, an endemic to a number of Indonesian islands and northern New Guinea, and *E. urophylla*, *E. wetarensis* and *E. orophila* (Pryor *et al.*, 1995) which are found in Timor and adjoining islands. Pryor and Johnson (1971), in their classification of the eucalypts, divide the genus into seven informal subgenera (*Corymbia*, *Blakella*, *Eudesmia*, *Gaubaea*, *Idiogenes*, *Monocalyptus* and *Symphyomyrtus*), based on shared diagnostic morphological characters. *Symphyomyrtus*, the largest informal subgenus contains about 300 species (Johnson and Briggs, 1983), and it is within *Symphyomyrtus* that the most important eucalypts in terms of wood and pulp production are placed (Eldridge *et al.*, 1993; Griffin, 1989).

2.2 Plantations

Eucalypt plantations are grown world wide, with an estimated six million hectares of land planted with eucalypts in 1985, the larger portion of which is planted in Brazil (Eldridge *et al.*, 1993). Eucalypts are grown to produce a wide array of products including timber and timber products such as sawn timber, fuelwood, poles and charcoal, foliage and flowers for the cut flower industry, oils which are used for pharmaceutical and industrial purposes, and floral nectar for the honey industry (McComb *et al.*, 1996). Furthermore, a large proportion of eucalypt plantations are

grown to provide pulpwood for the paper industry. It is an expanding industry and the demand for good quality eucalypt pulpwood continues to increase (Eldridge *et al.*, 1993; Potts and Potts, 1986; Tibbits, 1986). The use of plantations to provide for constant wood requirements is an alternative, sustainable wood source that can reduce the need to log natural eucalypt forests. This not only aids in natural forest and habitat preservation but helps retain a wider genetic reserve within eucalypt species which can be incorporated into future eucalypt plantations (Eldridge *et al.*, 1993; Griffin, 1989). Furthermore, continual growth of eucalypts in plantations can play a role in carbon sequestration thereby countering green-house gas emissions (Borough *et al.*, 1998). Two eucalypt species grown in plantations in temperate regions of the world to produce pulpwood are *E. globulus* and *E. nitens*. *Eucalyptus globulus*, which grows naturally in Tasmania and small areas of Victoria, has been widely planted around the world, with a total global plantation area of at least 2 million ha (Tibbits *et al.*, 1997). There is increasing interest in growing *E. nitens*, a native of Victoria and New South Wales, due to its frost hardiness (Eldridge *et al.*, 1993), and there is at least 220,000 ha devoted to *E. nitens* plantations world wide (Tibbits *et al.*, 1997). Both these species belong to the informal subgenus *Symphyomyrtus* (Pryor and Johnson, 1971), and have been reported to have superior growth rates at a young age compared to other plantation grown eucalypts (Tibbits, 1986). Overseas, *E. globulus* plantations are cut on a coppice rotation of between 8-12 years, usually two or three times, with *E. nitens* usually cut only once, as it has limited coppicing ability (Eldridge *et al.*, 1993). Coppicing however, is not utilised in Australia for either *E. nitens* or *E. globulus* with new plantations derived from seedlings to incorporate new genetic material.

2.3 Genetic improvement

Eucalypt trees incorporated into plantations are often grown from seed collected from natural stands as domestication of species through most genetic improvement

programmes still requires further development (Eldridge *et al.*, 1993; Tibbits, 1989). There is considerable genetic diversity within eucalypt species (Griffin, 1989), therefore seed collection from natural stands may incorporate trees with poor phenotypic characteristics into plantations. Furthermore, genetic gain will be minimal, as often little selection, by way of culling poor trees, or no selection has been carried out (Moncur and Boland, 2000). Eucalypt species show genetic variation both within and between populations, or provenances. In widespread species, much of the genetic variation between populations can be attributed to major geographic disjunctions within the distribution of the species (Potts and Wiltshire, 1997). In *E. globulus*, wide-scale geographic variation can be seen in differences in bark thickness, wood density, flowering precocity and leaf morphology (Dutkowski and Potts, 1999), as well as timing of vegetative phase change (Jordan *et al.*, 2000). As eucalypts are grown around the world in various environmental conditions, careful planning is required to establish plantations containing trees most suited to the area and which will maximise the desired good quality product. This end result can be achieved by establishing genetic improvement programmes.

Field trials containing trees established from seed collected from different provenances is the first step in identifying the variation present within provenances. For example, Pederick (1979) has shown, through provenance testing, that some *E. nitens* provenances are superior in cool, moist temperate sites, and others are better in sub-tropical highlands. *E. nitens* provenances also show differences in frost resistance (Tibbits and Reid, 1987). Once the variation of interest is identified, continued improvement needs a broad genetic base of suitable provenances maintained, plans for selection and mating each generation, and a means of mass propagation of the selected material (Eldridge and Griffin, 1990). The earliest improvement programmes for *E. globulus* began in the late 1960s in Portugal and Australia with the majority established in the late 1980s and early 1990s. Australia

was the first to establish an improvement program for *E. nitens* in the mid 1970s with most programmes commencing in the early 1990s (Tibbits *et al.*, 1997).

The occurrence of natural hybrids and their subsequent success compared to pure species has also lead to the development of eucalypt hybridisation programmes (Potts and Potts, 1986). These programmes aim to improve tree products by producing new gene combinations in hybrid trees. Hybridisation programmes have been established for both *E. nitens* and *E. globulus* (Tibbits, 2000).

Some improvement programmes have reached the stage of establishing eucalypt seed orchards as a means of producing large quantities of genetically improved seed (Eldridge *et al.*, 1993; Tibbits, 1989). Most seedling seed orchards overseas have been established using open pollinated seed from superior trees in existing plantations. A field trial is set up for progeny testing and over time inferior families and individuals are eliminated, and the remaining trees form the seed orchard (Eldridge *et al.*, 1993). In Australia, seedling seed orchards have been established from provenance trials derived from seed collected from superior trees in natural stands (Eldridge *et al.*, 1993). Some improvement programmes have reached the stage where grafts or cuttings from plus (superior) trees are used to establish clonal seed orchards (Eldridge *et al.*, 1993). Seed from both seedling and clonal seed orchards is used to establish plantations that aim to produce both superior quality and yield of wood. Controlled cross-pollinations on seed orchard trees would ensure outcrossed seed but this process can be time consuming and costly (Williams *et al.*, 1999). Recently Harbard *et al.* (1999) and Williams *et al.* (1999) have developed techniques to control pollinate eucalypt flowers in a single visit to each flower. Both studies have reported a significant increase in the number of seed per capsule compared to that achieved from open pollination. These methods have the potential to reduce the cost of pollination and further increase genetic gain in seed from seed orchards compared to open pollination. However, the use of open pollinated seedling seed orchards as a means of producing eucalypt seed is likely to continue in the

future as many species are difficult to propagate vegetatively, and investment in new technologies cannot be justified (Borralho, 1997; Griffin, 1989; Moncur and Boland, 2000).

It has been noted that the yield of seed from open pollinated eucalypt seed orchards has been low in some cases, and that self-fertilisation within orchards is occurring (Moncur *et al.*, 1995). Establishment of seed orchards in locations where pollen vectors are highly active may improve pollination and hence seed yield (Eldridge and Griffin, 1983), as pollen vectors have been suggested to be a limiting factor in open pollinated seed orchards (Moncur *et al.*, 1995). Differences in flowering times for eucalypts within a species but from different provenances occur in *E. regnans* and *E. globulus* (Griffin, 1989), and in *E. grandis* (Hodgson, 1976c). It is possible that within multi-provenance seed orchards differences in flowering times reduce the likelihood of outcrossing (Griffin, 1989; Hodgson, 1976c).

Another problem with seedling seed orchards is that the time to first flowering and seed production can be several years, so that achieving genetic gain can be slow (Griffin, 1989; Turnbull and Pryor, 1984). For example, it takes about five years for *E. nitens* to produce its first few buds under natural conditions, and production then increases gradually with age (Moncur and Hasan, 1994). Similarly, *E. globulus* grown in plantations has a generation time of five years (Turnbull and Pryor, 1984). Studies aimed at both reducing the time to first flowering and increasing flower numbers have been undertaken using the growth retardant paclobutrazol [(2RS,3SR)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)pentan-3-ol]. Mature *E. nitens* trees collar drenched once only with paclobutrazol have shown up to three years of enhanced umbel production (Moncur *et al.*, 1994). Further, Moncur and Hasan (1994) found that paclobutrazol does not work on *E. nitens* alone but in combination with winter chilling requirements. Applying paclobutrazol to *E. globulus* seedlings by foliar spraying resulted in flower production at age nineteen months and increased reproductive output was also achieved (Hasan and Reid, 1995). Paclobutrazol

appears to have the potential to effectively reduce generation time and increase reproductive output in eucalypts species, whilst producing seed on smaller, easier to harvest trees.

In view of the potential problems eucalypt breeders face, it is imperative that the reproductive biology and breeding system of the particular species to be grown is well understood in order to successfully manage a seed orchard (Eldridge, 1978; Eldridge and Griffin, 1983).

2.4 Reproductive biology/ecology

2.4.1 Floral morphology

Eucalypt flowers are in general bisexual. Few eucalypt species have single flowers with most species having inflorescences of 3, 7, 11, or even 15 or more flowers, borne on a common stalk, the peduncle. When in bud, a eucalypt flower has its reproductive organs enclosed within a hypanthium (a hollow receptacle) and an operculum which is a protective cap for the reproductive organs. Drinnan and Ladiges (1991) found that in the subgenus *Symphyomyrtus* there is usually an inner operculum derived from the rudimentary corolla, similar to the subgenera *Idiogenes*, *Eudesmia* and *Monocalyptus* where the operculum is a single structure. There is also an outer operculum usually derived from a rudimentary calyx primordium. The operculum is shed at anthesis (flower bud opening) exposing the reproductive organs. In species where two opercula are present the outer operculum may be shed at an early stage in bud development or be retained until anthesis (Drinnan and Ladiges, 1991).

Stamens are numerous and are inserted around the top of the hypanthium on the staminophore in alternating rows (Brooker and Kleinig, 1990). The stamens consist of two parts; the slender filament, which is the point of attachment to the hypanthium, and the anther, which houses the developing male gametophyte. During

development, the anther divides distally to form two bisporangiate lobes (Davis, 1968; Davis, 1969).

The female reproductive organ consists of a terminal stigma which subtends the style, which in turn widens at its base to become the ovary.

The stigma is the site of pollen adherence to the pistil, and the stigmatic surface can be divided into two categories, dry or wet, depending on whether a fluid secretion is present when the stigma is in its receptive state (Heslop-Harrison and Shivanna, 1977). All eucalypt flowers have wet (Heslop-Harrison and Shivanna, 1977), and papillate stigmas (Boland and Sedgley, 1986). Most eucalypts within *Symphyomyrtus* have stigmas described as blunt in shape, with lobed surfaces and short papillae (Boland and Sedgley, 1986).

The eucalypt style is covered by a cuticle (Anderson, 1984) and contains a lobed canal of varying length which may or may not reach the base of the style. Furthermore, the number of canal lobes appear to be related to the number of ovary chambers present (Boland and Sedgley, 1986). The canal is surrounded by transmitting tissue through which the pollen tubes grow intercellularly (Knox, 1984). The transmitting tissue narrows towards the base of the style thereby directing pollen tubes into the centre of the ovary (Ellis and Sedgley, 1992).

The inferior or partly superior ovary contains 2-7 locules (McComb *et al.*, 1996), each with numerous ovules and ovulodes attached in rows to a placenta. There is one placenta per locule that extends from a central vertical column. Ovulodes are congenitally sterile ovules and the positioning of ovules and ovulodes has taxonomic importance (Carr and Carr, 1962). Boland *et al.* (1980) also recognises the presence of non-viable ovules within locules. A mature ovule consists of an outer and inner integument, which subtends from the nucellar region, and an embryo sac in the centre. The outer integument overgrows the inner such that a micropyle is formed in a zig-zag shape which is the passage of entry into the ovule for a pollen tube (Davis, 1968; Davis, 1969). During embryo sac development, divisions from the megaspore

mother cell, conforming to *Polygonum* type development (Mauseth, 1988), result in the formation of three antipodal cells, two polar nuclei, which may fuse to form a polar fusion nucleus, and an egg apparatus containing an egg cell and two synergid cells. The antipodal cells are located at the chalazal end of the embryo sac, the two polar nuclei are located in the centre, and the egg apparatus is positioned at the micropylar end of the embryo sac. However, by embryo sac maturity, the three antipodal cells have degenerated leaving the embryo sac with four cells and five nuclei (Davis, 1968; Davis, 1969; Sedgley, 1989; Sedgley *et al.*, 1989). If a pollen tube grows into the embryo sac via one of the two synergid cells and releases two sperm nuclei, double fertilisation occurs if one sperm nucleus fuses with the egg nucleus, forming a zygote, and the second sperm nucleus fuses with the two polar nuclei. Normal development occurs if the zygote develops into a multicellular embryo, and free nuclear then cellular endosperm proliferates in the embryo sac lumen from the fusion of the two polar nuclei with a sperm nucleus (Sedgley, 1989; Sedgley and Granger, 1996).

A eucalypt flower matures into a woody capsule which generally contains seeds, chaff, which has developed from the ovulodes, non-fertilised ovules and non-viable ovules. The seeds are released only after the loculi have widened and the valves, formed by the loculi, have opened (Cremer, 1965). Cremer, (1965) identified three ways in which the loculi widen, with *E. globulus* valves opening when the loculi widen due to centrifugal shrinkage of the surrounding tissue.

E. globulus is one of the few species that has inflorescences comprising single flowers which are often lacking a peduncle and pedicel (stalk arising from the summit of the peduncle attaching the bud), or if present are very short. The operculum is 7-15 mm long, 14-17 mm wide, and the hypanthium is 10-12 mm long, 14-17 mm wide. Fruits are 10-21 mm long, 14-24 mm wide (Chippendale, 1988). *E. nitens* has an inflorescence of 7 flowers on a slightly flattened peduncle of 6-15 mm long, with pedicels absent. The operculum is conical, 2-3 mm long, 3-4 mm wide,

and the hypanthium is cylindrical or angular, 3-4 mm long and wide. Fruits are 4-7 mm long, and 4-6 mm wide (Chippendale, 1988).

2.4.2 Mating system

Eucalypt flowers are protandrous, that is the stamens ripen and release their pollen a number of days before the stigma of the same flower becomes receptive (Pryor, 1976). This may prevent self-pollination from the same flower but does not prevent pollination from other flowers on the same tree. Many species have been shown to have a mixed mating system, setting selfed as well as outcrossed seed following open pollination (Eldridge and Griffin, 1983; Hodgson, 1976a; Moran and Bell, 1983). The outcrossing rate in eucalypt species varies widely (Potts and Wiltshire, 1997). Estimates of the effective outcrossing rate have ranged between 0.69 and 0.86 in ten eucalypt species growing in natural populations and assessed using isozyme markers (Moran and Bell, 1983). In a seed orchard of *E. regnans*, Moran *et al.* (1989) reported that the outcrossing rate was 0.91 compared to 0.74 for a nearby natural population, again using isozyme analysis for assessment. This difference was thought to be due to the break-up of family structure within the seed orchard, which is present in natural populations, thereby reducing mating between relatives. McComb *et al.* (1996) reported that all species within *Eucalyptus* are self-fertile to some extent. Whilst there are no estimates of outcrossing rate in native stands of *E. nitens*, native *E. globulus* stands have been reported to have outcrossing rates between 0.48 and 1.00 (Hardner *et al.*, 1996). The lower outcrossing rates were obtained from isolated trees as opposed to open and closed forests as there were no near neighbours to effect cross-pollination. Seed orchards of *E. globulus* and *E. nitens* have been reported to have outcrossing rates of 0.77 and 0.75 respectively (Moncur *et al.*, 1995). Although eucalypts in general have a mixed mating system, the genus is considered to be overall preferentially outcrossing due to selection against selfs at several growth stages (Eldridge *et al.*, 1993; Griffin *et al.*, 1987; Hardner and Potts, 1995).

2.4.3 Self-incompatibility

An early definition of self-incompatibility was developed by de Nettancourt (1977; 1984) as 'the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination'. This definition, therefore, inferred that self-incompatibility mechanisms operate pre-fertilisation. With this view in mind de Nettancourt (1977; 1984) and Lewis (1979) outlined five systems of self-incompatibility in plants. One system, heteromorphic incompatibility, operates with groups of individual plants within a population having different floral architecture and pollen cytology such that selfing is inhibited. This system relies on phenotypic differences between individuals but other systems are the result of recognition of gene products, usually from a group of genes collectively called the S gene, or a combination of S genes and other genes. Gametophytic incompatibility operates by pollen tube inhibition in the style when a pollen grain contains an allele of the S gene that is also present in the style of the pollinated flower. Another form of gametophytic incompatibility occurs in grasses, with two genes involved, the S gene and the Z gene. Sporophytic incompatibility operates at the stigma to prevent pollen tube growth into the style. This system operates when a pollen grain contains two alleles of an S gene and either one allele is dominant or there is co-dominance between alleles. Self-incompatibility occurs when the stigma has the same active allele. Both authors also note the apparently odd report by Cope (1962) where the site of self-incompatibility in *Theobroma cacao* is in the embryo sac, and is referred to as ovarian inhibition. Male nuclei enter the embryo sac but fail to fuse with the egg cell or the polar nuclei.

Since this time, much work has been undertaken to further understand how self-incompatibility mechanisms operate. Mulcahy and Mulcahy (1983) even proposed a new system to explain gametophytic incompatibility. They hypothesised the concept that instead of an S gene there are many incompatibility supergenes operating with complementary and extensive pollen-style interactions. Reviews by Dumas and Knox (1983) and Gaude and Dumas (1987) identify that in both gametophytic and

sporophytic incompatibility, the key event in the rejection response is the production of callose. Abnormal callose distribution in both stigmas and pollen occurs in sporophytic incompatibility, and only in pollen and pollen tubes in gametophytic incompatibility.

Due to an increasing number of reports of self-incompatibility mechanisms operating in the ovary, Seavey and Bawa (1986) introduced the concept of late-acting self-incompatibility systems. This concept includes both late pre-zygotic and early post-zygotic self-incompatibility mechanisms. Sedgley (1994) points out that without detailed investigation it is difficult to distinguish between late pre- and early post-zygotic mechanisms. Further, she suggests post-zygotic mechanisms may have quite a significant role in the breeding systems of woody horticultural species.

To include the possibility of both pre- and post-zygotic barriers to selfing, Sedgley and Griffin (1989) have defined self-incompatibility within a species as 'a genetically controlled mechanism which reduces the prevalence of inbreeding depression in a population'. Hence, if significantly less seed is produced following selfing compared with outcrossing, a plant may be considered self-incompatible. Kenrick (1986) distinguishes between self-incompatible and partially self-incompatible. If the ratio of fruit yield after self-pollination to fruit yield after cross-pollination is less than 0.2, or between 0.2 and 0.9, then plants are self-incompatible and partially self-incompatible respectively.

Individuals within a single eucalypt species can range from fully self-compatible to self-incompatible (Ellis and Sedgley, 1992; Potts and Savva, 1988). The rare, Tasmanian endemic, *E. morrisbyi* has been found to be self-incompatible with the frequency of fully self-incompatible individuals (>80%) being the highest reported to date (Potts and Savva, 1988). Tibbits (1988) has found a mean reduction of approximately 72% in the number of seeds per capsule following self-pollination compared with cross-pollination in *E. nitens*. *E. globulus* has been shown to have a

mean 67% reduction in seed set following self-pollination compared with cross-pollination (Hardner and Potts, 1995).

2.4.4 Mechanisms of self-incompatibility

Only recently have attempts to determine the mechanisms that control self-incompatibility in eucalypts been made, with various mechanisms, both pre- and post-zygotic, now reported. In *E. woodwardii* Sedgley and Smith (1989) found that self-pollen tubes grew the length of the style and entered the ovary but ovule penetration was reduced compared to cross-pollen tubes. Post-zygotic seed abortion in *E. woodwardii* controls seed number only to a limited capacity (Sedgley, 1989). Similar observations were made on *E. spathulata* and *E. platypus*, with lower levels of pollen tube penetration and fertilisation following selfing compared with outcrossing, with the few fertilised ovules generally failing to achieve zygote division (Sedgley and Granger, 1996). Preferential outcrossing in *E. regnans* is a post-zygotic mechanism, as Sedgley *et al.* (1989) found no difference in ovule penetration and fertilisation, and in seed development to 16 weeks after self- and cross-pollination. Ellis and Sedgley (1993) found flowers on some trees of *E. leucoxylon* to be male sterile, which would ensure outcrossing in those trees. Ellis and Sedgley (1992) made investigations into pollen tube growth in the pistils of *E. cladocalyx*, *E. spathulata* and *E. leptophylla*, all of which were found to be either fully or partially self-incompatible. They found that there was no reduction in ovule penetration following selfing compared with outcrossing in *E. cladocalyx* and *E. leptophylla* indicating a post-zygotic mechanism of self-incompatibility. However, in *E. spathulata* they found a reduction in self-pollen tubes penetrating ovules in two trees but not in another which indicated both a pre- and post-zygotic mechanism occurring within the species. To date there have been no studies undertaken with the aim of determining the mechanism of self-incompatibility in either *E. globulus* or *E. nitens*.

