

**OVERCOMING BIOLOGICAL BARRIERS TO CONTROL-
POLLINATED SEED PRODUCTION IN *EUCALYPTUS***

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PREFACE

The experimental work described in this thesis was carried out at the Shaw Research Centre of Sappi Forests, situated in Tweedie, South Africa, from January 2003 to December 2008, under the supervision of Professor Steven D. Johnson (University of KwaZulu-Natal).

The results have not been submitted in any form to another University and except where the work of others is acknowledged in the text, are the result of my own investigation.

Tasmien Nadine Horsley

I certify that the above statement is correct.

Professor Steven D. Johnson
Supervisor

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A DEDICATION

*'The future belongs to those
who believe in the beauty
of their dreams.'*

Eleanor Roosevelt

My thesis is dedicated to Fluffy, Lady, Lassy, Scrappy and Pippin, who have given me indescribable pleasure and unconditional love.

I also dedicate this thesis to those family members and friends who although no longer here in body, will always be with me in spirit.

ABSTRACT

The overall aim of this PhD study was to develop protocols to improve the efficiency of eucalypt controlled pollinations (CPs) in order to make it more cost-effective for forestry companies to perform them on a commercial scale on small-flowered species. To achieve this, three research areas were explored, namely pollen handling, breeding systems and controlled pollination technique. Study species were *Eucalyptus grandis*, *E. dunnii*, *E. smithii*, *E. nitens*, *E. urophylla* and *E. macarthurii*.

The first specific aim of the study was to identify a suitable liquid *in vitro* germination medium for reliably testing pollen viability of all six study species. Six levels of sucrose [0, 10, 20, 30, 40 and 50% (w/v)] were tested, both with (0.15 mg l⁻¹) and without boric acid. The optimal sucrose concentration was found to be 30% (w/v), with boric acid stimulating pollen tube growth. A second aim was to determine temperatures suitable for the short-, medium- and long-term storage of *E. smithii*, *E. nitens* and *E. grandis* pollen. Pollen samples were stored at room (25°C), refrigerator (4°C), freezer (-10°C) and liquid nitrogen (-196°C) temperatures, and pollen viability tested every two months over a 12-month period. There was a rapid decline in the germination of pollen stored at 25°C, while temperatures cooler than 4°C appeared to maintain pollen viability for the duration of the 12-month study. Recommendations were thus to use a refrigerator for short-term (< 2 months), a freezer for medium-term (up to 10 months) and cryopreservation for longer-term storage.

In the second part of the study, breeding systems of *E. urophylla* and *E. grandis* were examined by studying pollen-tube growth in the style after single-donor self- and cross-pollinations. Results showed that, in addition to both species exhibiting reduced seed yields following self-pollination, pollen tubes from self-pollen took significantly longer than those from cross-pollen to grow to the base of the style. This suggested the presence of both late-acting self-incompatibility and cryptic self-incompatibility (CSI) as possible mechanisms responsible for outcrossing in these two species. In a follow-up study, the siring ability of self- and cross-pollen was examined after single- and mixed-donor pollinations were performed on *E. grandis*. Once again, single-donor cross-pollinations resulted in a significantly higher number of seeds compared to self-pollinations. In addition, microsatellite molecular markers

revealed that 100% of the progeny from mixed (self + outcross) pollinations were outcrossed, confirming the competitive advantage of cross-pollen. To date, CSI has never been associated with *Eucalyptus*, making this the first study to suggest its presence in the genus.

For the final study area, three CP-techniques were compared, namely the Conventional method, One Stop Pollination (OSP) and Artificially Induced Protogyny (AIP), in *E. grandis*, *E. smithii* and *E. macarthurii* maternal parents. Although the AIP technique produced the highest seed yields in all three species, it also resulted in high self- and foreign-pollen contamination (determined using microsatellite markers). This necessitated exploration of different methods of isolating the pollinated flower, and this led to the identification of a novel method which uses sodium alginate gel. Flowers hand-pollinated and isolated with sodium alginate produced progeny that were 100% outcrossed with the applied pollen, confirming the superiority of this innovative isolation technique compared to the currently used exclusion bag. Sodium alginate isolation also increased the efficiency of CPs as the gel was naturally shed, removing the need for operators to return to the tree to remove the isolation material, and thereby reducing the cost per seed.

Application of these results should make commercial CP-seed production of small-flowered eucalypts a practical reality.