2.4.5 Eucalypt pollinators and their effect on mating systems

Eucalypt pollen, when exposed from the anthers, is generally aggregated into sticky lumps. Hence, wind is not a vector for eucalypt pollen transfer, and pollination is effected by animals, in particular birds and insects (Eldridge *et al.*, 1993; Pryor, 1976). Eucalypt flowers produce nectar, and this serves as an attractant and a reward to floral visitors (Eldridge *et al.*, 1993). The flowers do not appear to be specialised towards particular floral visitors, as a large number of different floral visitors to single species have been reported. For example, Hingston and Potts (1998) observed 71 insect species and seven bird species visiting flowers of *E. globulus*. Similarly, Horskins and Turner (1999) observed 74 invertebrate species visiting flowers of *E. costata*. Both these studies found that honey bees (*Apis mellifera*) were the most frequent visitors. However, only eight species including the honey bee actually made contact with the stigmas of *E. globulus* whilst foraging on the nectar as their bodies were large enough to make contact with the stigma. Furthermore, insects rarely moved between trees, possibly contributing to self-pollination (Hingston and Potts, 1998). In a study on honey bees, Paton (1997) found that individual bees forage in very small areas or particular bushes for long periods of time, again possibly aiding self-pollination. This information suggests that for *E. globulus* insects may not play as large a role in pollinating flowers (either self- or cross-pollination) as in other eucalypt species with smaller stigmas. Furthermore, the effectiveness of insects as pollinators is reduced by their drop in activity during cold weather conditions, frequently experienced in Tasmania (Hingston and Potts, 1998). Hence, they argue that bird species are more likely to be the major pollinators of *E. globulus* and the major means of outcrossing. However, Moncur (1995) found that placing bee hives in a seed orchard of *E. nitens*, which has smaller stigmas than *E. globulus*, did not increase seed production, but increased the outcrossing rate. Bee hives added to a seed orchard of *E. globulus* increased seed production but had no effect on outcrossing rate (Moncur *et al.*, 1993).

2.5 Inbreeding depression

Selfing, or mating between closely related parents, can lead to inbreeding depression (ID). This can be seen as a reduction in fitness of the resultant inbred progeny compared to outcrossed progeny. Stebbins (1950) made comment that species which are generally cross-pollinated show severe signs of ID following selfing, with no or very weak offspring produced. On the other hand, those species that contain closely related individuals that self often, show little sign of ID. Charlesworth and Charlesworth (1987) suggest that species that generally self-pollinate may still suffer ID but it might not be evident until selfed progeny are compared to progeny derived from a cross with another population. In their review Charlesworth and Charlesworth (1987) reveal that ID can be very severe in gymnosperms but at the time there was little information on angiosperms with most studies on angiosperms restricted to herbs. Furthermore, studies rarely considered ID at more than one life stage of the progeny. They suggest that ID had a role in the development of self-incompatibility, and conclude that ID affects growth, survival, and fertility of progeny, therefore ID can act to reduce the genetic value of inbred progeny. Johannsson *et al.* (1998) has shown that further to reducing the fertility of progeny, ID can negatively affect the performance of the microgametophytes the inbred progeny produce.

Sometimes inbreeding can purge populations of mutant deleterious alleles such that ID will be reduced (Barrett and Charlesworth, 1991; Byers and Waller, 1999; Husband and Schemske, 1996). However, in small populations, purging by inbreeding may not be effective enough to reduce ID (Byers and Waller, 1999) and can cause a decrease in fitness which can only be overcome by outcrossing again (Barrett and Charlesworth, 1991). Furthermore, purging ID by selfing can increase the probability of extinction (Hedrick, 1994). In a review, Husband and Schemske (1996) conclude that in species which usually self-fertilise, ID becomes evident in late life stages of progeny, whereas generally outcrossing species can display the

effects of ID early or late in life. Furthermore, late-acting ID can be very difficult to purge, even with extreme inbreeding.

Studies on the effects of ID in eucalypts have accumulated in recent years. ID can manifest as a reduction in seed set (Hardner and Potts, 1995; Potts and Savva, 1988; Sedgley and Smith, 1989; Tibbits, 1989), seed viability (Hardner and Potts, 1995), and germination percent (Eldridge and Griffin, 1983; Hardner and Potts, 1995). ID can also be seen as a reduction in the vigour, size and fertility of inbred progeny compared to that of outcrossed progeny (Sedgley and Griffin, 1989). Survival can also be affected. For example *E. gunnii* selfed seedlings have shown significantly higher mortality after just one year (Potts *et al.*, 1987), and *E. regnans* selfed trees had either died or were suppressed after 13 years, compared with outcrossed trees (Eldridge and Griffin, 1983). Hardner and Potts (1997) evaluated the ID for survival in *E. regnans* selfed trees to be a very high 67 % after 15 years.

In *E. globulus*, ID has been reported as 74 % for viable seeds per flower and per capsule, 67 % for total seeds per capsule, and 25 % for seed viability following selfing (Hardner and Potts, 1995). Self-fertilised *E. globulus* trees have significant ID for height and diameter by 19 months after planting the seedlings, raised in a nursery, into a plantation (Hardner and Potts, 1995). Furthermore, ID increased with age to 43 months with a 26 % ID for height and 24 % ID for diameter. Hardner *et al.* (1998) in another study used measures of tree height and diameter at breast height to calculate the conic volume of *E. globulus* and found that ID for this value had occurred at four years of age. Self-pollination in *E. nitens* results in reduced capsule set and seed yield (Tibbits, 1989), reduced seed viability and a higher incidence of abnormality and mortality in developing seedlings (Tibbits, 1988). One year old *E. nitens* trees have been found to have ID for height (ID = 27 %), which increased to 28 % by two years of age, but dropped to 14 % by four years of age (Hardner and Tibbits, 1998). ID for diameter at breast height in 4 year old *E. nitens* trees was 22 % (Hardner and Tibbits, 1998). *E. nitens* has shown no ID in survival to 9 years of age (Hardner and

Tibbits, 1998). Survival of *E. globulus* trees was investigated by Hardner and Potts (1995) to 43 months of age, and trees from selfed seed showed no significant ID. However, there was a trend over time for the difference in survival between trees from open pollinated seed and trees from outcrossed seed to decrease, and the difference between trees from outcrossed seed and trees from selfed seed to increase.

2.6 Pollen competition

Early studies into pollen competition found a correlation between gametophytic and sporophytic growth rates. For example, Mulcahy (1974) conducted hand pollinations on *Zea mays* using pollens of known tube growth rates. The ovules fertilised by faster growing pollen tubes produced offspring that were faster growing compared to offspring from ovules fertilised by slower growing pollen tubes. Shortly after, Mulcahy (1979) proposed the pollen competition, or gametophytic selection hypothesis for angiosperms. The hypothesis proposes that the presence of enclosed carpels combined with insect pollination provide a mechanism that intensifies selective pressures among the microgametophytes. Mulcahy (1979) put forward three propositions, which combined form the basis for the hypothesis. Firstly, competition between microgametophytes is intensified due to enclosed carpels and insect pollination which reduce random variation on pollen competition. Secondly, pollen tubes differ in their growth rates due to genetic differences among the individual microgametophytes. Finally, there must be an overlap in the expression of genes between microgametophytes and sporophytes otherwise selection of microgametophytes wouldn't lead to any differences in the sporophytic generation.

Since this time, evidence has accumulated in support of Mulcahy's hypothesis. In a study on isozymes present in *Lycopersicon esculentum*, the tomato, Tanksley *et al.* (1981) found that about 60 % of genes encoding for the enzyme systems studied were present in both gametophyte and sporophyte. Shortly after, Willing and Mascarenhas (1984) studied hybridisation between mRNAs synthesised by the

gametophyte and cDNAs from the sporophyte in *Tradescantia paludosa*. Their results are in accordance with Tanksley *et al.* (1981) as they found about 60 % of the sequences of genes being expressed were present both in the gametophyte and sporophyte.

Many field experiments have also been conducted to investigate the possibility of pollen competition. Stephenson (1986) conducted hand pollinations of different pollen loads on *Cucurbita pepo*, the common zucchini. The vigour of the resultant progeny increased when the number of pollen grains applied to stigmas was greater than ovule numbers in the flower. Stephenson (1986) concluded that under high pollen loads only fast growing pollen tubes effected ovule fertilisation. Winsor *et al.* (1987) found that zucchini selectively abort fruit fertilised with a low pollen load. So zucchini exhibits both pre-zygotic (pollen competition) and post-zygotic (selective fruit abortion) mechanisms to increase the quality of its offspring. Stephenson *et al.* (1988) also reveal that the smaller seeds produced from high levels of pollen competition in zucchini are more vigorous in measures of progeny vigour than the larger seeds produced from low levels of pollen competition. More recently, Snow and Spira (1991b) conducted hand pollination with pairs of individual pollens on several females of *Hibiscus moscheutos*. They were the first to show that differences between growth rates of the two pollen types were consistent across maternal genotypes. This suggests that whenever pollen tubes are competing in nature, differences in pollen tube vigour could result in sexual selection.

Hand pollination studies investigating pollen competition have only provided evidence for the potential impact of pollen competition in plant species (Snow, 1986). Investigations into pollen competition in natural populations with natural pollination rates were conducted by Snow (1986) on *Epilobium canum*, a perennial wildflower in the U.S. She found that pollen competition does occur in nature and that both differences in pollen tube growth rate and the rate at which pollen arrives on the stigma can influence the intensity of pollen competition. Winsor *et al.* (2000)

have taken their studies a step further with *Curcubita foetidissima* and have shown that pollen deposition in excess of that required for full seed set occurs often in nature. Furthermore, they have shown that progeny from flowers naturally pollinated in excess of full seed set requirements are more vigorous than progeny from flowers that receive less pollen than that which is required for full seed set.

There are now several studies that have been conducted to investigate the possibility of pollen competition, with much information accumulating in support for Mulcahy's hypothesis (Hormaza and Herrero, 1992; Hormaza and Herrero, 1994). However, Hormaza and Herrero (1994) point out that there is still no clear picture as to how widespread and inclusive pollen competition is. Furthermore, a better understanding of pollen competition in vivo is required and may be useful in large scale screening where selection procedures to date limit practical plant breeding (Hormaza and Herrero, 1992; Hormaza and Herrero, 1994). Within *Eucalyptus*, Griffin *et al.* (1987) have shown that in *E. regnans* it is possible to have both self and outcrossed seed occurring naturally within a single capsule. They also conducted hand pollinations using a mixture of self- and cross-pollen on *E. regnans* and, by conducting isozyme analysis on the resultant progeny, showed that some individuals showed selective fertilisation in favour of cross-pollen. A similar result was found in *E. grandis* based on morphological marker assessment of resultant progeny (Hodgson, 1976b). However, to date, these are the only investigations regarding pollen competition within *Eucalyptus*.

2.7 Implications for forestry industry

At present there appears to be little difference between selfed and outcrossed seedlings for both *E. globulus* and *E. nitens* until some months after transplanting into a plantation. Although some seedling abnormalities such as green or subglaucous foliage instead of the normal glaucous leaves, and dwarfed habit (Potts and Jordan, 1994), and a chlorophyll deficiency (Patterson *et al.*, 2000) have been

detected in *E. globulus*, culling selfs in the nursery stage before planting out is not considered a viable option (Hardner and Potts, 1995). It is therefore necessary to determine a method of ensuring cross-pollination within seed orchards thereby producing outcrossed seed to eliminate the possibility of selfed seedlings being incorporated into plantations (Eldridge and Griffin, 1983). Due to the negative results of inbreeding depression on plantation eucalypts, a reduction in selfed seed from seed orchards will help maximise the pulp production per unit area of land. Hence, there is a requirement by plantation growers for improved outcrossed seed from eucalypt seed orchards.

2.8 Conclusions

Until knowledge on how to most effectively manage seed orchards is gained and put into practise, demand for improved outcrossed seed will continue to be greater than supply from seed orchards. Furthermore, there will be a lag in supply of genetically improved seed due to the time required for selected superior trees or genotypes in managed seed orchards to reach a seed producing age. It would clearly be advantageous to have seed orchards comprising only self-incompatible trees, from provenances with superior wood properties and similar flowering times. Exclusively self-incompatible trees would mean that all seed produced would be outcrossed.

Seed yield will be maximised if a greater understanding of pollinating systems for individuals species is attained and considered in new seed orchard establishment. The inclusion of bee hives into existing orchards could be a worthwhile consideration. To achieve eucalypt seed orchards comprising only self-incompatible trees, a greater understanding of the mechanisms of self-incompatibility is required for each species. At present there is only limited information for a few eucalypt species. With the knowledge of self-incompatibility mechanisms, existing seed orchards could be screened in order to establish which trees are self-incompatible. Outcrossed seed could then be collected, whilst other trees could be used as a pollen

source only. Ultimately, open pollinated seed orchards should be established with only self-incompatible trees of a wide genetic base, which have the same flowering time and occur in areas of high pollinator activity. Alternatively, differences in flowering time and low pollinator activity would need to be overcome in the future with efficient controlled pollination techniques.

Chapter 3

Characterisation of the self-incompatibility mechanism in *Eucalyptus nitens*

3.1 Introduction

Self-incompatibility mechanisms have been established for only a few eucalypt species, with mechanisms ranging from late pre-zygotic control to post-zygotic control of selfed seed production (Ellis and Sedgley, 1992; Sedgley, 1989; Sedgley and Granger, 1996; Sedgley *et al.*, 1989; Sedgley and Smith, 1989). This evidence supports the notion that late pre- and post-zygotic incompatibility systems are not as rare as once thought (Seavey and Bawa, 1986).

Eucalyptus nitens is valued as a forestry species as it has a fast growth rate (Tibbits, 1986), produces wood suitable for pulping (Turnbull and Pryor, 1984) and shows frost tolerance (Eldridge *et al.*, 1993; Turnbull and Pryor, 1984). Plantations are generally established by planting out seedlings that have been germinated from seed. In order to supply growers, *E. nitens* seed orchards have been established as a means of producing genetically improved material. *E. nitens* trees, however, become reproductively mature at 5 years of age and typically produce few and infrequent flowers (Moncur and Hasan, 1994; Moncur *et al.*, 1994), producing small seed crops (Eldridge *et al.*, 1993; Turnbull and Pryor, 1984). Consequently, genetic improvement through breeding programs has been slow.

Moncur *et al.* (1995), studying a reproductively mature *E. nitens* seed orchard in Tasmania, Australia, reported seed production levels of 5.1 seeds per capsule following open pollination, and a mean outcrossing rate of 75 per cent. The

production of self-pollinated seed is of concern to eucalypt breeders due to the detrimental effects of inbreeding. Self-pollination in *E. nitens* results in reduced capsule set and seed yield (Tibbits, 1989), reduced seed viability and a higher incidence of abnormality and mortality in developing seedlings (Tibbits, 1988). At later developmental stages, selfed *E. nitens* trees show inbreeding depression for the growth parameters of height, diameter at breast height, trunk basal area and volume, and show reduced flower bud production at reproductive maturity (Hardner and Tibbits, 1998).

To avoid inbreeding problems, eucalypt breeders aim to maximise outcrossing within seed orchards (Griffin and Cotterill, 1988). An understanding of the breeding system of the species is important in achieving this goal (Eldridge, 1978). Eucalypt species are preferentially outcrossing, although self-pollinated seed are often produced (Griffin *et al.*, 1987). Within a species, individuals can range from fully self-compatible to fully self-incompatible (Ellis and Sedgley, 1992; Potts and Savva, 1988). Seed orchards comprising only self-incompatible trees would eliminate inbreeding problems, and knowledge of the self-incompatibility mechanism of an individual species may assist in identifying self-incompatible trees.

At present, there is a lack of information relating to the reproductive processes operating in the *E. nitens* pistil. Growth of *E. nitens* pollen tubes has been studied in *E. globulus* styles (Gore *et al.*, 1990), but not in *E. nitens* flowers. An embryological study on *E. nitens* considered the structure of embryos from mature seeds (Bandyopadhyay and Hamill, 2000) but early stages of embryological development were not investigated. The current study investigated pollen tube growth in *E. nitens* pistils and early embryo development following controlled self- and cross-pollinations to elucidate the self-incompatibility mechanism in this species.

3.2 Materials and methods

3.2.1 Plants

Five mature *E. nitens* trees (located in a seed orchard at Bream Creek, south-eastern Tasmania (42° 48' 05" lat. / 147° 49' 49" long.)) were selected as pollen recipients. These trees had been previously labelled to identify their position within the orchard, and these labels have been retained in this study. All trees showed evidence of previous capsule retention and had similar flowering times. The self-incompatibility status of the trees was unknown.

3.2.2 Pollinations

Individual flowers were emasculated just prior to anthesis, which was identified by a change in colour of the operculum from green to yellow through to pink, and eventually by operculum lift. Pollen shed before anthesis was not observed. The opercula were removed and individual flowers emasculated with a curved scalpel blade, taking care not to damage the staminal ring. An average of five flowers was emasculated and isolated from pollen contamination within a single wax-coated paper bag. Open flowers and green buds were removed prior to emasculation. Each pollination bag was randomly assigned one of two pollen treatments (self- or cross-pollination). Seven days after emasculation the pollination bags were opened, the pollen treatment was applied to all flowers within the bag, and the bag re-sealed. Four trees had twenty pollination bags, and the fifth tree had thirteen pollination bags, for each pollen treatment.

3.2.3 Pollen

The two pollen treatments were pollen from the same tree (self-pollination) or a pollen mixture (cross-pollination). The pollen used in both pollen treatments was

collected at the time of flower emasculation, then stored in gelatine capsules over silica gel in glass vials at -20°C until required. Pollen mixtures were made up by collecting pollen from equal numbers of flowers from nine *E. nitens* trees unrelated to the females (family history to grandparent level known) which were also located in the seed orchard. Pollen treatments were applied to the stigmas of flowers using separate fine bristled paint brushes for each pollen treatment.

The viability of pollen was tested by streaking pollen samples onto semi-solid agar media (Potts and Marsden-Smedley, 1989). Samples were incubated at approximately 25°C for 24 h, then viewed with a light microscope to observe pollen grain germination and pollen tube growth. All pollen samples had abundant pollen tube growth.

3.2.4 Field harvests and microscopy

Two weeks after pollination, one flower was harvested from each pollination bag, and the bag re-sealed. At 4 weeks after pollination, one flower was harvested from ten randomly chosen pollination bags, for each treatment and tree. A further harvest took place 6 weeks after pollination, with a single flower harvested from five randomly chosen pollination bags, for each treatment and tree.

The style and one locule dissected from all flowers harvested 2 weeks after pollination were placed into Carnoy's fixative (Sass, 1958) for a minimum of 24 hours. Fixed material was re-hydrated through an alcohol series (90 %, 70 % and 30 %), softened in 0.1N sodium hydroxide at 60°C for five hours, and stained overnight with decolourised aniline blue (Martin, 1959). The cuticle of each style was scored longitudinally, and individual styles squashed onto microscopy slides in 80 % glycerol. All ovules from each locule were mounted onto microscope slides.

Fluorescence microscopy was used to determine the number of pollen tubes at the base of each style. Every ovule was scored as either penetrated or not penetrated by a pollen tube.

One locule from all flowers harvested at 2, 4 and 6 weeks after pollination was processed for bright field microscopy following the method described by Feder and O'Brien (1968). Ovules were fixed in 3 % glutaraldehyde in 0.025M phosphate buffer, pH 7.2, for a minimum of 48 hours. Once fixed, ovules were dehydrated through a series of alcohols. The alcohol series comprised methoxy-ethanol, ethanol, propanol and butanol. Individual ovules were dissected from locules whilst in ethanol. Ovules were infiltrated overnight in a 1 : 1 mixture of butanol : glycol methacrylate (GMA). Following further infiltration individual ovules were embedded in GMA in gelatine capsules and polymerised at 60°C for 2 days. Only ovules from three self- and three cross-pollinated flowers from three trees harvested 2 weeks after pollination, and ovules from a single self- and cross-pollinated flower from the same trees 4 weeks after pollination were processed through to embedding in GMA. The remaining intact and fixed locules were dehydrated through methoxy-ethanol to ethanol and stored for observation.

Serial, longitudinal sections (LS), 3 μm thick, were cut through all embedded ovules. No correction was made for bilateral symmetry as this was minimal in this material. Sections were stained with Periodic acid-Schiff's reagent followed by Toluidine blue O (PAS/TBO). Sections were observed on a Zeiss Axiophot microscope using bright field optics for evidence of fertilisation. Data were recorded about the state of the nucellus cells, the presence or absence of an embryo sac, and the contents and the appearance of contents of each embryo sac. A calibrated microscope eyepiece was used to measure the length and diameter of each ovule and its embryo sac.

3.2.5 Seed set

Pollination bags were removed following the 6 week harvest and all remaining capsules were left to mature. All capsules remaining at 12 months after pollination were harvested and allowed to release their seed. The number of seed in each capsule was counted to provide an estimate of the degree of self-incompatibility in each experimental tree. Self-incompatibility (%) was determined using the following formula:

$$SI = ((V_{CP} - V_{SP}) / V_{CP}) * 100$$

where

SI = self-incompatibility,

V_{CP} = viable seed per flower cross-pollinated and

V_{SP} = viable seed per flower self-pollinated.

3.2.6 Statistical analysis

3.2.6.1 Seed set

Wilcoxon 2-sample tests were used to investigate the effect of pollen treatment on seed set per flower per tree. As it was common for more than one capsule to be harvested from within a pollination bag, average bag data were analysed. *P* values were considered statistically significant when below 0.05. This was the case throughout this study.

3.2.6.2 Pollen tubes and ovule penetration

A statistical model including terms for the fixed effects of tree, pollen treatment and the interaction between tree and pollen treatment was fitted to the pollen tube count and ovule penetration data from the five trees using PROC MIXED of SAS (SAS, 1992). This procedure uses a Maximum Likelihood (REML) method to fit the model. Main effect and interaction Least Square Means were calculated and pair-

wise contrasts undertaken. Data on the number of pollen tubes in the style were *log*10 transformed prior to analysis to attain normality.

3.2.6.3 Ovule fertilisation

To investigate whether the proportion of fertilised and healthy ovules differed between treatments within each tree, data on fertilisation status collected 2 weeks after pollination were pooled and subjected to Chi-square analysis.

3.2.6.4 Ovule dimensions

To determine if ovule and embryo sac lengths and widths differed with respect to fertilisation status, pollen treatment and harvest time, all data collected for each of the four traits was analysed using PROC MIXED. The model included the fixed effects of tree, fertilisation status, pollen treatment, harvest time and their interactions. Only ovule and embryo sac length data had significant interaction terms involving the effect, tree. Hence, individual pair-wise contrasts were run for length data to determine if ovule and embryo sac lengths differed between fertilisation status, for each tree, pollen treatment, and harvest time.

3.3 Results

3.3.1 Seed set and self-incompatibility

Trees ranged in self-incompatibility levels from 25.8 to 93.6 % (Table 3.1). All trees except one set significantly more seed per flower following cross- compared with self-pollination. There was a trend of reduced self-pollinated capsule retention with increasing self-incompatibility levels.

Table 3.1. Number of flowers pollinated and capsules set, and mean number of seed per flower following self- and cross-pollination, with level of self-incompatibility. *P* values represent comparisons between the mean number of self- and cross-pollinated seeds set per flower pollinated. SP, self-pollinated; CP, cross-pollinated; SI, self-incompatibility.

Tree code	Flowers SP	Capsules set	Mean seed set per SP flower	Flowers CP	Capsules set	Mean seed set per CP flower	*P*	SI (%)
1,7	61	2	0.3	54	36	4.7	<0.0001	93.6
1,8	65	21	0.7	58	31	4.5	0.0013	83.9
2,27	86	12	0.4	83	50	5.1	<0.0001	93.2
7,29	28	20	3.1	27	14	4.2	0.3206	25.8
8,22	61	51	4.1	46	35	6.9	0.0072	40.3

Note: Calculations to determine self-incompatibility estimates used seed set values with 2 decimal places. These values have been rounded to 1 decimal place in this table.

Plate 3.1

A. Fluorescence image of a squashed *Eucalyptus nitens* style harvested 2 weeks after self-pollination and stained with aniline blue. Following pollen grain germination (pg), pollen tubes (pt) travelled the length of the style. Bar = 400 µm.

B. Fluorescence image of a dissected *Eucalyptus nitens* ovule harvested 2 weeks after self-pollination and stained with aniline blue. A pollen tube (pt) penetrated the ovule and entered the micropyle. Bar = 200 µm.

C. Longitudinal section of a degenerating *Eucalyptus nitens* ovule 4 weeks after self-pollination, stained with PAS/TBO, showing a collapsed embryo sac surrounded by degenerated nucellus cells (dn). Bar = 200 µm.

D. Longitudinal section of the micropylar end of a *Eucalyptus nitens* embryo sac 2 weeks after cross-pollination, stained with PAS/TBO, showing zygote (z) and persistent synergid (s). Note presence of filiform apparatus (fa) of both persistent and degenerated synergid. Bar = 20 µm.

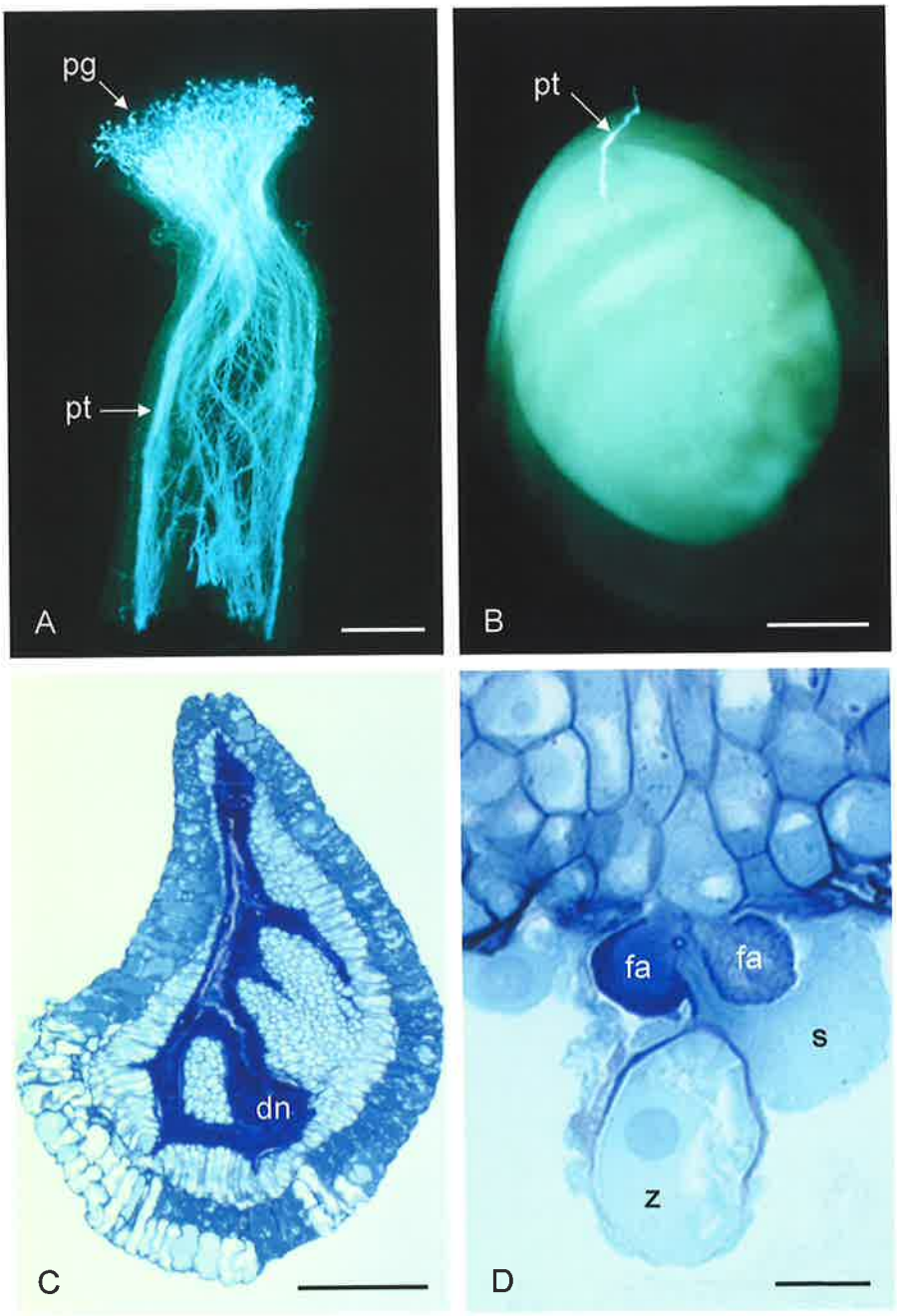

pg
pt
A
pt
B
dn
C
fa
fa
s
z
D

Table 3.2. Mean number of pollen tubes in styles averaged across self- and cross-pollinated flowers for each tree.

Tree code	Average pollen tubes	Standard error	Sig.
1,7	34.2	4.1	a
1,8	46.8	4.2	b
2,27	57.8	4.4	b
7,29	67.9	5.3	c
8,22	34.4	4.0	a

Note: Different letters indicate a significant difference (P=0.05).

3.3.2 Pollen tubes in styles

Pollen grains had germinated on stigmas and pollen tubes had grown the full length of the style by 2 weeks after pollination (Plate 3.1A). Pollen tube numbers between treatments across all five trees were not significantly different (P = 0.52). There was an average of 47.9 pollen tubes in styles following cross-pollination and an average of 48.5 pollen tubes in styles following self-pollination. The number of pollen tubes present varied significantly (P <0.001) between trees and averages ranged from 34.2 to 67.9 (Table 3.2). There was no significant interaction between pollination treatment and tree (P = 0.15).

3.3.3 Ovary arrangement and ovule penetration

Flowers generally contained three locules in the ovary, with four or two locules occurring rarely. Within a locule, there was an average of 10.7 ovules. Pollen tubes had reached ovules by 2 weeks after pollination in all five trees, with pollen tubes observed to have penetrated ovules through the micropyle (Plate 3.1B). Analysis of pollen tube penetration data revealed that there was no significant difference in the proportion of ovules penetrated by a pollen tube either, between pollen treatments (P = 0.13), or between trees (P = 0.21). Cross-pollination resulted in 53 % of ovules being penetrated by a pollen tube, with 48 % of ovules penetrated following self-pollination.

3.3.4 Ovule structure

Sectioning work was conducted on about 30 ovules for each pollen treatment per tree, and results were expressed as percentages. Fertilisation had occurred by 2 weeks after pollination. There was either evidence of fertilisation, with ovules either healthy or showing signs of degeneration, or rarely, ovules had embryo sacs that had degenerated and collapsed. When an embryo sac was observed to have collapsed it

Table 3.3. Anatomical details of ovules from both self- and cross-pollinations, at 2 and 4 weeks after pollination. % values represent the percentage of ovules with each feature averaged over three locules for each pollen treatment at 2 weeks after pollination and for 1 locule for each pollen treatment 4 weeks after pollination.

			% Ovules with						% Ovules		
Flowers harvested	Tree code	Pollination treatment	Nucellus degeneration	Nuclear endosperm healthy	Nuclear endosperm degenerating	Zygote healthy	Zygote degenerating	Persistent synergid	Fertilised healthy	Fertilised degenerating	Fertilisation status unknown
2 weeks after pollination	1,7	self	0	8	92	46	17	83	8	92	0
		cross	0	90	10	90	0	97	90	10	0
	2,27	self	0	13	87	13	10	100	10	90	0
		cross	9	88	9	84	3	91	81	19	0
	8,22	self	0	26	71	41	3	82	26	71	3
		cross	4	82	18	86	4	96	79	21	0
4 weeks after pollination	1,7	self	100	0	45	0	9	27	0	45	55
		cross	73	36	64	36	0	45	36	64	0
	2,27	self	90	10	60	10	20	20	10	60	30
		cross	60	40	50	40	0	40	40	50	10
	8,22	self	78	11	78	11	11	78	11	67	22
		cross	50	50	50	50	0	38	50	50	0

Plate 3.2

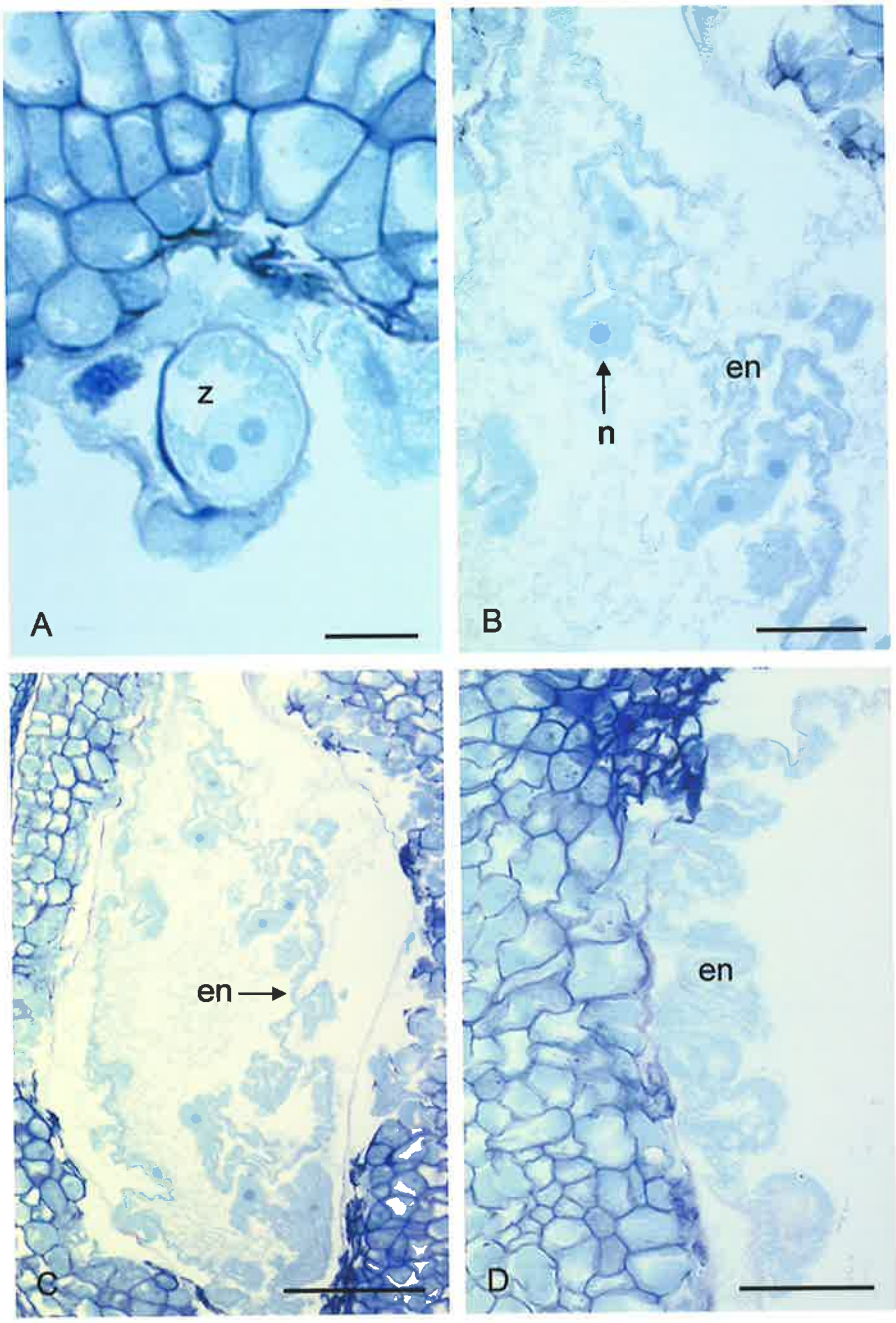
Longitudinal sections of *Eucalyptus nitens* ovules stained with PAS/TBO and observed with bright field optics.

A. Micropylar end of embryo sac at 2 weeks after cross-pollination showing zygote (z) with divided nucleus. Bar = 20 μm.

B. Embryo sac 2 weeks after cross-pollination showing nuclei (n) of free nuclear endosperm (en). Bar = 40 μm.

C. Embryo sac 2 weeks after cross-pollination showing free nuclear endosperm (en) forming a ring of cytoplasm in the embryo sac lumen. Bar = 100 μm.

D. Embryo sac 2 weeks after self-pollination showing degenerating endosperm (e) with no nuclei visible. Bar = 50 μm.


z
A
n
en
B
en
C
en
D

was not possible to determine whether it had been fertilised or not (Table 3.3) (Plate 3.1C).

Ovules were considered fertilised and healthy when the embryo sac contained an intact zygote, frequently adjacent to a single or occasionally two persistent synergid cells, which were located at the micropylar end of the embryo sac (Plate 3.1D). In two self- and two cross-pollinated zygotes, the nucleus had divided to give two distinct nuclei (Plate 3.2A). Healthy fertilised ovules also had free nuclear endosperm, composed of several nuclei joined by cytoplasm (Plate 3.2B). The free nuclear endosperm formed a ring within the embryo sac in longitudinal sections (Plate 3.2C). Finally, ovules classified as healthy, contained nucellus cells with no signs of degeneration (loss of cell integrity and dark staining).

Fertilised but degenerating ovules typically had endosperm present with no (or very few) nuclei present (Plate 3.2D). In these ovules zygotes were sometimes present and often showed signs of degeneration, including loss of shape and definition of the cell wall and nucleus (Plate 3.3A). Embryo sacs occasionally appeared smaller, indicating they were starting to collapse, and a few ovules had degenerating nucellus cells (Plate 3.3B). By 4 weeks after pollination two self- and two cross-pollinated ovules had spherical multicellular embryos (Plate 3.3C). Occasionally a pollen tube could be seen, having grown in the micropyle towards the embryo sac (Plate 3.3D).

3.3.5 Ovule fertilisation

At 2 weeks after pollination most ovules showed evidence of fertilisation. The percentage of fertilised, healthy ovules was greater following cross- than self-pollination in all three trees. Analysis of the total number of fertilised, healthy ovules within each tree revealed that there were significantly more fertilised, healthy ovules following cross- than self-pollination (P <0.0001 for each tree). By 4 weeks after

Plate 3.3

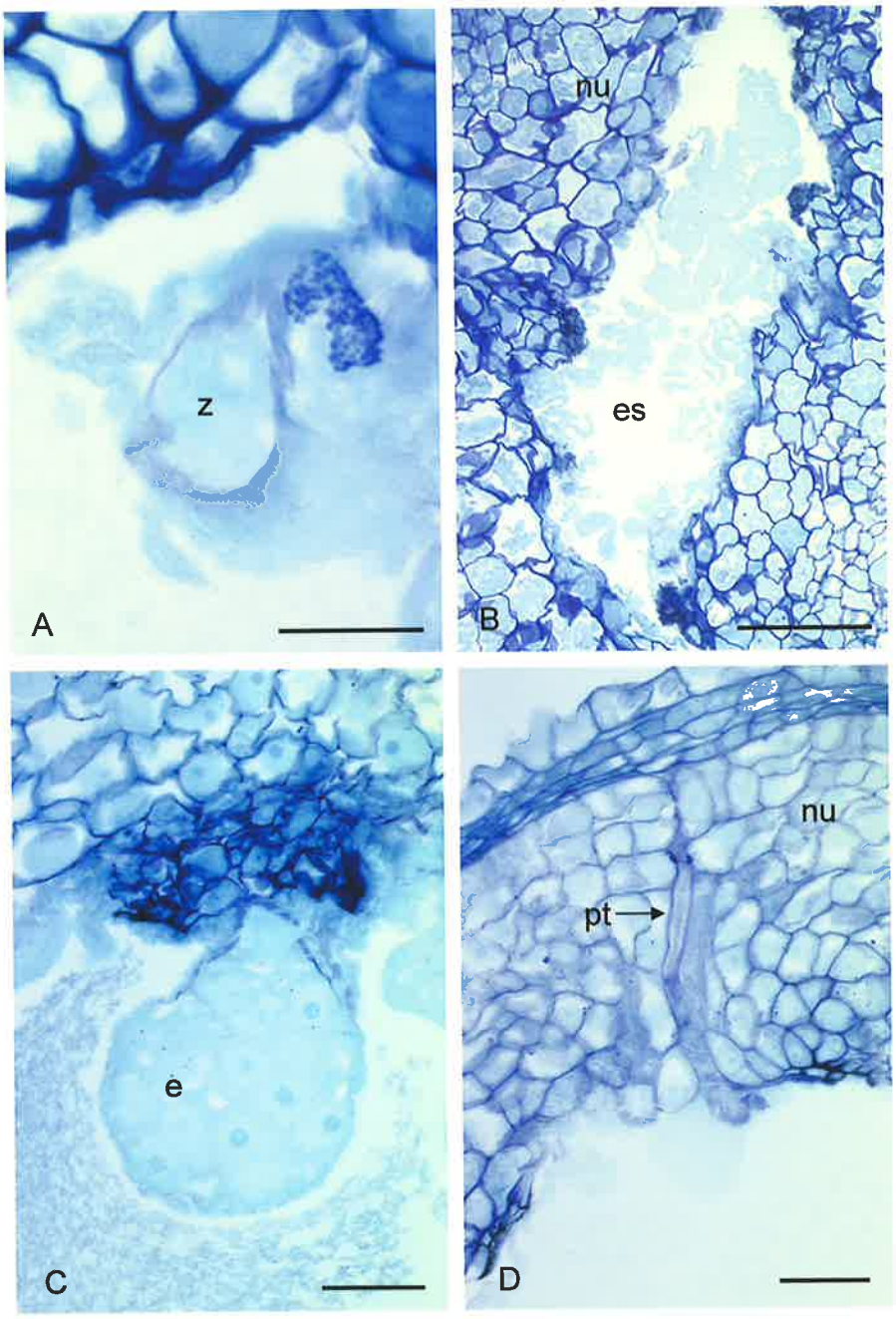
Longitudinal sections of *Eucalyptus nitens* ovules stained with PAS/TBO.

A. Micropylar end of embryo sac 2 weeks after self-pollination showing degenerating zygote (z) with no nucleus visible. Bar = 20 μm.

B. Collapsing embryo sac (es) and degenerating nucellus cells (nu) 4 weeks after self-pollination. Bar = 100 μm.

C. Micropylar end of embryo sac 4 weeks after cross-pollination showing multicellular embryo (e). Bar = 40 μm.

D. Micropylar end of ovule 2 weeks after cross-pollination showing a pollen tube (pt) in the micropyle surrounded by nucellus tissue (nu). Bar = 40 μm.


z
A
nu
es
B
e
C
nu
pt
D

pollination, the percentage of fertilised, healthy ovules was less than at 2 weeks after pollination, for all three trees and both pollen treatments, except for the self-pollen treatment in tree 2,27 which remained the same. Many ovules that were initially fertilised had ceased developing and were degenerating, however, there was still a trend for more fertilised, healthy ovules following cross- compared with self-fertilisation.

3.3.6 Ovule dimensions

Fertilised, healthy ovules were wider in cross-section and had wider embryo sacs than ovules that were degenerating, for all trees at both harvest times, regardless of pollen treatment (P <0.0001).

At 2 weeks after pollination, fertilised, healthy ovules were longer than those that were not fertilised and healthy in all three trees, regardless of pollen treatment, except the self-pollen treatment for tree 1,7, where no significant difference was found (Table 3.4). Ovule lengths had increased by 4 weeks after pollination, with fertilised, healthy ovules still longer than unfertilised or degenerating ovules, except the self-pollen treatment for tree 8,22, where no difference was found.

Embryo sac lengths were longer for fertilised, healthy ovules compared to those that were not, at 2 weeks after pollination. No differences were found following self-pollination. By 4 weeks after pollination embryo sac lengths were longest in fertilised, healthy ovules, in all trees regardless of pollen treatment, except the self-pollen treatment for tree 8,22, where no significant difference was found (Table 3.5).

Observation of locules harvested 6 weeks after pollination enabled distinction between developing and non-developing ovules for all three trees (Plate 3.4A and B)

Table 3.4. Mean ovule lengths and widths. *P* values represent comparisons between the ovule length of 'fertilised and healthy' and 'not fertilised and healthy' ovules. Separate *P* values not obtained for ovule width as an overall analysis revealed healthy, fertilised ovules were significantly wider than ovules that were not fertilised and healthy ($P < 0.0001$). SE, standard error of the mean (~30 values).

Flowers harvested	Tree code	Treatment	Ovule length (µm)					Ovule width (µm)			
			Fertilised and healthy	SE	Not Fertilised and healthy	SE	*P*	Fertilised and healthy	SE	Not Fertilised and healthy	SE
2 weeks after pollination	1,7	Self	890	88	845	27	0.6294	540	66	554	20
		Cross	942	24	700	72	0.0017	566	18	413	54
	2,27	Self	980	72	828	24	0.0468	613	54	547	18
		Cross	1052	24	917	51	0.0176	845	47	580	38
	8,22	Self	971	42	847	25	0.0113	618	31	581	19
		Cross	1002	27	837	51	0.0044	651	20	593	38
4 weeks after pollination	1,7	Self	-	-	1029	38	-	-	-	619	28
		Cross	1600	62	920	47	<0.0001	845	47	566	35
	2,27	Self	1560	125	1044	42	0.0001	840	94	631	31
		Cross	1485	62	982	51	<0.0001	845	47	580	38
	8,22	Self	980	125	1040	44	0.6508	780	94	660	33
		Cross	1430	62	1085	62	0.0001	855	47	740	47

Table 3.5. Mean embryo sac lengths and widths. *P* values represent comparisons between the embryo-sac length of fertilised and healthy and not fertilised and healthy ovules. Separate *P* values not obtained for embryo sac width as an overall analysis revealed embryo sacs within healthy, fertilised ovules were significantly wider than embryo sacs within ovules that were not fertilised and healthy ($P < 0.0001$). SE, standard error of the mean (~30 values).

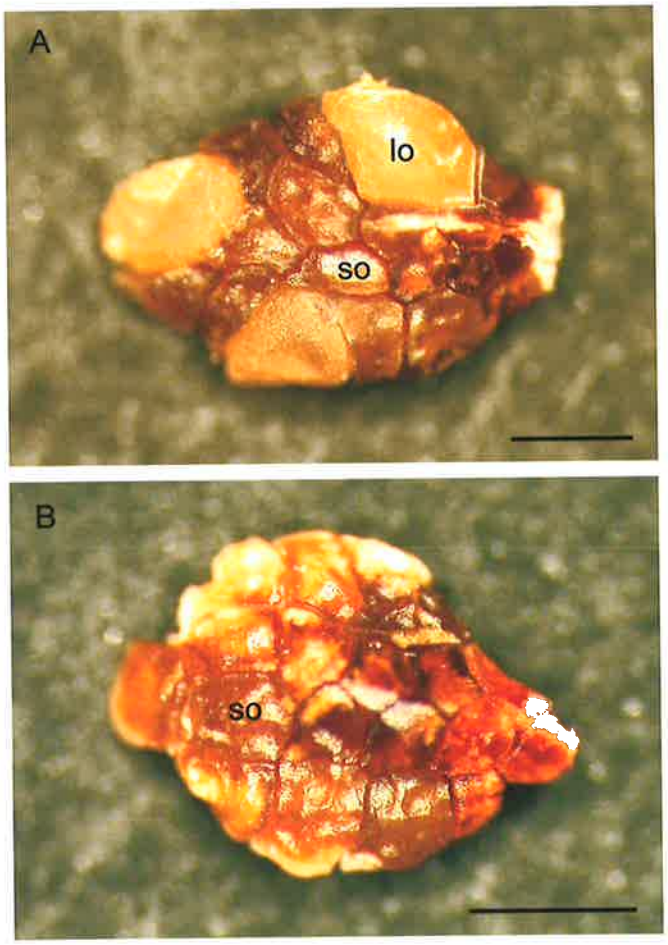
Flowers harvested	Tree	Treatment	Embryo sac length (µm)					Embryo sac width (µm)			
			Fertilised and healthy	SE	Not Fertilised and healthy	SE	*P*	Fertilised and healthy	SE	Not Fertilised and healthy	SE
2 weeks after pollination	1,7	Self	420	89	440	27	0.8305	230	60	229	18
		Cross	542	25	367	73	0.0236	263	17	207	49
	2,27	Self	567	73	451	24	0.1346	307	49	291	16
		Cross	608	25	460	52	0.0105	305	17	237	35
	8,22	Self	556	42	502	25	0.2804	276	28	297	17
		Cross	581	27	447	52	0.0747	311	18	290	35
4 weeks after pollination	1,7	Self	-	-	406	48	-	-	-	95	25
		Cross	1090	63	360	48	0.0002	380	42	149	32
	2,27	Self	1060	126	509	48	<0.0001	480	85	113	28
		Cross	975	63	452	57	<0.0001	475	42	143	35
	8,22	Self	560	126	428	45	0.3239	420	85	151	30
		Cross	965	63	470	63	<0.0001	390	42	195	42

Plate 3.4

Dissected locules from *Eucalyptus nitens* flowers harvested 6 weeks after self-pollination.

A. Locule showing large ovules (lo) and small ovules (so). Bar = 1 mm.

B. Locule showing only small ovules (so). Bar = 1 mm.


A
lo
so
B
so

3.4 Discussion

The five trees used in this study varied in their ability to prevent the production of selfed seed. Trees 1,7 and 2,27 appeared to have the strongest barriers to selfed seed production, as indicated by their 93.6 and 93.2 % self-incompatibility levels respectively. Even though self-incompatibility levels ranged widely between the five trees, all retained more capsules and produced more seeds following cross- compared with self-pollination, demonstrating preferential outcrossing, a reproductive trend common to *Eucalyptus* (Eldridge *et al.*, 1993).

Pollen tubes grew down styles of all five trees equally well following self- and cross-pollinations. Thus, no barrier to selfing appeared to be operating in the style of *E. nitens*. This result is consistent with findings from other eucalypt species (eg. Sedgley and Granger, 1996; Sedgley *et al.*, 1989). There was variation in the number of pollen tubes present in styles between trees, but as the mean number of pollen tubes was greater than the mean number of ovules per flower (47.9 pollen tubes / style vs 32.1 ovules / flower), pollen tube numbers were not found to limit seed set.

Whilst there were, on average, more pollen tubes than ovules, only about 50 % of ovules were observed to have been penetrated by a pollen tube, with no difference between pollen treatments. Pollen treatments had no effect on the proportion of ovules penetrated by a pollen tube, indicating that the barrier to selfed seed production was operating beyond the ovule penetration stage.

Fertilisation had taken place by 2 weeks after pollination with most ovules fertilised, leading to the conclusion that the barrier to selfed seed production operated post-zygotically. Furthermore, a greater percentage of self- compared to cross-fertilised ovules was showing signs of degeneration by 2 weeks after pollination, and there were fewer healthy self- compared to cross-fertilised ovules and many more

degenerated ovules following selfing, at 4 weeks after pollination. Hence, self-fertilised ovules appeared to be aborted relatively early in the post-fertilisation seed development process.

Examples of species with late-acting self-incompatibility mechanisms have been accumulating in recent years (Seavey and Bawa, 1986), however, relatively few studies have investigated embryological processes and confirmed that fertilisation has occurred before ovule failure. Within *Eucalyptus*, Sedgley *et al.* (1989) found fertilisation had occurred in *E. regnans* following both controlled self- and cross-pollinations, with no difference in embryo and endosperm development up to 16 weeks after pollination. Other examples include *Capparis retusa*, where some selfed ovules initiated embryo development, but by 96 h after pollination, were smaller than cross-pollinated ovules (Bianchi and Gibbs, 2000). *Chorisia chodatii*, *C. speciosa*, *Tabebuia caraiba* and *T. ochracea* all form resting zygotes and undergo initial endosperm divisions following self- and cross-pollinations before selfed flowers abscise (Gibbs and Bianchi, 1993). It is possible that more species will be found to have post-zygotic failure of selfed ovules where ovule development is investigated in detail.

Neither sporophytic nor gametophytic stylar based self-incompatibility systems (de Nettancourt, 2001) are likely to be operating in *E. nitens*, as near-complete self-fertilisation was demonstrated. Instead, a late-acting self-incompatibility system, as proposed by Charlesworth (1985) and Seavey and Bawa (1986), appears to be operating to reduce selfed seed production. At present there are two main hypotheses proposed to explain late-acting rejection events; a post-zygotic self-incompatibility response of an undefined genetic nature, and early-acting inbreeding depression. Currently, it is difficult to experimentally determine which system is operating within a species (see Charlesworth, 1985; Sage *et al.*, 1994; Seavey and Bawa, 1986). Seavey and Bawa (1986) suggested uniform ovule abortions may indicate a self-incompatibility response whereas ovule abortions occurring at various stages of

embryo development would be indicative of inbreeding depression. Some authors have favoured the inbreeding depression hypothesis as self-fertilised ovules have been found to abort over the course of seed development (Bertin *et al.*, 1989; Wiens *et al.*, 1987). However, Waser and Price (1991) argue that for the high levels of self-incompatibility observed within species, the large number of lethals an individual would require in a heterozygous condition to result in such few, if any, selfed seed, is unrealistic. Most self-fertilised *E. nitens* ovules showed evidence of degeneration by 4 weeks after pollination, and as most self-fertilised ovules had begun to degenerate within the first few weeks following pollination, instead of being spread over the entire seed development time, ovule breakdown may be a self-incompatibility response.

Whilst nearly all ovules showed evidence of fertilisation 2 weeks after pollination, only about 50 % of ovules were observed to have been penetrated by a pollen tube 2 weeks after pollination. This discrepancy suggests that, in some penetrated ovules the pollen tube was not visible. Unevenness in pollen tube fluorescence has been reported in styles of *Tecona grandis* (Tangmitcharoen and Owens, 1997) and at the ovule level in *Dolichandra cynanchoides* (Gibbs and Bianchi, 1999), and this may also be the case in *E. nitens*. Alternatively, the physical action of removing ovules from the placenta may have caused pollen tubes to be pulled out of the micropyles of some ovules. Whilst it seems reasonable to compare pollen treatments at the ovule penetration stage, these data suggest methods used for pollen tube observation at the ovule level may not reflect actual penetration levels. This problem highlights the importance of extending studies to consider embryological development for a clearer representation of reproductive success.

A reduction in the number of healthy, selfed ovules was expected as the trees were, to differing degrees, self-incompatible. However, some healthy, cross-fertilised ovules aborted between 2 and 4 weeks after pollination, with further abortions assumed to occur between 4 weeks after pollination and seed set. A similar result

was found in *E. regnans* (Sedgley *et al.*, 1989). As angiosperms are known to invest maternal resources into seed development following fertilisation (Westoby and Rice, 1982) it is possible that the reproductive strategy in *E. nitens* is to produce fewer, more competitive seeds. Selection against inferior offspring genotypes due to pollen source variability (Wiens, 1984; Wiens *et al.*, 1987) may have occurred such that resources were directed to potentially superior offspring (Westoby and Rice, 1982).

Ovule size was greatest in healthy, developing ovules, and at six weeks after pollination, locules from self- and cross-pollinated flowers showed size differences in ovules (no data collected but photos were taken). However, it is likely that, at later stages of development, healthy, developing ovules will be easier to detect. It currently takes about a year to determine the self-incompatibility status of a tree. Controlled self- and cross-pollinations are conducted and comparisons between seed set numbers are made. This information on ovule size may prove useful for breeders by reducing the time required to determine the likelihood that trees will set selfed seed. Although larger-scale verification is required, it may be possible to determine the ability of individual trees to produce selfed seed by conducting self- and cross-pollinations, harvesting flowers as early as 2 months after pollination, followed by inspections of ovule size. The fewer developing, larger ovules following self-pollinations, the more self-incompatible an individual tree is likely to be.

In conclusion, *E. nitens* trees vary greatly in their ability to produce self-pollinated seed. Such variation in selfed seed set highlights the importance of determining the self-incompatibility level of individual trees within *E. nitens* seed orchards to maximise the production of outcrossed seed. Self-incompatibility appears to be controlled by post-zygotic abortion of selfed ovules, and differences in ovule size following pollination may assist in assessing the potential of individual trees to set selfed seed.

Chapter 4

Pollen tube growth and seed set in *Eucalyptus globulus*

4.1 Introduction

Only a few eucalypt species have been studied to determine the site of action of the self-incompatibility mechanism. These studies have revealed differing mechanisms ranging from late-prezygotic to post-zygotic mechanisms (Ellis and Sedgley, 1992; Sedgley and Granger, 1996; Sedgley *et al.*, 1989; Sedgley and Smith, 1989).

Eucalyptus globulus is now widely planted around the world, mostly for pulpwood for paper production. There is a demand for genetic improvement and at present the genus is largely seed propagated (Griffin and Cotterill, 1988; Griffin *et al.*, 1987; van Wyk, 1977) as temperate eucalypt species are difficult to propagate vegetatively (Griffin, 1989). Seed orchards have been established to produce genetically improved seeds in large quantities but seed orchards have been failing to reach their potential, both in seed quality and quantity (Griffin and Cotterill, 1988; Griffin *et al.*, 1987). Moncur *et al.* (1995) reported on self-fertilisation occurring in an *E. globulus* seed orchard, with the orchard having a mean outcrossing rate of 77 %. Selfed eucalypt seed results in inbreeding depression in the resultant generation at several growth stages and this is detrimental to the plantation industry (Eldridge and Griffin, 1983; Hardner and Tibbits, 1998; Hardner and Potts, 1995; Hardner and Potts, 1997; Potts *et al.*, 1987).

In order to successfully manage a eucalypt seed orchard it is important that the reproductive biology and breeding system of the species are well understood (Eldridge, 1978). Very little research has been conducted to investigate reproductive processes in *E. globulus* pistils. Trindade *et al.* (2001) found that outcross *E. globulus* pollen grains were hydrated by 8 h after pollination, pollen tubes were detected 24 h after pollination and a few had reached the base of the style after 7 days. Gore *et al.* (1990) determined that *E. globulus* outcross pollen tubes grow an average of 1.4 mm per day through the transmitting tissue down the style and grow into the ovary between five and 14 days after pollination. The mechanism of self-incompatibility in *E. globulus* is unknown. The current method of determining whether individual trees are self-incompatible involves conducting controlled pollinations with self- and outcross-pollen, waiting about a year for seeds to mature and then making comparisons between seed set from the self- and outcross pollinations. The aim of this study was to investigate pollen tube growth in self-incompatible *E. globulus* pistils in order to gain an understanding of the mechanism of self-incompatibility.

4.2 Materials and methods

4.2.1 Trees

The experiment was conducted on five wild mature *E. globulus* trees located south of Hobart, Tasmania, with pollinations carried out during September and October 1999. These five trees were chosen due to prior knowledge of their high self-incompatibility status, their floral abundance and accessibility for hand pollination. All trees had previously produced seed crops for several years, and had been allocated numbers for identification purposes that have been retained in this study.

4.2.2 Pollinations

Flowers on each tree were emasculated just before anthesis by removing the lifting operculum and the stamens before dehiscence, but leaving the staminal ring intact. The emasculated flowers were isolated in wax-coated paper bags. As eucalypt flowers are protandrous with about a week between anther dehiscence and stigma receptivity (Pryor, 1976) the isolation bags were opened between 5 and 7 days after emasculation, the flowers were pollinated with either one of two treatments (self- and cross-pollination) and the bags reclosed. The isolation bags were left covering the flowers for 8 weeks, by which time the stigmas had dried out.

A minimum of 54 flowers per treatment per tree were pollinated for early harvesting and a further 20 flowers per treatment per tree were pollinated to be left for seed set. The two treatments were pollen from flowers from the same tree (self-pollination) and cross-pollination. Cross-pollen was collected as a mixture of pollen from 10 different, unrelated *E. globulus* trees. Trees were considered genetically unrelated to the experimental trees if they were located at a distance greater than 500 m from the experimental trees (Hardner *et al.*, 1998). Pollen was collected from flowers opened in the laboratory and subsequently stored in gelatine capsules at –20 °C over silica gel.

Pollen viability tests were conducted on the stored pollen before use, to ensure pollen was viable. Pollen samples were streaked onto semi-solid agar media (Potts and Marsden-Smedley, 1989), allowed to incubate at about 25 °C for 24 h and then viewed with a light microscope for pollen germination and pollen tube growth.

4.2.3 Field harvests and microscopy

Between 18 and 24 flowers were harvested per treatment per tree at 2 weeks after pollination and between 7 and 12 flowers were harvested per treatment per tree at four weeks after pollination. The style was dissected from each flower harvested 2 weeks after pollination and one locule per flower from both 2- and 4-week harvests

was sampled. All dissected material was processed for fluorescence microscopy as previously described in Chapter 3, except samples were placed in sodium hydroxide at 60°C for half an hour only.

Styles were scored longitudinally to sever the outer cuticle and squashed onto microscope slides. A single count of the number of pollen tubes in each style was made about two-thirds down the length of the style.

The total number of ovules present within each locule was recorded along with the total number of locules present within each flower for samples harvested 2 weeks after pollination. Ovules from each locule from both 2- and 4-week harvests were dissected off the placenta and placed individually onto microscope slides. Every ovule was scored as either penetrated or not penetrated by a pollen tube.

4.2.4 Seed set

All capsules remaining at maturity (12 months after pollination) from the 20 flowers per treatment per tree pollinated for capsule maturation were harvested and allowed to dry out in the laboratory and release their seed. The number of viable seeds in each capsule was counted. Seeds were considered potentially viable if they were rounded and solid as opposed to flat and consisting only of outer integument. Seed-set data was used to determine the level of self-incompatibility as described in Chapter 3.

4.2.5 Statistical analysis

All data were analysed using Genstat 5 release 4.1, 4th Edition, (1998).

4.2.5.1 Pollen tube growth in styles

The effect of pollen treatment on pollen tube numbers in styles, from data pooled over all five trees, was analysed by the restricted maximum likelihood (REML) method. The statistical model included terms for the fixed effect of pollen treatment,

the random effect of tree and the random interactions between tree and area within tree and between tree and person pollinating. The significance of the pollen treatment was tested by a Wald test.

To investigate the effect of pollen treatment on pollen tube numbers within each tree, data were analysed separately for each tree by REML. The statistical model included terms for the fixed effect of pollen treatment and the random effects of area within tree and person pollinating. Wald tests were used to test the significance of the pollen treatment.

4.2.5.2 Ovary arrangement

Means and standard errors of the number of ovules in a locule were calculated. The means were extrapolated to the whole flower level.

4.2.5.3 Ovule penetration by pollen tubes

Pollen tube penetration data from ovules harvested 2 weeks after pollination and pooled over all five trees were subjected to an emperical logit transform to attain normality and analysed by a weighted REML method to test the effect of pollen treatment on the proportion of ovules penetrated. The statistical model included terms for the fixed effect of pollen treatment, the random effect of tree and the random interaction between tree and area within tree. The significance of the pollen treatment effect was tested by a Wald test.

To investigate the effect of pollen treatment on the proportion of ovules penetrated at 2 weeks after pollination within each tree, data were analysed for each tree separately by the REML method. The statistical model included terms for the fixed effect of pollen treatment and the random effect of area within tree. The significance of the pollen treatment was tested by a Wald test.

Data on ovule penetration by a pollen tube collected at 4 weeks after pollination was pooled over all five trees, square-root transformed to attain normality and

analysed for the effect of pollen treatment by the REML method. Terms included in the model were the fixed effect of pollen treatment and the random effect of tree. A Wald test was used to test the significance of the pollen treatment. The data were back-transformed to calculate means.

To investigate the effect of pollen treatment on the proportion of ovules penetrated within each tree, data were analysed for each tree separately by the REML method. The statistical model included the fixed effect of pollen treatment with no random effects. A Wald test was used to test the significance of the pollen treatment.

4.2.5.3 Seed set

The effect of pollen treatment on seed set per flower per tree was investigated by a Wilcoxon 2-sample test.

4.3 Results

4.3.1 Seed set

Capsule retention following cross-pollination was greater overall than that following self-pollination, with cross-pollination producing significantly more seed per flower (Table 4.1). In all trees, there was significantly greater seed set following cross-pollination. Self-incompatibility levels varied between trees from 100% self-incompatible, with no selfed seed produced, to 76 % self-incompatible with a relatively small amount of seed produced following self-pollination.

4.3.2 Pollen tube growth in styles

Large numbers of pollen tubes were observed near the base of styles in flowers from both pollen treatments 2 weeks after pollination. Pollen grains germinated in abundance on stigmas and pollen tubes travelled down the style, converging at the

Table 4.1. Mean number of seeds per flower following self- and cross-pollination with level of self-incompatibility.

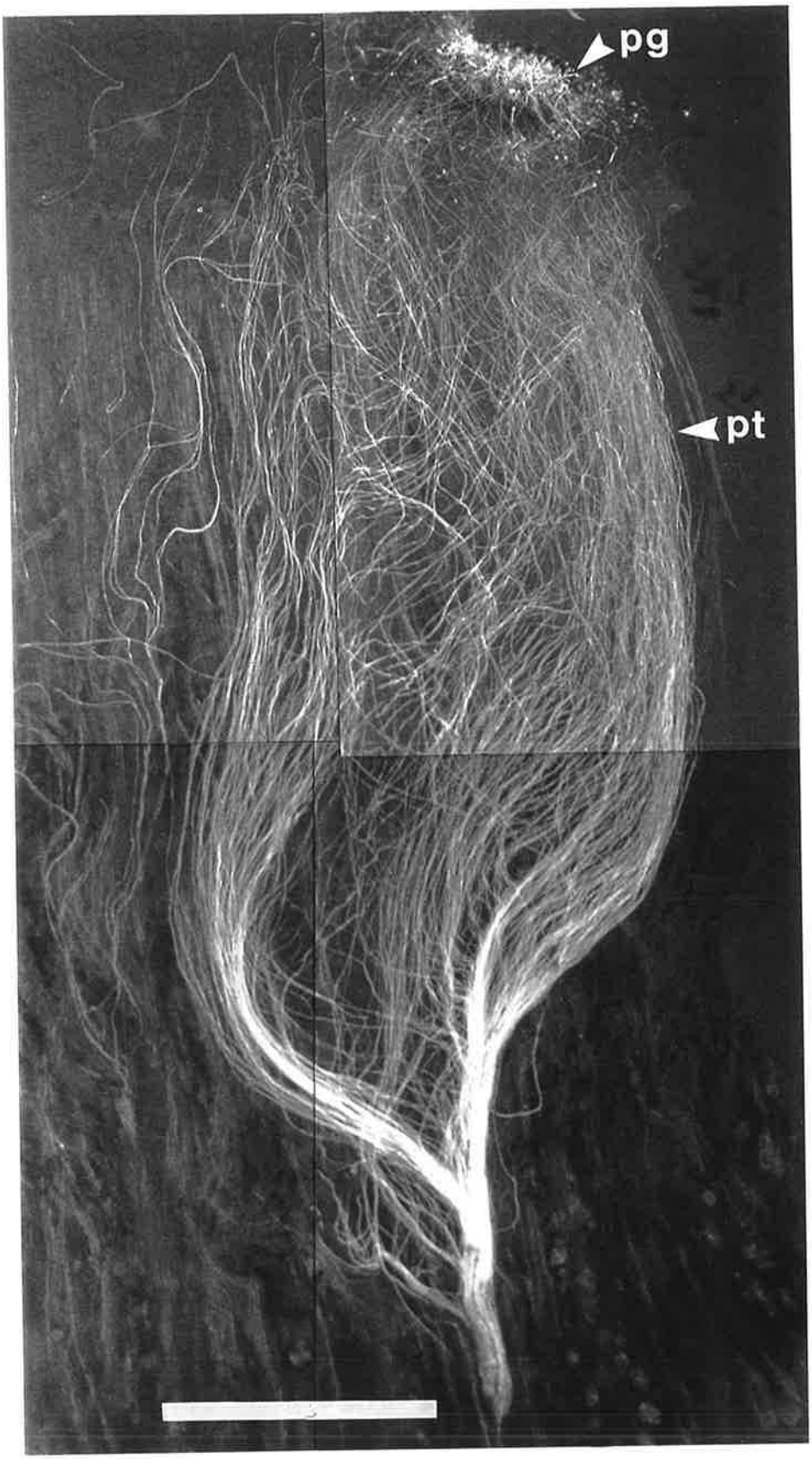
SP, self-pollinated. CP, cross-pollinated. SI, self-incompatibility.

Tree	SP flowers	Capsules set	SP seeds per number of SP flowers	CP flowers	Capsules set	CP seeds per number of CP flowers	*P*	SI (%)
319	20	13	5.0	20	14	20.6	0.0250	76.0
503	19	1	0.2	21	8	28.0	0.0106	99.4
509	20	1	0.0	20	12	15.2	0.0001	100.0
537	20	0	0.0	20	9	14.4	0.0021	100.0
538	20	4	0.6	20	11	17.7	0.0013	96.6

Note: The mean number of seeds produced is presented on a per flower pollinated basis to allow for comparison between pollen treatments in overall seed production success.

Plate 4.1

Fluorescence micrograph of a squashed *Eucalyptus globulus* style harvested at 2 weeks after cross-pollination and stained with aniline blue. Following pollen grain germination (pg), pollen tubes (pt) travelled the length of the style and converged as the transmitting tissue narrowed. Bar = 1 mm.


pg
pt

base of the style as the transmitting tissue narrowed (Plate 4.1). There was no significant difference in pollen tube numbers between treatments from pooled data over all five trees ($\chi^2 = 1.2$, df = 1, $P = 0.273$). The cross-pollination treatment had an average of 287.7 pollen tubes present and the self-pollination treatment had an average of 269.9 pollen tubes present. There was also no significant difference in the number of pollen tubes present between the self- and cross-pollination treatments within trees (Table 4.2). The number of pollen tubes varied from a mean of 176.5 in self-pollinated flowers from tree 503 up to 524.2 in cross-pollinated flowers from tree 537. Tree 537 had considerably larger numbers of pollen tubes than the other four trees.

4.3.3 Ovary arrangement

Within trees, there was variation in the number of locules present within the ovary, with either four or five locules present. Mean ovule numbers per locule and per flower, when flowers contained either four or five locules, are presented in Table 4.3. Tree 503 had the largest number of ovules on average, with more than 300 ovules per flower, and tree 319 had the smallest number of ovules, with fewer than 200 ovules per flower. The number of ovules per locule was roughly similar between flowers with four or five locules within a tree. When expanded up to a whole flower, all trees had a greater number of ovules when the ovary contained five locules than when it contained four locules.

4.3.4 Ovule penetration by pollen tubes

All five trees had some ovules penetrated by a pollen tube 2 weeks after pollination. Pollen tubes were observed to have penetrated ovules at the micropyle and had grown through the nucellus tissue toward the embryo sac (Plate 4.2). Combined data from all five trees revealed that there was no significant difference between treatments ($\chi 2 = 1.6$, df = 1, $P = 0.206$). At the individual tree level, tree

Table 4.2. Mean number of pollen tubes in styles from self- and cross-pollinated flowers. *n*, number of styles.

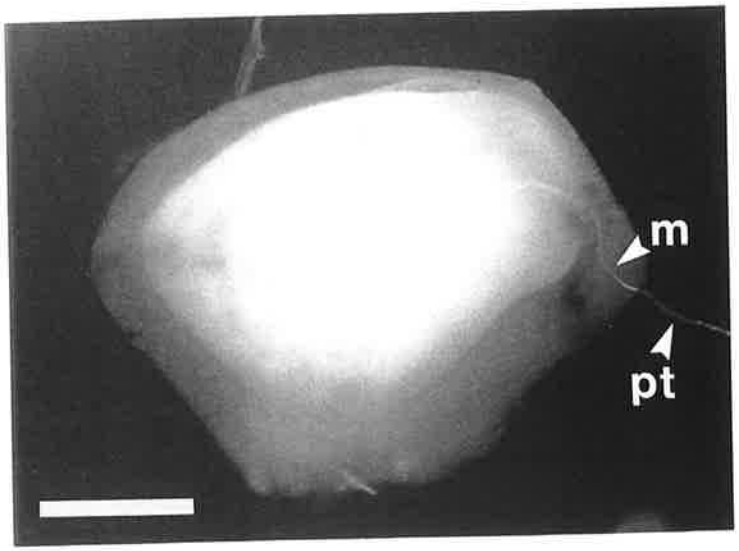
Tree	Mean number of pollen tubes		Standard error	*P*
	Selfed styles (*n*)	Crossed styles (*n*)		
319	223.6 (20)	186.5 (20)	31.59	0.10
503	176.5 (18)	212.2 (21)	44.79	0.27
509	281.4 (24)	318.0 (24)	73.01	0.25
537	492.6 (17)	524.2 (16)	35.10	0.53
538	188.4 (18)	224.7 (21)	33.32	0.44

Table 4.3. Mean number of ovules present per tree and per locule. Per flower data is an extrapolation from the corresponding per locule data. SE, standard error of the mean.

Tree	4-loculed flowers		5-loculed flowers	
	Number of ovules per locule ± SE	Number of ovules per flower	Number of ovules per locule ± SE	Number of ovules per flower
319	42.87 ± 0.91	171.48	37.33 ± 2.00	186.65
503	76.33 ± 2.34	305.32	68.08 ± 2.20	340.40
509	59.80 ± 1.54	239.20	59.10 ± 1.18	295.50
537	65.74 ± 1.71	262.96	64.40 ± 2.56	322.00
538	56.83 ± 1.13	227.32	51.89 ± 1.12	259.45

Plate 4.2

Fluorescence micrograph of a dissected *Eucalyptus globulus* ovule harvested at 2 weeks after cross-pollination and stained with aniline blue. A pollen tube (pt) penetrated the ovule at the micropyle (m). Bar = 250 μm.


m
pt

509 had significantly greater ovule penetration ($P = 0.03$) in cross-pollinated flowers than in self-pollinated flowers and the reverse trend was seen in tree 537 ($P = 0.007$) (Table 4.4). The other three trees showed no difference between treatments. Proportions of ovules penetrated ranged from 28 % in the self-pollination treatment from tree 319 to only 2.1 % in the self-pollination treatment from tree 509, with tree 503 only slightly higher.

By four weeks after pollination, data pooled from the 4-week harvest revealed that there was significantly greater ovule penetration in the cross-pollination treatment than in the self-pollination treatment ($\chi^2 = 5.8$, df = 1, $P = 0.016$). The cross-pollination treatment resulted in an average of 13.6 % ovules penetrated compared to an average of 9.5 % following self-pollination. At the individual tree level, tree 319 had a significantly higher proportion of ovules penetrated by a pollen tube ($P = 0.02$) in the cross-pollination treatment; however, there was no difference found in the other four trees (Table 4.4). The total number of selfed ovules penetrated was similar at 4 weeks as it had been at two weeks after pollination in trees 319 and 537; but the proportion of selfed ovules penetrated in trees 319 and 537 fell between 2 and 4 weeks after pollination. This was because of varying ovule numbers in flowers with different numbers of locules. Tree 503 showed greater ovule penetration at 4 weeks after pollination compared with the earlier harvest.

4.4 Discussion

Four out of the five trees were either 100 % fully self-incompatible or greater than 99 % self-incompatible, indicating a very strong mechanism was operating to prevent selfed seed from being produced. Tree 319 was the only tree to consistently allow selfed seed to develop, but mean seed numbers were still significantly lower than for outcrossed flowers.

Table 4.4. Mean ovule penetration by a pollen tube from flowers self- and cross-pollinated, both 2 and 4 weeks after pollination. *P* values represent comparisons between self- and cross-pollination on the proportion of ovules penetrated. SE, standard error of the mean.

Flowers harvested	Tree	Mean number of penetrated ovules per flower following:		Mean percentage of penetrated ovules per flower following:		*P*
		self-pollination ± SE	cross-pollination ± SE	self-pollination	cross-pollination	
2 weeks	319	44.31 ± 5.86	33.69 ± 7.06	28.0	26.0	0.650
after	503	5.92 ± 4.72	10.92 ± 4.74	3.2	3.4	0.210
pollination	509	6.00 ± 3.57	11.08 ± 6.31	2.1	5.7	0.030
	537	44.71 ± 7.75	29.07 ± 4.11	20.3	12.0	0.007
	538	6.46 ± 2.66	17.25 ± 3.41	7.0	9.7	0.250
4 weeks	319	39.80 ± 6.49	53.30 ± 7.35	17.6	32.8	0.02
after	503	43.67 ± 8.59	52.70 ± 8.09	14.3	14.8	1.00
pollination	509	8.00 ± 4.34	18.55 ± 6.89	1.1	3.9	0.22
	537	37.2 ± 4.38	31.64 ± 3.07	13.7	11.2	0.19
	538	21.64 ± 3.74	28.89 ± 6.34	7.1	12.0	0.12

There was no difference in the number of pollen tubes present in the style between treatments, indicating that the style is not the site of self-rejection. This differs from the more common self-incompatibility mechanisms in plants, which operate in the stigma or style and involve expression of alleles from the S-gene complex (de Nettancourt, 1977; Lewis, 1979). While there was variation in pollen tube numbers between trees, the average numbers was similar to the number of ovules present within a flower; hence, pollen tubes are not a limiting factor to seed production following cross-pollination. Trindade *et al.* (2001) reported a dramatic reduction in the number of outcross pollen tubes reaching the base of *E. globulus* styles, compared with the number of pollen grains observed on the stigma; however, the data for these observations were not shown. They suggest that pollen tube development is controlled by mechanisms present in the pistil. This reduction in pollen tube numbers with increasing distance down the style could possibly be explained by a limitation of space for pollen tubes in the lower style region as the transmitting tissue narrows (Ellis and Sedgley, 1992). Self-incompatibility studies on other eucalypt species reveal no control mechanism for self-incompatibility operating in the style (Ellis and Sedgley, 1992; Sedgley, 1989; Sedgley and Granger, 1996; Sedgley and Smith, 1989). Even though Sedgley and Smith (1989) found fewer pollen tubes reaching the lower style than were present in the upper style in both crossed and selfed *E. woodwardii* flowers, the number of pollen tubes reaching the base of the ovary was not restrictive to seed set. Greater than 200 pollen tubes were recorded in the lower style compared with a mean maximum of 43.5 ovules that were penetrated by a pollen tube. Furthermore, Sedgley (1989) found a mean of 160.5 normal ovules present within an *E. woodwardii* ovary, which is less than the number of pollen tubes present in the lower style. This suggests that although there may be a reduction in the number of pollen tubes down the style of a eucalypt flower, it may not necessarily demonstrate a control mechanism that has any effect on the seed-set potential of the flower. Furthermore, the above data were collected following

controlled self- and cross-pollinations and thus may not necessarily reflect events in open-pollinated flowers.

There was variation in the number of ovules in flowers between and within trees, however, in all cases the number of ovules was far greater than the number of ovules penetrated by a pollen tube and consequently the number of seed produced. This is a common theme among eucalypts and suggests that it may be more beneficial to produce a few, competitive offspring than many weaker offspring (Westoby and Rice, 1982). This trend has also been reported for 35 Northern American woody outcrossing perennials that have an average of 33 % of ovules developing into mature seeds (Wiens, 1984). Furthermore, eucalypt ovaries contain sterile ovule-like structures, termed ovulodes (Carr and Carr, 1962). It has been suggested that ovulodes, present in large numbers and situated at the apex of capsules, may act to protect ovules from fungal infection and insect predation (Sedgley, 1989). This may be so; however, insect predation on developing eucalypt ovules and seeds has been reported (Andersen, 1989; Drake, 1981). The present study also found capsules with seed damaged by insects (data not shown). It is possible that large numbers of ovules help ensure that some will develop normally to seed maturity and dispersal. While individual flowers may produce few seed relative to the number of ovules present, a eucalypt tree as a whole may produce large quantities of seed.

Ovule penetration by pollen tubes revealed variation between trees and between harvests. For example, tree 503 had greater pollen tube penetration at 4 weeks after pollination than 2 weeks after pollination. This suggests that while some pollen tubes had reached ovules 2 weeks after pollination, pollen tubes continued to penetrate ovules after this time. Gore *et al.* (1990) found that *E. globulus* pollen tubes reached ovaries between 5 days and 2 weeks after pollination, with pollen tubes located in, or on the surface of, the tissue surrounding the locules. It appears that some pollen tube growth from the tissue surrounding the locules to ovule micropyles could occur beyond 2 weeks after pollination. The increase in pollen tube penetration over time is

also reflected in the combined tree data of this study, which revealed no difference between treatments at 2 weeks after pollination. By 4 weeks after pollination, however, combined data revealed slightly greater pollen tube penetration of ovules in the outcross treatment. This suggests that there is a late pre-zygotic mechanism operating in *E. globulus* to reduce the number of ovules penetrated by self pollen tubes at least in some trees. A similar result was found in *E. woodwardii* (Sedgley and Smith, 1989). In other genera, reduced ovule penetration by self pollen tubes has been reported in *Hemerocallis thunbergii* (Brewbaker and Gorrez, 1967), *Medicago sativa* (Brink and Cooper, 1938; Cooper and Brink, 1940; Sayers and Murphy, 1966), *Crocus vernus* subsp. *vernus* (Chichiricco, 1990), *Thryptomene calycina* (Beardsell *et al.*, 1993) and *Tecona grandis* (Tangmitcharoen and Owens, 1997). This varied list of species adds strength to the idea that late-acting self-incompatibility mechanisms in plants are not as rare as earlier thought (Seavey and Bawa, 1986).

It is interesting to note that the number of ovules penetrated by each pollen type varied between the five trees, which may reflect a level of variability within this species. Alternatively, it may suggest that ovule penetration data for some trees underestimate the true mean. An underestimation could be due to uneven fluorescence of pollen tubes, making observation difficult in some cases. A lack of uniformity in pollen tube fluorescence was noted in *Tecona grandis* styles (Tangmitcharoen and Owens, 1997), and at the stage of ovule penetration in *Dolichandra cyanchoides* (Gibbs and Bianchi, 1999). It is unlikely that there is genetic differentiation for patchiness of staining between the two pollen treatments used in this study, as both self- and cross-pollen tubes were visually similar, enabling a comparison between the pollen treatments to be made.

While this study produced evidence for late pre-zygotic control over the production of selfed seeds via reduced ovule penetration from self pollen tubes, the difference found was small compared with the scale of the difference between selfed

and outcrossed seed set. This indicates that there is more than one site of arrest of selfed seed development and that the major mechanism operates after pollen tube penetration of ovules. There must be a further reduction of selfed seed development, such as failed or incomplete fertilisation following self pollen tube penetration of ovules, or post-zygotic abortion some time between self-fertilisation and seed set, or both. Pre- and post-zygotic barriers to selfing operate in *E. spathulata* and *E. platypus* (Sedgley and Granger, 1996) and, when considered together with evidence in this study, highlight that self-incompatibility within *Eucalyptus* can be a complex issue. Furthermore, investigating events after pollen tube penetration of ovules is required to gain a more complete understanding of self-incompatibility mechanisms. Such a study would help to explain why, for example, tree 319 allows more self seed to develop compared to the other trees investigated in this study.

From a plant breeder's perspective, the differences found by fluorescence microscopy were too small to categorise an individual tree as self-incompatible for inclusion into a seed orchard seed production program. Whilst this work has not identified a screening method that could reduce the current time of 1 year for self-incompatibility identification, it has provided important information relating to the initial stages of the self-incompatibility mechanism. Chapter 5 investigates developmental stages following pollen tube penetration of ovules, including pre- and post-fertilisation stages to elucidate the self-incompatibility mechanism and assist in identifying a reliable and quick method for screening *E. globulus* trees for self-incompatibility.

Chapter 5

Early ovule development in *Eucalyptus globulus*

5.1 Introduction

The genus *Eucalyptus* has a breeding system that is preferentially outcrossing; however, selfed seed production commonly occurs (Eldridge *et al.*, 1993; Griffin *et al.*, 1987). Within eucalypt species individual trees can range from self-compatible to completely self-incompatible (Ellis and Sedgley, 1992; Potts and Savva, 1988). Moncur *et al.* (1995) reported the outcrossing rate in an *E. globulus* seed orchard to be 77%, confirming the presence of self-compatible trees. Ideally seed orchards should contain only self-incompatible trees to eliminate the production of selfed seed. Otherwise, seed should only be harvested from trees within a seed orchard that have been identified as self-incompatible. Identifying the mechanism of self-incompatibility within a species may enable development of a relatively quick test for self-incompatibility in individual trees.

Self-incompatibility mechanisms have traditionally been found to operate in styles with either a sporophytic or gametophytic incompatibility system (de Nettancourt, 1977; de Nettancourt, 1984; Lewis, 1979). More recently, evidence has been accumulating for late pre- and post-zygotic self-incompatibility systems (Seavey and Bawa, 1986). At present the self-incompatibility mechanism has only been determined for a few eucalypt species, and these range from late pre-zygotic control to post-zygotic control (Ellis and Sedgley, 1992; Sedgley, 1989; Sedgley and Granger, 1996; Sedgley *et al.*, 1989; Sedgley and Smith, 1989). Reduced ovule penetration following self-pollination compared to cross-pollination was found in *E. globulus* (Chapter 4), but this difference did not completely account for the level of

self-incompatibility known to exist in the trees studied. The present study aimed to determine the self-incompatibility mechanism in *E. globulus* by investigating embryological processes. A further aim was to attempt to develop a test for screening individual *E. globulus* trees for self-incompatibility.

5.2 Materials and methods

5.2.1 Plants and pollinations

This experiment was conducted on three of the same trees used as females in Chapter 4 (trees 319, 503 and 537), which were wild, mature trees located south of Hobart, Tasmania. The level of self-incompatibility of trees 319, 503 and 537 was found to be 76, 99.6 and 100 % respectively (Chapter 4).

Flowers were emasculated just prior to anthesis, and as eucalypt flowers are protandrous, flowers were isolated individually from pollen contamination in wax-coated paper bags. Seven days after emasculation, stigmas had secreted a sticky exudate indicating receptivity to pollen, so isolation bags were opened, flowers pollinated with either self or cross pollen, and the bags re-closed. Pollinations were conducted using the same pollen source described in Chapter 4, either self pollen collected from the individual experimental tree, or a cross pollen mixture from 10 *E. globulus* trees unrelated to trees 319, 503 and 537. Flowers used were spread throughout all accessible lower branches and the pollen treatments were randomly applied. Pollinations were conducted on all three trees simultaneously throughout September and early October 1999, the same time as pollinations described in Chapter 4.

5.2.2 Field harvests and microscopy

Five flowers were harvested per treatment per tree at 4, 6 and 8 weeks after pollination. One locule was dissected from each flower and the number of locules

present in each flower was recorded. The tissue processing method used was similar to that described by Feder and O'Brien (1968). The dissected locules were fixed as described in Chapter 3. Once fixed, one locule per treatment from trees 319, 503 and 537 from the 4-week harvest, and one locule per treatment from tree 319 from the 6-week harvest were processed for embedding in GMA as described in Chapter 3. The remaining locules were dehydrated through methoxy-ethanol to ethanol and stored for observation.

Serial, longitudinal sections (LS) 3 μm thick were cut through every ovule per locule embedded for tree 319, but only 35 ovules per locule for tree 503 and 25 ovules per locule for tree 537 due to the large number of ovules present per locule. Sections were stained with Periodic acid-Schiff's reagent and Toluidine blue O (PAS/TBO) and observed using a Zeiss Axiophot microscope in bright field mode. The state of the nucellus cells was observed and recorded, along with the contents of each embryo sac. From embryo sac observations each ovule was classified as fertilised or not fertilised.

The length and diameter of every ovule and its embryo sac was measured using a calibrated microscope eyepiece.

5.2.3 Statistical analysis

Data analysis was performed using Genstat 5 release 4.1, 4th edition. Two-sample t-tests were performed on the parameters ovule and embryo sac lengths and widths between ovules that were fertilised and those that were not, for both self- and cross-pollen treatments from each tree. Data from the self-pollen treatment of tree 537 were excluded from the analysis due to the small sample size of fertilised ovules. Data on ovule length and width, and embryo sac length and width were pooled separately from the observations collected 4 weeks after pollination. Due to the unbalanced nature of the pooled data sets, the data were analysed using Restricted Maximum Likelihood (REML). The statistical model included terms for the fixed

effects of pollen treatment, fertilisation status of ovules (fertilised or not) and the interaction between pollen treatment and fertilisation status, and the random effects of tree and the interaction between tree and flowers pollinated. The significance of the fixed effects was tested using Wald tests.

5.3 Results

5.3.1 Ovule structure

Ovules consisted of an outer integument that surrounded the inner integument to form the micropyle, with the inner integument surrounding the nucellus and embryo sac. By 4 weeks after pollination all three trees had some ovules with nucellus cells that were degenerating (darkly stained), and embryo sacs that were collapsing (embryo sac lumen still present) (Plate 5.1A) or that had collapsed completely (no embryo sac lumen present). By 6 weeks after pollination, the number of ovules with collapsed embryo sacs and nucellus degeneration had increased from that observed at 4 weeks (Table 5.1). When an embryo sac was present, ovules were clearly unfertilised if the embryo sac contained egg apparatus (egg cell with two synergid cells with filiform apparatus) and a central cell with two polar nuclei either fused (Plate 5.1B) or separate (Plate 5.1C). Ovules were also categorised as unfertilised when the egg apparatus or the central cell was degenerating or had completely disappeared, or if the embryo sac had collapsed. This was done to discriminate between healthy, fertilised ovules that had a chance of developing to seed maturity, and those ovules that did not.

Fertilisation had occurred by 4 weeks after pollination in all three trees for both treatments. Zygotes were observed at the micropylar end of the ovule as thick-walled single cells with a distinct nucleus (Plate 5.1D) or they were beginning to form and take shape. By 6 weeks after pollination, zygotes were obvious with a single nucleus; however, three zygotes had developed to the extent that the nucleus had divided to

Plate 5.1

Longitudinal sections of *Eucalyptus globulus* ovules, stained with PAS/TBO.

A. Degenerating ovule at 6 weeks after self-pollination showing the embryo sac (es) and degenerating nucellus (dn). Bar = 200 μm.

B. Micropylar end of the embryo sac at 4 weeks after cross-pollination showing synergids (s), egg cell (e) and central cell (c). Bar = 25 μm.

C. Micropylar end of the embryo sac at 4 weeks after cross-pollination showing polar nuclei (n). Bar = 25 μm.

D. Micropylar end of the embryo sac at 6 weeks after cross-pollination showing zygote (z). Bar = 25 μm.

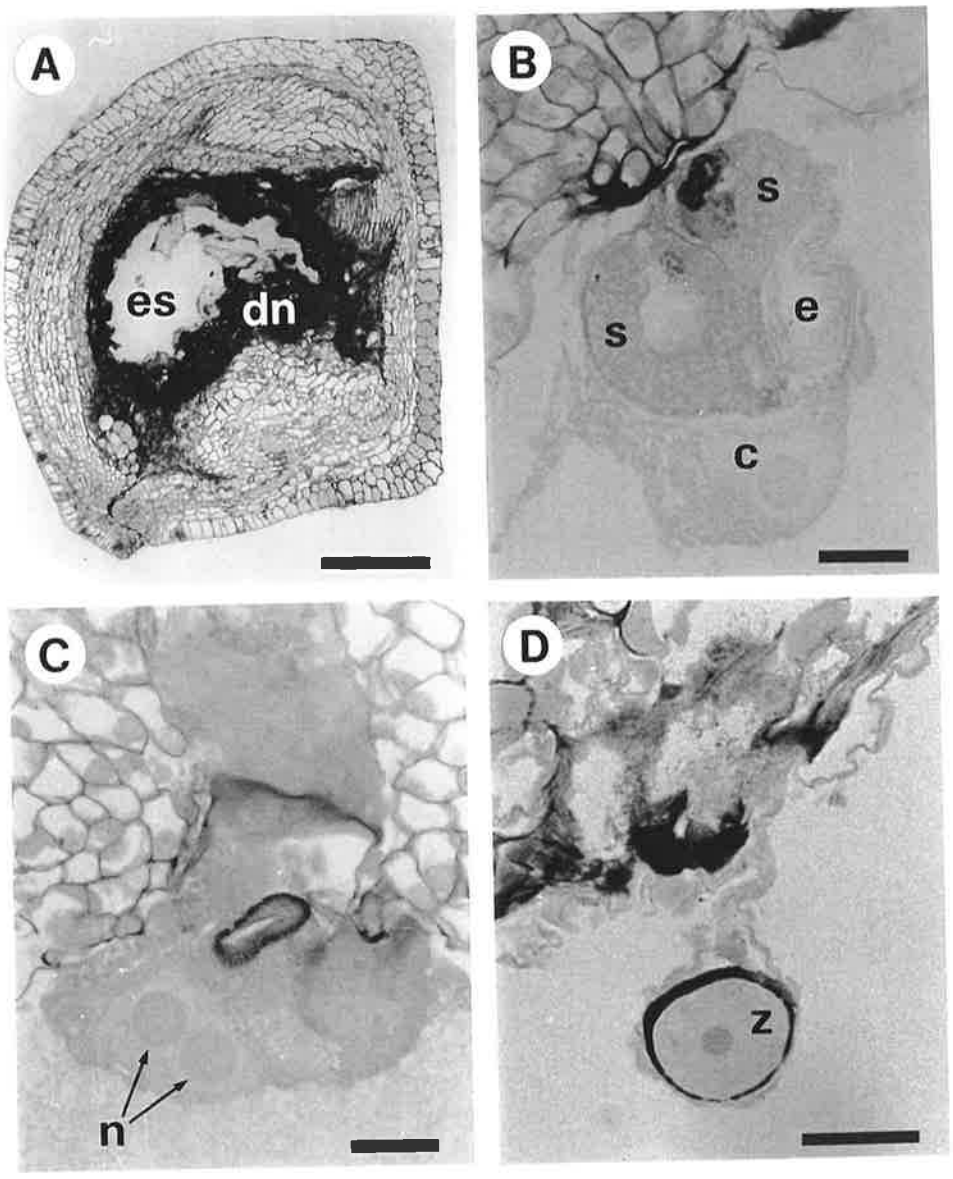

A
es
dn
B
s
s
e
c
C
n
D
z

Table 5.1. Anatomical details of ovules from both self- and cross-pollinations, at 4 and 6 weeks after pollination. n = number of ovules for which a particular feature was present, with percentage in parentheses.

				n (%)					
Flowers harvested	Tree	Pollination treatment	No. of ovules observed (total ovules in locule)	Embryo sac still present	Nuclear endosperm	Zygote	Persistent synergid	Fertilisation	Nucellus degeneration
4 weeks after pollination	319	self	42 (42)	38 (90)	10 (24)	10 (24)	12 (29)	13 (31)	21 (50)
		cross	38 (38)	38 (100)	19 (50)	11 (29)	16 (42)	20 (53)	1 (3)
	503	self	35 (63)	33 (94)	15 (43)	12 (34)	25 (71)	15 (43)	8 (23)
		cross	35 (94)	29 (83)	15 (43)	14 (40)	22 (63)	15 (43)	15 (43)
	537	self	25 (60)	14 (56)	1 (4)	1 (4)	2 (8)	1 (4)	16 (62)
		cross	25 (67)	23 (92)	6 (24)	4 (16)	7 (28)	6 (24)	3 (12)
6 weeks after pollination	319	self	29 (29)	22 (76)	5 (17)	6 (21)	6 (21)	6 (21)	25 (86)
		cross	35 (35)	19 (54)	12 (34)	12 (34)	10 (29)	12 (34)	23 (66)

give two distinct nuclei (Plate 5.2A). Many zygotes were adjacent to a persistent synergid with filiform apparatus. Fertilised ovules had free nuclear endosperm present. This was seen as either two large nuclei resulting from the first division following fusion of one sperm nucleus with the two fused polar nuclei (polar fusion nucleus), or as several smaller nuclei, joined by cytoplasm (Plate 5.2B). The nuclear endosperm formed a ring in longitudinal sections within the embryo sac lumen (Plate 5.2C). Ovules were considered fertilised and healthy if a zygote and free nuclear endosperm were present, when the egg apparatus or the central cell was degenerating or had completely disappeared, or if the embryo sac had collapsed. This was done to discriminate between healthy, fertilised ovules that had a chance of developing to seed maturity, and those ovules that did not.

5.3.2 Ovule fertilisation

At 4 weeks after pollination the proportion of ovules fertilised following self-pollination ranged from 4 % in tree 537 to 43 % in tree 503. Following cross-pollination, ovule fertilisation ranged from 24 % in tree 537 to 53 % in tree 319 (Table 5.1). These data were not amenable to statistical analysis. In trees 319 and 537, a greater proportion of ovules were fertilised following cross-pollination than self-pollination. There was no difference in the proportion of ovules fertilised between treatments in tree 503. Tree 319 had a smaller proportion of fertilised ovules in both treatments at 6 weeks compared with 4 weeks after pollination, indicating that some fertilised ovules had ceased to develop and had degenerated. There was still a greater proportion of fertilised ovules in the cross-pollination treatment compared with the self-pollination treatment.

E. globulus flowers have either four or five locules, and the number of ovules per locule varies (Chapter 4). As the ovules sectioned were from a whole or part of a single locule, the proportions of ovules from both fertilised and unfertilised

Plate 5.2

Longitudinal sections of *Eucalyptus globulus* ovules, stained with PAS/TBO.

A. Micropylar end of the embryo sac at 6 weeks after cross-pollination showing zygote (z) with divided nucleus. Bar = 25 μm.

B. Embryo sac at 6 weeks after cross-pollination showing nuclei (n) of free nuclear endosperm (en). Bar = 50 μm.

C. Ovule at 6 weeks after cross-pollination showing free nuclear endosperm (en) forming a ring of cytoplasm in the embryo sac lumen. Bar = 500 μm.

D. Micropylar end of ovule 6 weeks after cross-pollination showing a pollen tube (pt) in micropyle surrounded by nucellus tissue (nu). Bar = 50 μm.

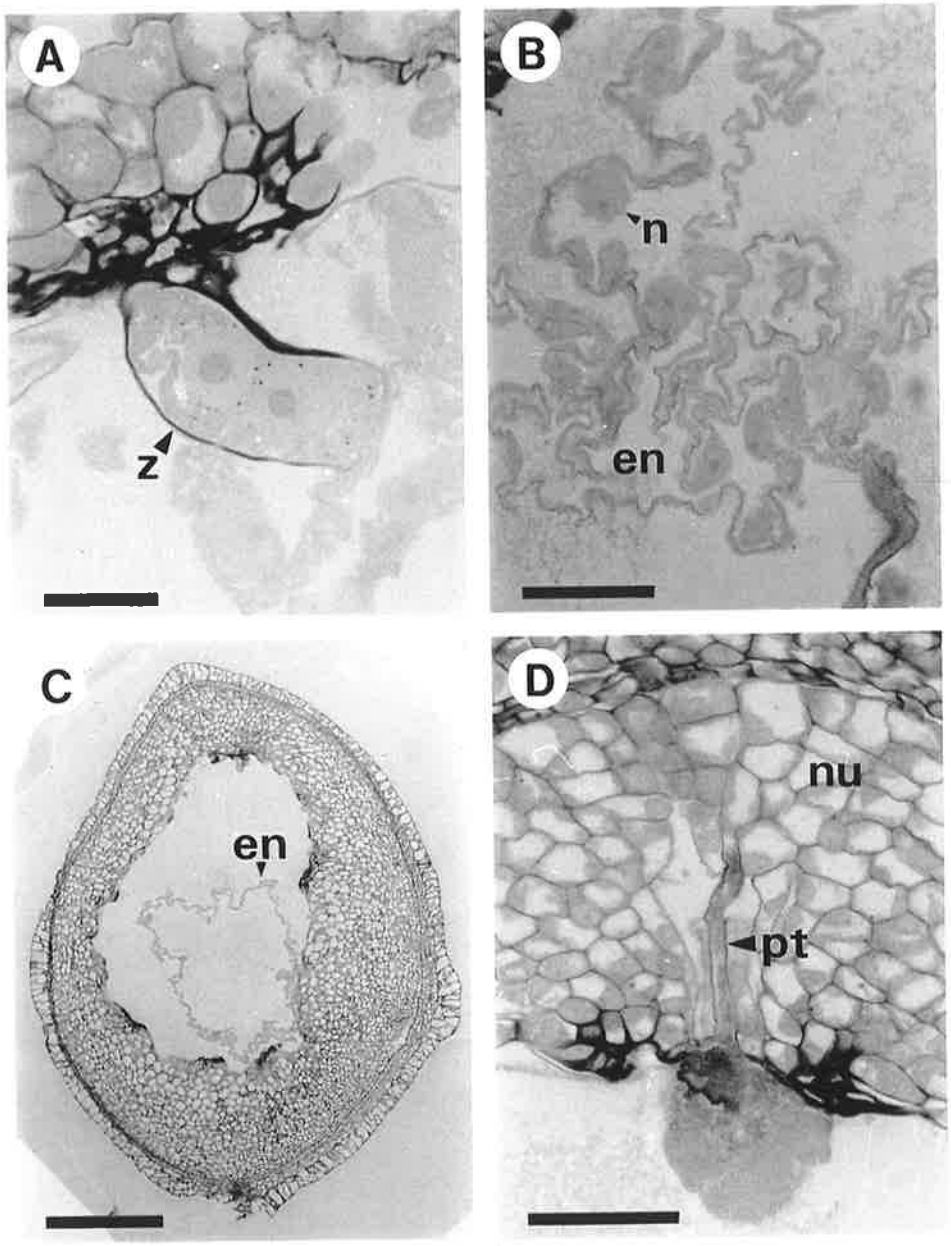

A
z
B
n
en
C
en
D
nu
pt

Table 5.2. Number of ovules fertilised at 4 and 6 weeks after pollination, and mean number of seeds per flower, following self- and cross-pollination. Fertilisation data extrapolated from that collected from a single locule or part locule. Variables for which no data were collected are represented by -. The apparent discrepancy in data between tables 5.1 and 5.2 is due to different flowers containing different numbers of locules and different numbers of ovules in each locule.

	Number of fertilised ovules per flower				Mean number of seeds per capsule 12 months after pollination following:	
	4 weeks after pollination following:		6 weeks after pollination following:			
	Selfing	Crossing	Selfing	Crossing	Selfing	Crossing
Tree 319	52.0	100.0	30.0	48.0	7.6	31.7
Tree 503	125.1	201.4	-	-	3.0	73.5
Tree 537	9.6	64.3	-	-	0.0	35.9

Note: The mean number of seeds produced is presented on a per capsule harvested basis (not on a per flower pollinated basis as in Table 4.1), to allow for a direct comparison over time within developing capsules.

categories were extrapolated to a whole flower, and total numbers per flower were compared (Table 5.2). These derived data were not amenable to statistical analysis. These data reveal that whilst tree 503 may have had a similar proportion of fertilised ovules, there was a greater number of fertilised ovules in the cross-pollination treatment compared with the self-pollination treatment. Furthermore, in all three trees a greater number of ovules were fertilised in both pollen treatments at 4 weeks after pollination, and in tree 319 at 6 weeks after pollination, compared with the mean number of ovules that developed into mature seeds (Table 5.2) (mean mature seed numbers from Chapter 4).

5.3.3 Ovule dimensions

In all trees, fertilised ovules from cross-pollination treatments were longer and wider than non-fertilised ovules (Table 5.3). This pattern was not consistent following self-pollination. Tree 319 showed no significant difference in ovule lengths and widths at both 4 and 6 weeks after pollination. Whilst not significant, there was a trend for increased ovule lengths and widths in fertilised ovules compared with non-fertilised ovules in tree 319. Statistical significance was indicated by *P* values rather than standard deviations.

In all trees from both pollen treatments at both 4 and 6 weeks after pollination, embryo sacs were longer in fertilised ovules compared with non-fertilised ovules, except for the self-pollen treatment in tree 319, 4 weeks after pollination. Fertilised ovules also had wider embryo sacs compared with non-fertilised ovules for both pollen treatments over all trees, but no difference was found in the self-pollen treatment from tree 319, 6 weeks after pollination (Table 5.4). Again, although measurements were not significant in tree 319, the trend was for fertilised ovules to be larger.

Table 5.3. Mean ovule lengths and widths. Variables for which data were unavailable are represented by -. fert., fertilised, sd, standard deviation of the mean

Flowers harvested	Tree	Treatment	Ovule length (μm)					Ovule width (μm)				
			fert.	sd	not fert.	sd	*P*	fert.	sd	not fert	sd	*P*
	319	self	1054	171	1023	189	0.620	759	150	672	209	0.060
		cross	1027	145	907	117	0.010	744	147	619	90	0.004
4 weeks	503	self	953	116	878	75	0.028	704	97	639	76	0.033
after		cross	926	103	852	76	0.019	654	71	581	86	0.011
pollination	537	self	1110	-	889	148	-	770	-	652	131	-
		cross	1097	187	933	130	0.023	813	112	681	118	0.024
6 weeks	319	self	1580	452	1105	189	0.050	1080	408	726	149	0.090
after		cross	1973	235	1032	169	<0.001	1357	276	714	191	<0.001
pollination												

Table 5.4. Mean embryo-sac lengths and widths. Variables for which data were unavailable are represented by -. fert., fertilised, sd, standard deviation of the mean.

			Embryo-sac length (μm)					Embryo-sac width (μm)				
Flowers harvested	Tree	Treatment	fert.	sd	not fert.	sd	*P*	fert.	sd	not fert.	sd	*P*
4 weeks after pollination	319	self	465	91	395	181	0.120	375	130	212	121	<0.001
		cross	532	58	407	147	0.002	387	57	298	146	0.020
	503	self	423	83	338	94	0.011	257	51	187	84	0.009
		cross	391	56	321	66	0.004	261	47	213	60	0.022
	537	self	480	-	415	150	-	340	-	210	114	-
		cross	547	155	268	129	<0.001	377	81	139	87	<0.001
6 weeks after pollination	319	self	820	373	377	89	0.030	420	346	162	86	0.130
		cross	1195	181	334	218	<0.001	770	127	90	120	<0.001

Table 5.5. Average ovule length and width, and embryo sac length and width for fertilised and non-fertilised ovules at 4 weeks after pollination among all 3 trees.

	Ovule length (μm)	Ovule width (μm)	Embryo sac length (μm)	Embryo sac width (μm)
Fertilised ovules	1003.4	735.0	463.7	314.5
Non-fertilised ovules	915.8	640.7	381.8	210.1
P	<0.001	<0.001	<0.001	<0.001

Combining data collected 4 weeks after pollination from all trees showed that the interaction between pollen treatment and fertilisation status was not statistically significant. There was no effect of pollen treatment on ovule size, but all four ovule measurements were statistically larger in fertilised ovules compared with non-fertilised ovules (Table 5.5).

Visual observation of locules harvested at 6 and 8 weeks showed that several ovules in the cross-pollinated flowers had increased greatly in size (Plate 5.3A), whereas self-pollinated flowers had small, relatively uniform ovules (Plate 5.3B). Tree 319, the most self-compatible tree, was occasionally observed to have some large ovules following self-pollination, but fewer than following cross-pollination.

5.4 Discussion

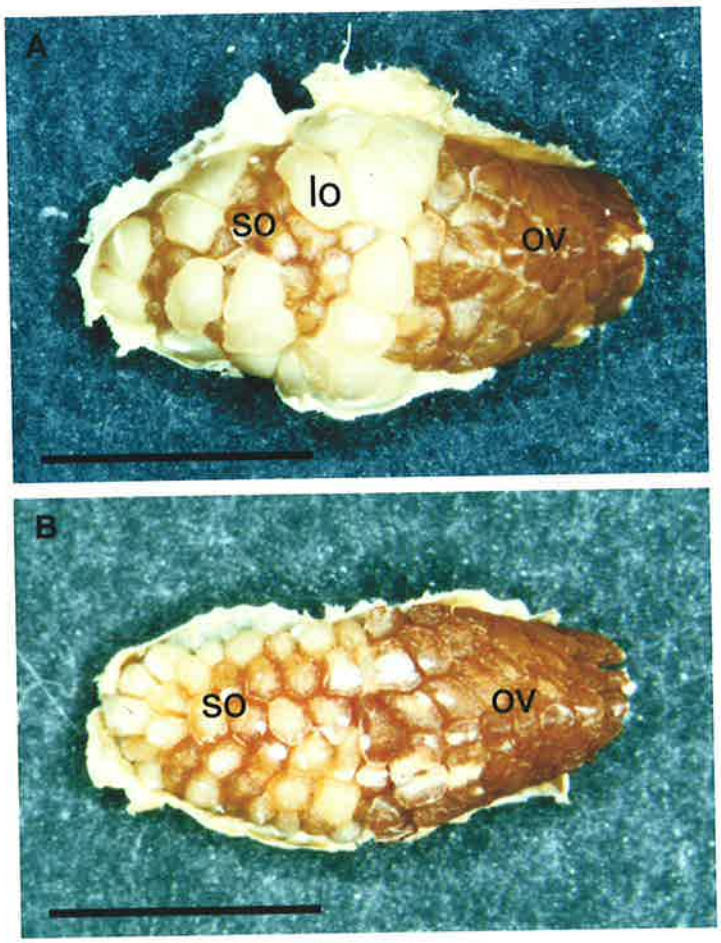
The three trees used in this study were found to have self-incompatibility levels of 76 % (tree 319), 99.4 % (tree 503), and 100 % (tree 537) (Chapter 4). There was also no difference found in pollen tube numbers in styles of self- and cross-pollinated flowers, and a small but statistically significant decrease in ovule penetration by self pollen tubes (9.5 %) compared with cross pollen tubes (13.6 %) over five trees collectively (Chapter 4). As fertilisation had already occurred at 4 weeks after pollination, the exact timing of fertilisation events remains unclear. It is possible that fertilisation occurred before 4 weeks and a resting zygote was seen at this first sample time. Furthermore, it is possible that some ovules with little or no embryo sac left at 4 weeks may have been fertilised before degeneration took place. Earlier harvest times of both control pollinated and unpollinated flowers would help determine a more precise time sequence of events. The present study found a greater total number of healthy, fertilised ovules for all three trees following cross-pollination compared with self-pollination at 4 weeks after pollination, and in tree 319 at 6 weeks after pollination. At 6 weeks after pollination, there were fewer healthy, fertilised ovules than at 4 weeks for both pollen treatments, and a further

Plate 5.3

Dissected *Eucalyptus globulus* locules 6 weeks after pollination, viewed with a dissecting microscope.

A. Locule from a cross-pollinated flower showing large ovules (lo), small ovules (so) and ovulodes (ov). Bar = 500 μm.

B. Locule from a self-pollinated flower showing only small ovules (so) and ovulodes (ov). Bar = 450 μm.


A
lo
so
ov
B
so
ov

reduction in developing ovules must have occurred after this time to produce the final seed set numbers. This information suggests that the self-incompatibility mechanism in *E. globulus* does not occur in the style, but is evident in a slight reduction in pollen tube penetration of ovules and post-zygotic abortion. In short, the self-incompatibility mechanism appears to be both late pre- and post-zygotic. The difference in the level of self-incompatibility between the three trees appears to be determined by the number of selfed ovules successfully fertilised that increase in size and develop on until seed maturity.

One mechanism that may account for late-acting, pre-zygotic self-incompatibility before ovule penetration is that there may be a reduction in the availability of fertile ovules due to the absence of a required stimulus for normal ovule development following self-pollination (Sage *et al.*, 1999). It is unclear whether this is occurring in *E. globulus* as embryo sacs were investigated from 4 weeks after pollination.

Late-acting self-incompatibility mechanisms occurring after pollen tube penetration have been reported to occur in 35 plant species from diverse families (Gibbs and Bianchi, 1999). Within *Eucalyptus*, post-zygotic self-incompatibility mechanisms have been reported in *E. regnans* (Sedgley *et al.*, 1989), *E. cladocalyx* and *E. leptophylla* (Ellis and Sedgley, 1992). However, embryological development was not studied for the latter two species, so late pre-zygotic barriers, after pollen tube penetration of ovules, cannot be ruled out. Other species found to have post-zygotic self-incompatibility mechanisms following examination of embryological development include *Capparis retusa* (Bianchi and Gibbs, 2000), *Chorisia chodatii*, *C. speciosa*, *Tabebuia caraiba*, and *T. ochracea* (Gibbs and Bianchi, 1993). The above examples clearly show that as the breeding systems of more species are studied in detail at an embryological level, the self-incompatibility mechanisms operating in these species will be better understood.

There was a reduction in healthy, fertilised ovules over time in both cross- and self-pollinated flowers. Late-acting self-incompatibility mechanisms can explain the reduction in apparently healthy self-fertilised ovules, but the reason for the reduction in healthy cross-fertilised ovules is not as clear. Angiosperms are known to invest maternal resources into seed development after fertilisation has taken place (Westoby and Rice, 1982). Perhaps the reproductive strategy employed in *E. globulus* is to allocate resources into producing fewer larger, more competitive seeds, than many smaller seeds. Following controlled cross-pollinations on *E. globulus*, it was observed that when capsules produced few seeds, the seeds were much larger compared with those produced when capsules contained many seeds (pers. obs.). The number of fertilised ovules following both self- and cross-pollinations at 16 weeks after pollination in *E. regnans* was much higher than mature seed numbers (Sedgley *et al.*, 1989). These authors suggested that the reproductive strategy was for fewer, more competitive offspring. Trees may select against fertilised ovules whose zygotes or embryos are of an inferior genotype due to pollen source variability (Wiens, 1984; Wiens *et al.*, 1987) by directing available resources to potentially superior offspring (Westoby and Rice, 1982). Both pollen source and resource allocation were considered to be important factors in the production of fewer seed compared with fertilised ovules following both self- and cross-pollination in *E. spathulata* and *E. platypus* (Sedgley and Granger, 1996). The level of selection within *E. camaldulensis* fruits of a self-pollinated tree was dependent on the number of seeds and ovules per capsule (James and Kennington, 1993), which also suggests the involvement of pollen source and resource allocation. Whilst the cross-pollen mixture used in this experiment was presumed to be genetically unrelated to the pollen recipients, some gene combinations may have resulted in less vigorous progeny which were subsequently aborted. Within cross-pollinated, fertilised *E. globulus* ovules there appears to be a slight lack of synchrony in zygote development at 6 weeks after pollination, and not all large ovules observed at 6 and 8 weeks were

of a similar size. Perhaps the ovules that develop faster and are larger at an early age are the ovules that survive to seed maturity.

The percentage of ovules fertilised in both pollen treatments in tree 503 at 4 weeks after pollination was 43 %. This figure is much greater than the mean ovule penetration in tree 503 4 weeks after self-pollination (14.3 %) or cross-pollination (14.8 %) (Chapter 4). These figures were analysed separately for within-tree differences but were not statistically significant. The discrepancy between fertilisation and ovule penetration suggests that the pollen tube observations at the ovule level were an underestimate of the true value. A similar discrepancy was also found in *E. nitens* (Chapter 3). Dissection of ovules from the placenta may have resulted in some pollen tubes being removed from some of the ovules. It is also possible that uneven fluorescence of pollen tubes occurred, thereby preventing observation. The phenomenon of uneven fluorescence of pollen tubes has been reported previously in styles of *Tecona grandis* (Tangmitcharoen and Owens, 1997), and at the ovule level in *Dolichandra cynanchoides* (Gibbs and Bianchi, 1999). As both self- and cross-pollen tubes were visually similar in *E. globulus*, it would seem unlikely that genetic differentiation for patchiness of staining between the two pollen types existed. Therefore, although pollen tube penetration data are an underestimate, comparison between the two pollen types can still be made. It is possible that staining sections to show pollen tubes prior to staining with PAS/TBO may provide a better estimate of pollen tube penetration. The difference between pollen tube penetration and ovule fertilisation highlights the importance of examining individual embryo-sacs for evidence of fertilisation to gain a complete understanding of the breeding system of a species.

This study has shown that fertilised ovules appear larger, on average, than non-fertilised ovules. Furthermore, there appear to be clear differences in the size of ovules following cross-pollination at 6 and 8 weeks after pollination, with no such trend following self-pollination in the most self-incompatible trees. These size

differences suggest that the degeneration of fertilised selfed ovules occurs uniformly and very early in the 12 month development to seed maturity. This also suggests that the degeneration of fertilised selfed ovules is indeed a self-incompatibility response, as opposed to early inbreeding depression which occurs at many developmental stages following fertilisation to seed maturity (Charlesworth, 1985; Seavey and Bawa, 1986).

The differences in size observed in ovules at 6 and 8 weeks after pollination may be used to determine whether an individual tree is 100 % self-incompatible (no data collected at 8 weeks after pollination but photos were taken). Whilst larger-scale verification is required, it is proposed that a tree may be considered 100 % self-incompatible if, at 6-8 weeks following controlled self- and cross-pollinations, selfed flowers consistently reveal no observable differences in ovule size, compared with cross-pollinated flowers. It presently takes about a year, using seed set numbers following controlled pollinations, to determine the level of self-incompatibility in a tree. This new method would greatly reduce the time required for ascertaining the level of self-incompatibility in *E. globulus*.

Chapter 6

Pollen competition does not affect the success of self-pollination in *Eucalyptus globulus*

6.1 Introduction

Whilst there is no doubt that selfed seed production is occurring within *E. globulus* seed orchards, estimates of the potential of individual trees to produce selfed seed may be exaggerated when self-compatibility is assessed using separate controlled self- and cross-pollinations. Such crossing does not take into account that cross-pollinations may out-compete self-pollinations during mixed pollinations. Individual flowers of *E. globulus* are protandrous by 5-7 days, but flowering within the canopy may last for over a month (Gore and Potts, 1995) allowing ample opportunity for mixed pollinations to occur under natural open pollination. Since Mulcahy (1979) first proposed the pollen competition hypothesis, much research has been conducted to investigate pollen competition and sexual selection in plants. Natural pollen deposition on stigmas has been found in excess of that required to fertilise all ovules, such that an opportunity for selection exists (Snow, 1986; Winsor *et al.*, 2000). Differences have been found in pollen tube growth rates and these differences are genetically determined (Snow, 1986; Snow and Spira, 1991a; Snow and Spira, 1991b) indicating that differences in pollen tube vigour could result in sexual selection when excess pollen deposition occurs. In addition, field experiments using different pollen loads have shown that large pollen loads result in more vigorous progeny (Stephenson *et al.*, 1986; Winsor *et al.*, 2000). A component of the genes exppressed in the gametophytic life-cycle phase are also expressed in the sporophytic phase (Tanksley *et al.*, 1981; Willing and Mascarenhas, 1984) which

supports the idea that pollen competition could result in differences in the sporophytic generation (Mulcahy, 1979). However, the precise way in which sexual selection in plants operates in nature, its evolutionary role, and how widespread it is, remain unclear (Grant, 1995; Hormaza and Herrero, 1994).

Isozyme studies have been widely used to determine the rates of self-fertilisation and cross-fertilisation following open pollination of *Eucalyptus* species; including *E. regnans* (Moran *et al.*, 1989), *E. camaldulensis* (James and Kennington, 1993), *E. urophylla* (House and Bell, 1994), *E. leucoxylon* (Ellis and Sedgley, 1993) and *E. globulus* (Hardner *et al.*, 1996; Patterson *et al.*, 2000). Isozymes have also been used to determine paternity following mixed-pollinations (self- and cross-pollen) in *E. regnans* (Griffin *et al.*, 1987). Both self and outcross seed were present within the one capsule, with three of the five *E. regnans* trees examined producing more outcrossed seed than expected based on separate pollinations (Griffin *et al.*, 1987). The greater outcrossed seed set in *E. regnans* was attributed to competitive interaction of embryo genotypes within a capsule and maternal resource allocation, not to competition between growing pollen tubes. In another mixed pollination study, *E. grandis* showed selective fertilisation in favour of cross-pollen based on morphological marker assessment (Hodgson, 1976b).

The present study aimed to determine whether pollen competition is important in *E. globulus* and specifically whether there is additional selection against self-pollinations in the presence of outcross-pollen in partially self-compatible trees.

6.2 Materials and methods

6.2.1 Plants

All trees used in this study were located in southern Tasmania and had produced seed crops for several years. The five trees (529, 536, 538, 532 and 72-279) on which controlled pollinations were performed, were selected because of their floral

abundance and accessibility. Furthermore, three of these five trees (529, 536 and 538) were known to set some self seed following controlled self-pollinations (B. Potts, pers. comm. 1999), and their isozyme genotypes at the glucose-phosphate-isomerase (GPI-2) locus were identified as suitable for the purposes of this study. A further two trees (507 and 739) were selected as pollen donors as they were found to have rare isozyme genotypes at the GPI-2 locus which could then be used to distinguish the paternity of seeds following mixed pollinations. These two trees were considered unrelated to those chosen as females, as they were located further than 500 m from all females. This assumption was made as Hardner (1998) studied *E. globulus* progeny vigour as an indirect measure of parental relatedness and found crosses from parents 21 m apart or closer resulted in inbred progeny but parents separated by only 250 m appeared to be as unrelated as parents separated by 100 km.

6.2.3 Pollinations

Pollinations were conducted during October through to December 1999. Individual flowers from the partially self-compatible trees 529, 536 and 538 were emasculated and bagged as described in Chapter 4. Twenty to twenty-six flowers were emasculated per pollen treatment per tree. Seven days after emasculation, at peak stigmatic receptivity, the isolation bags were opened, flowers pollinated, and the bags resealed. Fewer flowers were pollinated for some treatments due to a loss of flowers between emasculation and pollination (Table 6.1). The isolation bags were removed at about 6 weeks after pollination when styles had dried out and most had fallen from the flowers.

6.2.4 Pollen treatments

Trees used to test for pollen competition were 529, 536 and 538. The treatments for trees 529 and 536 were pollen from the same tree (self), a mixture of self pollen and pollen from tree 507 (mix 1), a mixture of self pollen and pollen from tree 739

(mix 2), pollen from tree 507 (marker 1), and pollen from tree 739 (marker 2). Tree 538 shared a common allele with tree 507 so only three pollen treatments were applied; pollen from the same tree (self), a mixture of self pollen and pollen from tree 739 (mix 2) and pollen from tree 739 (marker 2). Twenty to twenty-six flowers were pollinated, per tree, per treatment (Table 6.1).

All self pollens and pollen from tree 507 were collected fresh and stored in gelatine capsules over silica gel at -20°C. Pollen from tree 739 had been collected in the same way in previous years and was tested for viability before use. Pollen samples from tree 739 were streaked onto semi-solid agar media (Potts and Marsden-Smedley, 1989), allowed to incubate at approximately 25°C for 24 hours, and then viewed with a light microscope for pollen germination and pollen tube growth. Pollen tubes were clearly visible on the semi- solid agar media hence the pollen was considered viable and included in pollen mixes. Pollen mixes were made up with an equal weight of self-pollen and marker pollen which was mixed before storage in gelatine capsules.

6.2.5 Testing pollen mixtures

The proportional composition of all five pollen mixes (mixes 1 and 2 from each of 529 and 536 and mix 2 from 538) were independently determined by conducting controlled pollinations with the pollen mixes on two trees, 532 and 72-279, which were unrelated to any of the pollen parents. Twenty flowers were emasculated and pollinated (as described above) for each of the five pollen mixes per tree (Table 6.2).

6.2.6 Seed set

Pollinated flowers were left to mature into capsules, and all capsules remaining at maturity (12 months after pollination), were harvested. The number of viable seed in each capsule was counted.

Table 6.1. Number of flowers pollinated with each pollen treatment on trees 529, 536 and 538. GPI-2 genotypes for trees 529, 536 and 538, and marker pollens are presented in brackets. Marker 1 pollen is from tree 507 and marker 2 pollen is from tree 739.

Pollen treatment	Tree		
	529 (1,1)	536 (1,1)	538 (1,2)
self	25	25	20
mix 1 (self and marker 1)	24	25	-
mix 2 (self and marker 2)	25	26	24
marker 1 (2,2)	26	26	-
marker 2 (5,5)	25	26	22

Table 6.2. Number of flowers on trees 532 and 72-279 pollinated with pollen mixes used on trees 529, 536 and 538.

Pollen treatment	Tree	
	532 (1,1)	72-279 (1,1)
529 : 507 (mix 1)	20	20
529 : 739 (mix 2)	20	20
536 : 507 (mix 1)	20	20
536 : 739 (mix 2)	20	20
538 : 739 (mix 2)	20	20

The level of self-incompatibility for trees 529, 536 and 538 was determined using the following formula:

$$SI = ((V_{CP} - V_{SP}) / V_{CP}) * 100$$

where SI = self-incompatibility, V_{CP} = viable seed per flower cross pollinated (marker 2 and marker 1 pollinations for trees 529 and 536, and marker 2 pollinations for tree 538) and V_{SP} = viable seed per flower self pollinated.

6.2.7 Isozyme analysis

Paternity of seeds produced from mixed pollinations of self and marker pollen on trees 529, 536 and 538 was established by performing isozyme analysis on seedlings (Moran and Bell, 1983) (Fig. 6.1). A minimum of ten seeds per capsule harvested were set to germinate at 25°C on moist vermiculite which had been sprayed with "Aquasol" nutrient solution (Hortico). Seven day old seedlings were assayed for the enzyme system GPI-2 (EC 5.3.1.9) using cellulose acetate electrophoresis (Hebert and Beaton 1993). Seedlings were ground in 0.2 M Tris (pH 8.0) containing 10 % glycerol, 10 % PVP-40, 1 % Triton X100 and 0.1 % 2-mercaptoethanol. A continuous Tris Glycine, pH 8.5 buffer system was used to assay the enzyme system GPI-2. The gel staining method was similar to Hebert and Beaton (1993), except the amount of ß-nicotinamide adenine dinucleotide (NAD) was reduced from 1.5 mL to 300 µL, phenazine methosulphate (PMS) was doubled to 10 drops (~ 400 µL) and 2 mL water was substituted for 2 mL agar.

Paternity of seeds produced from pollinations on trees 532 and 72-279 (both 1,1 homozygotes for GPI-2) were also germinated and subjected to isozyme analysis as described above (Plate 6.1). Determination of seed paternity then enabled the proportional composition of the pollen mixes to be estimated, by averaging results across trees 532 and 72-279.

Fig. 6.1

Diagrammatic zymogram showing glucose-phosphate-isomerase (GPI-2) banding patterns and genotypes for trees, pollens, and possible progeny from mixed pollinations of self-pollen and marker pollen. Three bands are present for heterozygotes as GPI-2 is a dimer. 1 = trees 529 and 536; 2 = tree 538; 3 = pollen from tree 507 (marker 1); 4 = pollen from tree 739 (marker 2); 5 = self progeny for trees 529 and 536; 6 = cross progeny for trees 529 and 536 with pollen from tree 507; 7 = cross progeny for trees 529 and 536 with pollen from tree 739; 8-10 = self progeny for tree 538; and 11-12 = cross progeny for tree 538 with pollen from tree 739. Horizontal arrows mark alleles 1, 2 and 5. GPI-2 genotypes are indicated below banding patterns. Migration is anodal.

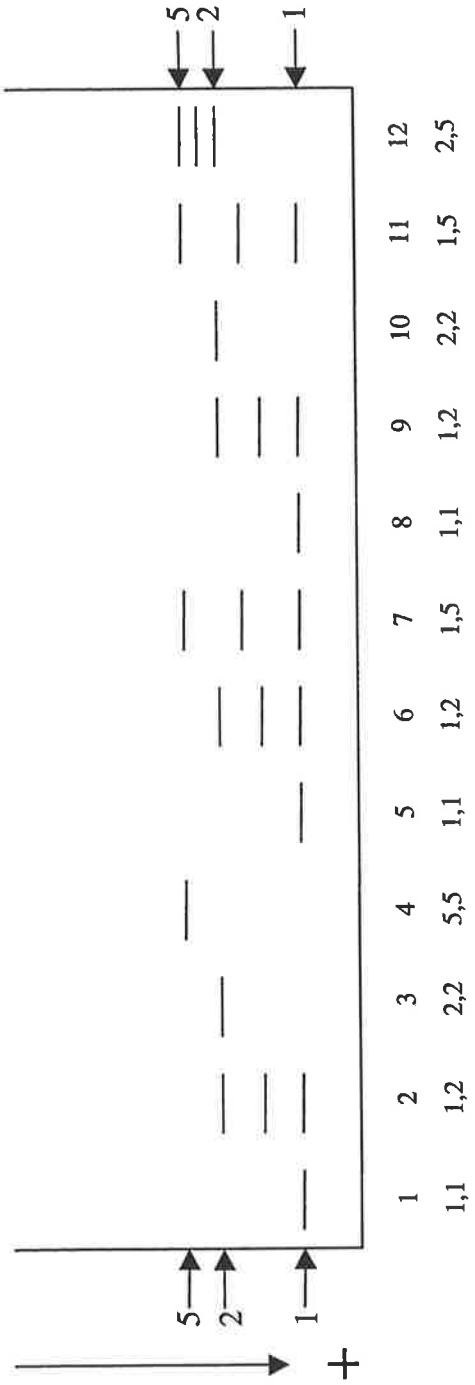
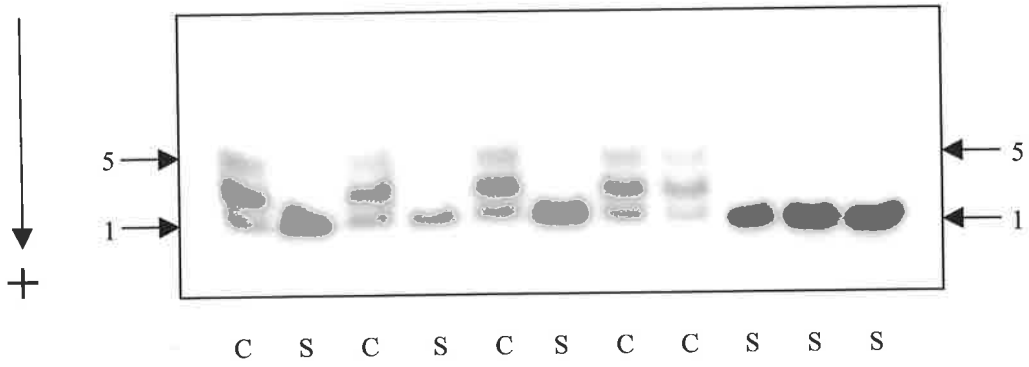

5
2
1
12 2,5
11 1,5
10 2,2
9 1,2
8 1,1
7 1,5
6 1,2
5 1,1
4 5,5
3 2,2
2 1,2
1 1,1
5
2
1
+

Plate. 6.1

Zymogram showing GPI-2 banding patterns and genotypes of seeds from tree 532 pollinated with the pollen mix of pollen from tree 529 and marker pollen from tree 739. C = seed with a 1,5 genotype, pollinated with marker pollen from tree 739 (cross-pollen component of pollen mix); S = seed with a 1,1 genotype, pollinated with pollen from tree 532 (self-pollen component of pollen mix). Horizontal arrows mark alleles 1 and 5. Migration is anodal.


5
1
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1
C S C S C S C C S S S

6.2.8 Statistical analysis

6.2.8.1 Seed set

Kruskall-Wallis tests were performed to determine if there were any differences in viable seed per capsule set between treatments for trees 529, 536 and 538. Comparisons for chosen treatments were performed using Wilcoxon Two-Sample tests.

6.2.8.2 Seed paternity

Contingency χ^2 tests were used to determine if the number of seeds that were outcrossed following mixed pollinations was significantly different to that expected, assuming no pollen competition. The isozyme data obtained from pollen mix 1 and mix 2 were pooled within trees 529 and 536 to improve robustness of the tests. Similarly, the data expected assuming no pollen competition, were also pooled. The number of seed expected to be selfed under no pollen competition for each individual tree and individual pollen mix was estimated using the following equation:

$$N_S = P_{SP} * (1- (SI / 100)) * S$$

where N_S = the number of seed expected to be selfed under no pollen competition, P_{SP} = the proportion of self pollen in the pollen mix, SI = self-incompatibility level of the tree and S = the number of seeds analysed. The number of seed expected to be outcrossed under no pollen competition was estimated with the following equation:

$$N_O = S - N_S$$

where N_O = the number of seed expected to be outcrossed under no pollen competition, S = the number of seeds analysed and N_S = the number of seed expected to be selfed under no pollen competition.

Contingency χ^2 tests were also used to test for differences in the proportion of each pollen (self and outcross pollen) within the pollen mixes as found following isozyme analysis on the germinated seeds resulting from the pollinations on trees 532 and 72-279.

6.3 Results

The level of self-incompatibility of trees 529, 536 and 538 was determined to be 57.2, 94.5 and 97.8 % respectively. Tree 529 was the most self-compatible tree with a mean of 11.4 seeds produced per self-pollinated flower (Table 6.3). Trees 536 and 538 were relatively less self-compatible with means of 1.3 and 0.6 seeds produced per self-pollinated flower.

All three trees revealed highly significant differences in viable seed per capsule set when the applied pollen treatments were compared, with trees 529, 536 and 538 having P values of 0.0005, <0.0001 and <0.0001 respectively. Individual comparisons between pollen treatments revealed that all comparisons made in trees 536 and 538 for viable seed per capsule showed highly significant differences. There was greater viable seed set per capsule in both marker pollens when compared with their associated pollen mix, and with cross- compared to self-pollen. The difference between pollen treatments in viable seed per capsule set in tree 529 was evident only in the cross- compared to the self-pollen treatment (Table 6.4).

For each tree, the number of selfed and crossed seeds, as determined by isozyme analysis, were combined for the two pollen mixes and the expected frequencies for the two classes were calculated (Table 6.5). Actual and expected frequencies in tree 529 were extremely close, with no statistical difference found. In tree 538, the expected frequencies for self seed production were lower than conventional for Contingency χ^2 analysis to be statistically acceptable, however there was no trend indicating any differences between actual and expected selfed and crossed seed set.

Table 6.3. Mean number of seeds per flower following controlled self- and cross-pollination, with level of self-incompatibility.

Tree	Mean viable seed per flower cross-pollinated	Mean viable seed per flower self-pollinated	Self-incompatibility (%)
529	26.7	11.4	57.2
536	23.4	1.3	94.5
538	27.6	0.6	97.8

Table 6.4. Means and *P* values from Wilcoxon Two-sample tests performed for specific comparisons within viable seed set per capsule. VSD/CAP = viable seed set per capsule. An overall analysis on viable seeds per capsule set data revealed trees 529, 536 and 538 had *P* values of 0.0005, <0.0001 and <0.0001 respectively.

Variable	Tree								
	529			536			538		
	mean	mean	*P*	mean	mean	*P*	mean	mean	*P*
VSD/CAP									
mix 1 vs marker 1	56.4	65.6	0.20	30.3	39.7	0.0088	-	-	-
mix 2 vs marker 2	46.6	31.7	0.06	30.5	43.8	0.0008	18.6	33.8	0.0002
outcross vs self	63.5	26.0	0.0002	41.8	3.6	<0.0001	33.8	4.0	0.0074

Table 6.5. Total number of selfed and crossed seeds determined by isozyme analysis (combined from the two pollen mixes for trees 529 and 536), expected frequencies and Chi-square probability values.

	Tree					
	529		536		538	
	self	outcross	self	outcross	self	outcross
Actual	40.0	185.0	12.0	295.0	2.0	134.0
Expected	47.6	177.4	5.4	301.6	1.4	134.6
P	0.213		0.004		0.634	

Note: Estimates of self-incompatibility were rounded to whole numbers for calculation of 'expected' frequencies.

Interestingly, in tree 536 there were greater numbers of selfed seed produced than expected.

The composition of each pollen mix was estimated based on the results summed across the two tester trees, 532 and 72-279 (Table 6.6). The proportion of self-pollen and cross-pollen in each version of pollen mix 1 containing 529 or 536 pollen was found to be consistent across the two tester trees. However, for pollen mix 2 significant differences in pollen ratios between the two trees were detected. For example, pollinations using pollen mix 2 involving 739 pollen mixed with pollen of 529, 536 or 538 resulted in different proportions of self and marker pollen between seeds from tree 532 and 72-279 with P values of 0.0026, <0.0001 and 0.0001 respectively. Tree 532 set more seed from the marker 2 pollen (739) compared to self-pollen from trees 529 and 536, with the reverse trend in tree 72-279. Furthermore, tree 532 set more seed from self-pollen from tree 538 compared to marker 2 pollen, with the reverse trend again found in tree 72-279.

6.4 Discussion

This study demonstrates that under mixed pollination, both self and cross pollen tubes successfully reach and fertilise the ovules of *E. globulus*. Further, the proportion of selfed seed produced following mixed pollinations was not significantly greater than expected from independent pollinations, which indicates that there is no evidence of pollen competition occurring in favour of outcrossed progeny in these trees. Tree 536 produced more selfed seed than expected, however, the biological significance of this difference may be minimal, due to the very small number of observed and expected selfed seed numbers when compared to the total sample size.

The paternity of seed set following mixed pollinations appears to be determined by the late-acting, mainly post-zygotic self-incompatibility mechanism which exists

Table 6.6. Proportions of self pollen and marker pollen in pollen mixes as determined by isozyme analysis on seeds resulting from mixed pollinations on trees 532 and 72-279.

Pollen mix	Pollen ratio (%)		*P*	Pooled ratio
	tree 532	tree 72-279		
529 : 507 (mix 1)	42 : 58	46 : 54	0.2977	44 : 56
529 : 739 (mix 2)	59 : 41	46 : 54	0.0026	53 : 47
536 : 507 (mix 1)	28 : 72	32 : 68	0.4576	30 : 70
536 : 739 (mix 2)	53 : 47	25 : 75	<0.0001	40 : 60
538 : 739 (mix 2)	44 : 56	62 : 38	0.0001	53 : 47

in *E. globulus* (Chapters 3 and 4). Selection against self-penetrated ovules would account for the observation of less seed per capsule following mixed compared to cross pollinations in trees 536 and 538, the trees less likely to produce selfed seed. That is, post-penetration abortion of selfed ovules would most likely account for ovule wastage and the reduction in seed per capsule. Whilst post-zygotic selection is seemingly wasteful compared to pre-zygotic selection, with respect to maternal resource allocation, selecting against inferior offspring genotypes may be more advantageous than selection only between pollen donor characteristics (Lloyd, 1980).

The lack of pollen competition in this study contrasts with that of Hodgson (1976b) who found there was greater than expected seed set in favour of outcrossed seed set following mixed (self and outcross) pollinations in *E. grandis*. Selective fertilisation was suggested as an explanation, however, as the self-incompatibility mechanism in *E. grandis* is unknown, the possibility of post-fertilisation selection, whether embryo abortion of inbred genotypes, or selective maternal resource allocation, cannot be ruled out. Griffin *et al.* (1987) also found evidence for competition in favour of outcrossing following mixed pollinations in *E. regnans*, and concluded that selection was dependent upon both embryo genotype and maternal resource allocation. This conclusion suggested post-zygotic control of the breeding system, a suggestion later confirmed by Sedgley *et al.* (1989). It should be noted however, that the results of Griffin *et al.* (1987) were not uniform, with only three out of the five trees studied providing evidence of competition in favour of outcrossing. In a study of selfed progeny of *E. camaldulensis*, James and Kennington (1993) found that the level of selection against homozygotes at a locus with an excess of heterozygotes declined in capsules that had large numbers of ovules or seeds. This decline in selection pressure was attributed to a greater level of maternal resources allocated to capsules with large ovule or seed numbers, a maternal strategy also noted by Lloyd (1980). It is therefore possible that the excessive application of

mixed pollen on *E. globulus* stigmas resulted in many more ovules fertilised in those particular capsules compared to others on the tree. This may have attracted disproportionate maternal resources allowing some selfed seed to develop such that pollen selection was not evident.

Eucalyptus globulus is a highly variable species, with 13 races identified (Dutkowski and Potts, 1999), and the trees used in this study represent only one race from southern / south-eastern Tasmania. As evidence based on controlled hand-pollinations may not reflect events that would follow natural pollination (Snow, 1986; Winsor *et al.*, 2000), an understanding of natural pollination events within the racial area would further elucidate the mechanisms controlling seed paternity. Both insects, in particular the introduced honey bee (*Apis mellifera*) and birds visit *E. globulus* flowers (Hingston and Potts, 1998). Bird species are believed to be the major contributors to outcrossing, with insects more likely to cause selfing (Hingston and Potts, 1998). The order in which self- and outcross-pollen is deposited on to *E. globulus* stigmas, the growth rate of self- and cross-pollen tubes, and the average number of pollen grains deposited on stigmas may provide useful information relating to the number of seed set per capsule and seed paternity.

The variable results for the proportional composition of pollen mix 2, determined by paternity analysis from seed set for each of the two tester trees, was an unexpected result. It is quite common for different, non-self pollen donors within a pollen mix to sire unequal seed numbers (Marshall and Diggle, 2001; Marshall and Oliveras, 2001; Snow and Spira, 1991a) but the differences observed have been consistent across maternal genotypes. Few studies have found that the ability of pollen donors to sire seed differs according to the maternal plant used (eg. Bertin, 1982; Bertin, 1985). The varying results across maternal genotypes found in this study, along with other examples, suggests it is possible that, in some cases, the maternal plant influences seed paternity. However it is possible that seed paternity patterns in the crosses on tester trees could be influenced by a level of relatedness

between the marker 2 pollen tree (739) and the tester tree 532. While such relatedness is unlikely, it can not be entirely ruled out nor can the possibility that the differential fertilisation results from inadequate mixing of the sticky pollen.

In conclusion, this study has shown that following mixed pollination, both self- and cross-pollen tubes reach *E. globulus* ovules, with paternity of seed set reflecting the level of self-incompatibility of each tree. The number of seeds set per capsule following mixed pollination was less than cross-pollination. These results suggest that both self- and cross-pollen tubes are able to penetrate ovules, with a late-acting self-incompatibility mechanism operating to abort self-penetrated ovules. This study has found no evidence to suggest that pollen competition occurs in favour of outcrossing in *E. globulus*. An understanding of the effect of natural self- and cross-pollen deposition on stigmas of *E. globulus* seed orchard trees would help to determine if self-incompatibility levels alone, or together with other factors, influences seed paternity. In the meantime, the importance of having very highly self-incompatible trees within *E. globulus* seed orchards to best avoid the production of selfed seed is emphasised.

Chapter 7

General Discussion

The outcome of this research has been an enhanced understanding of the mating systems occurring in *E. globulus* and *E. nitens*. The findings have contributed to increasing our limited knowledge of breeding systems within *Eucalyptus*, a large and diverse genus. Furthermore, this research may prove valuable to eucalypt breeders, by providing information that may directly aid in maximising the production of outcrossed seed. In doing so, the deleterious effects of inbreeding on growth would be eliminated making Australian plantations more profitable and hence competitive, potentially helping to reduce our $2 billion dollar trade deficit in forest products (Yainshet and Sledge, 2001).

The ability of both *E. nitens* and *E. globulus* trees to produce self-fertilised seed has been found to vary considerably. This is consistent with the generalised view that eucalypts have a mixed mating system (Eldridge *et al.*, 1993; Potts and Wiltshire, 1997). The range in selfing levels found in this study, combined with average selfing rates of 23 and 25 % in an *E. globulus* and *E. nitens* seed orchard respectively (Moncur *et al.*, 1995), highlight the possibility that many seed orchards of these species contain self-compatible trees.

Pollen tube growth in styles of both *E. globulus* and *E. nitens* was found to be similar following both self- and cross-pollination. *E. nitens* also had no statistical difference in ovule penetration by pollen tubes, with *E. globulus* only revealing a small difference in favour of cross-pollination. Not only does this mean both species have late-acting control over selfing, but also eliminates the possibility of a relatively quick screening test involving pollen tube observation in styles, for ascertaining the self-incompatibility status of individual trees.

This study found that the fluorescence method of visualising pollen tube penetration of ovules in both species was unrepresentative of the real proportion of ovules penetrated as determined by examination of ovules for fertilisation. As similar observations have been made across other genera, including *Tecona* (Tangmitcharoen and Owens, 1997) and *Dolichandra* (Gibbs and Bianchi, 1999), this result should act as a warning for researchers reporting on breeding systems of plant species, if only pollen tube growth is observed. If investigations are not taken to the embryological level, species that have late-acting mechanisms may be incorrectly defined. Furthermore, beyond suggesting that a species has a late-acting self-incompatibility system, it may not be possible to categorically say whether the system operates at a pre- or post-zygotic level, or both, without embryological investigation.

This study found that *E. nitens* has post-zygotic self-incompatibility and that post-zygotic control is responsible for most of the self-rejection in *E. globulus*. The number of species reported to have post-zygotic self-incompatibility systems is currently few compared to the many species reported with stylar inhibition of self pollen tubes (Seavey and Bawa, 1986). It is likely that as more researchers take the time for careful embryology studies, more species will be discovered with post-zygotic self-incompatibility systems.

Fertilised, healthy developing ovules were found to be larger than degenerating ovules by 6 weeks after pollination in both *E. globulus* and *E. nitens*. This difference was most noticeable by eye in *E. globulus* due to the large size of floral structures compared with the small flowers and ovules of *E. nitens*. This information may prove directly beneficial to seed orchard managers by reducing the time required to ascertain the compatibility levels of individual trees. The proposed screening method would involve conducting controlled self- and cross-pollinations on seed orchard trees, followed by a harvest of flowers at about 6-8 weeks after pollination, with locules then dissected out of flowers and the size of ovules observed. The fewer large

ovules observed following self-pollination compared with cross-pollination, the more self-incompatible a tree is likely to be. However, further testing is required to ascertain how many flowers to test and the best time to harvest them. Comparisons of results from an early harvest would need to be made with self-incompatibility estimates based on seed set. This proposed new method would see a reduction in the current time required to establish self-incompatibility levels in these species from about 1 year to about 2 months.

No evidence was found to suggest that cross-pollen can outcompete self-pollen for seed paternity in partly self-compatible *E. globulus* trees when both self and cross-pollen were applied to individual stigmas. Rather, seed paternity was consistent with the level of self-incompatibility of each individual tree. Seed orchard trees are highly likely to be pollinated with both self- and cross-pollen under open pollination. The finding that the presence of cross-pollen on a flower does not inhibit successful self-fertilisation in partly self-compatible trees further emphasises the need to determine the levels of self-incompatibility of each tree. To maximise genetic gain, seed should be preferentially harvested from the most self-incompatible trees to avoid harvesting self-fertilised seed. The more self-compatible trees can still be retained in seed orchards as pollen donors or in case seed supply is short.

The forestry industry has focussed on eucalypt improvement through selection, predominantly targeting traits such as growth rate and form (Pederick, 1979; Volker and Orme, 1988), wood density (Turner *et al.*, 1983) and, depending on the species, disease resistance (Purnell and Lundquist, 1986) and frost tolerance (Potts and Potts, 1986; Tibbits and Reid, 1987). If seed orchard trees were selected both for the above traits and self-incompatibility, maximum quality and quantity in plantations would be achieved through the production of genetically superior seed. This could be achieved by considering ovule size as proposed by this study. Alternatively, chemical emasculation by application of gametocides to achieve functional female trees has

also been proposed (Moncur and Boland, 2000). However, this approach would limit the number of trees within an orchard capable of being pollen donors.

It has been noted that mass propagation of eucalypts by cuttings or micropropagation are not currently feasible options. Indeed, clonal propagation of some eucalypts has proven difficult (Moncur and Boland, 2000). Despite this, grafted clonal seed orchards have now been established for several eucalypt species (Eldridge *et al.*, 1993). It would be useful to clonally propagate *E. globulus* and *E. nitens* trees that have both desirable genetic traits and high self-incompatibility, for seed orchard use. Seed orchards containing such clones would produce seeds of high genetic quality and all trees would also be useful as pollen donors, maximising the potential genetic gain.

There has been some debate as to whether post-zygotic abortion of self-fertilised ovules is actually a self-incompatibility response, or early-acting inbreeding depression. One criterion proposed to distinguish between the two is that a self-incompatibility response would result in abortion of self-fertilised ovules at a uniform point in time, whereas an inbreeding response should result in abortion of ovules throughout the seed development period (Seavey and Bawa, 1986). The timing of post-zygotic self-rejection has been identified for very few species. This project has identified that post-zygotic abortion of selfs plays a major role in the control of selfing, especially in *E. nitens*. The majority of abortions in both species were found to occur very early on in seed development, mostly within the first month of an approximate 12 month development period. This finding suggests that control of selfing is indeed a self-incompatibility response.

Future research should be directed at gaining information to further characterise post-zygotic self-incompatibility systems in flowering plants. We are beginning to understand the more commonly reported self-incompatibility systems that operate in the style, but there is currently no information relating to the genetic and molecular control of post-zygotic self-incompatibility.

As individuals within a eucalypt species can vary widely in their ability to set selfed seed, it would prove useful to gain an estimate of the heritability of self-incompatibility. In particular, reliable heritability estimates from controlled cross-pollinations between individuals of markedly different self-incompatibility, followed by cross-compatibility studies between the F_1 families would provide valuable information.

In addition to understanding the genetics of post-zygotic self-incompatibility, research should be directed towards determining the molecular mechanism of post-zygotic self-incompatibility. It would be useful to establish if control of self-incompatibility is due to a single gene, or multiple genes, and the number of alleles involved. Further, research could determine if there are similarities in the recognition or control systems operating in post-zygotic self-incompatibility and systems that are better understood, such as gametophytic or sporophytic self-incompatibility (Ebert *et al.*, 1989; Stone and Goring, 2001). Comparing differences in gene expression of healthy and aborting embryos may aid in isolating genes involved in the process and determining the pathway of events in post-zygotic abortion. The development of a molecular marker for self-incompatibility would benefit plant breeders concerned with producing outcrossed seed from self-incompatible trees. This might be achieved by conducting bulked segregant analysis (BSA) on the genomic DNA (Mekuria *et al.*, 2001; Michelmore *et al.*, 1991) from groups of both self-incompatible and self-compatible trees. Establishing a marker for self-incompatibility would enable a relatively quick test for self-incompatibility in young trees/ seedlings, as opposed to current methods that require trees to be of flowering age.

The challenge for future research in broadening our overall understanding of plant self-incompatibility systems lies in learning more about late-acting or post-zygotic self-incompatibility. Clear identification of species with post-zygotic self-incompatibility is needed, followed by genetic and molecular research to establish how post-zygotic self-incompatibility operates.

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Appendix 1: Publications

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.

Pound, L.M., Wallwork, M.A.B., Potts, B.M. and Sedgley, M. (2002). Self-incompatibility in *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *Australian Journal of Botany* **50**: 365-372.

Pound, L.M., Patterson, B., Wallwork, M.A.B., Potts, B.M. and Sedgley, M. (2003). Pollen competition does not affect the success of self-pollination in *Eucalyptus globulus* (Myrtaceae). *Australian Journal of Botany* **51**: 189-195.

Accepted:

Pound, L.M., Wallwork, M.A.B., Potts, B.M. and Sedgley, M. (2003). Pollen tube growth and early ovule development following self- and cross-pollination in *Eucalyptus nitens*. Sexual Plant Reproduction.

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, v. 89 (5), pp. 613-620.

Pound, L.M., Wallwork, M.A.B., Potts, B.M., and Sedgley, M., (2002) Self-incompatibility in *Eucalyptus globulus* ssp. *globulus* (Myrtaceae). *Australian Journal of Botany, v. 50 (3), pp. 365-372.*

Pound, L.M., Patterson, B., Wallwork, M.A.B., Potts, B.M., and Sedgley, M., (2003) Pollen competition does not affect the success of self-pollination in *Eucalyptus globulus* (Myrtaceae).
Australian Journal of Botany, v. 51 (2), pp. 189-195.

Appendix 2: Industry Communication

Pound, L. (2002). Self-incompatibility in *Eucalyptus globulus* and *E. nitens*. Seminar at 'SeedFest' 11 February. Melbourne, Victoria.