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ABBREVIATIONS

AIP	Artificially Induced Protogyny
ANCOVA	analysis of covariance
ANOVA	analysis of variance
CP	controlled pollination
CSI	cryptic self-incompatibility
DNA	deoxyribonucleic acid
DWAF	Department of Water Affairs and Forestry
FAA	formaldehyde-acetic acid fixative
FAO	Food and Agricultural Organisation
GLM	general linear model
H ₂ O	water
ISI	index of self-incompatibility
K ₃ PO ₄	potassium phosphate
LSI	late-acting self-incompatibility
NaOH	sodium hydroxide
OSP	One Stop Pollination
RH	relative humidity
SI	self-incompatibility
SRC	Shaw Research Centre
UV	ultraviolet
V _c	viable seed per flower cross-pollinated
V _s	viable seed per flower self-pollinated
w/v	weight by volume

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CHAPTER 1: INTRODUCTION

Eucalypts are the most widely planted hardwood trees in the world, occupying more than 17 million hectares globally (FAO 2000). While *Eucalyptus globulus* is the premier species for temperate zone plantations in Portugal, Spain, Chile and Australia, elite hybrid clones involving *E. grandis* and *E. urophylla* are extensively used by the pulp and paper industry in tropical regions of Brazil, South Africa, India and Congo because of their superior wood quality, rapid growth, disease resistance and high volumetric yield (Eldridge *et al.* 1993). In 2000, the FAO estimated a total of 17.9 million hectares of planted *Eucalyptus* worldwide, with India the largest planter at over 8 million hectares, followed by Brazil with 3 million hectares (FAO 2000). In South Africa, of the approximately 1.33 million hectares planted to forestry, around 524 000 hectares are dedicated to eucalypts (DWAF 2005).

The genus *Eucalyptus* contains in excess of 700 species, all belonging to the family Myrtaceae, and most being endemic to Australia (Johnson and Briggs 1984). It is divided into 8 subgenera, viz. *Blakella*, *Corymbia*, *Eudesmia*, *Symphyomyrtus*, *Idiogenes*, *Gaubaea*, *Monocalyptus* and *Telocalyptus* (Pryor and Johnson 1981). The subgenus *Symphyomyrtus* is not only the largest, including more than 300 species, but also the most widespread (Potts and Pederick 2000). The genus contains the tallest hardwood species in the world, reaching heights of over 100 m (Mace 1996). From north to south, the Australian climate changes from summer to winter rainfall and from warm to cooler seasons which, combined with the effect of variations in altitude, aspect and soils, has resulted in an immense diversity of habitats to which eucalypts have adapted. They show exceptional differentiation and there are often large genetic differences, both within and between species. This variation provides the basis for selection and breeding of variants adapted to a wide range of plantation environments (Potts and Pederick 2000).

Throughout the world, eucalypts are cultivated to meet a wide range of needs, including amenity plantings for shade and shelter. Young trees are a source of paper pulp, charcoal and fuelwood, poles, mining timber and fibreboard (Eldridge *et al.* 1993). Mature trees provide strong, durable timber, while all sizes are capable of use for other forest products such as volatile oils for pharmaceutical and industrial uses,

and honey (Boland *et al.* 1991). The greatest production of industrial eucalypt wood is for the pulp and paper industry, mainly bleached kraft pulp which is in demand because of the excellent properties it imparts to printing, writing and tissue papers, as well as its availability and price (Sidaway 1988).

Although eucalypt breeding is currently a dynamic and technically advanced operation, the challenge to the forestry industry is that eucalypts are still in the early stages of domestication when compared to crop species, with most eucalypt breeding programmes only one or two generations removed from the wild (Myburg *et al.* 2006). Improvement depends on the effectiveness in developing new, superior genotypes. In nature and most current forestry operations, new genotypes are created through a process of open pollination (Faegri and van der Pijl 1979), but the time frame is long and there is no control over the pollen parent.

Controlled pollination (CP) could be used to combine genetic material of selected elite trees to produce high quality, and consequently high value, seed (Frampton 1997). The attractiveness of this type of pollination is that there is more control over the parental genotypes. The technique has been used to improve seed yield, control the level of outcrossing in seed orchards, improve breeding through knowledge of both female and male parents, achieve interspecific hybridisation, and study self-incompatibility levels in *Eucalyptus* species (Harbard *et al.* 1999; Moncur 1995). However, the high cost of performing controlled crosses and the relatively low seed production often obtained has necessitated the use of lower quality, open pollinated seed for the establishment of the majority of commercial tree plantations.

The aim of this PhD study was to develop techniques to improve the efficiency of controlled pollinations in order to make them more cost-effective for forestry companies to perform on a commercial scale. In countries like Australia, Chile and Portugal, commercial CPs are routinely carried out on large-flowered eucalypts like *Eucalyptus globulus*. However, the technique still needs to be adapted for use on small-flowered species and for this reason, the present study focused on the following eucalypt species: *E. grandis*, *E. urophylla*, *E. smithii*, *E. nitens*, *E. dunnii* and *E. macarthurii*. All belong to the subgenus *Symphyomyrtus*. *Eucalyptus nitens*, *E. smithii*, *E. dunnii* and *E. macarthurii* are cold-tolerant species, belonging to the

section *Maidenaria* (Pryor and Johnson 1981). *Eucalyptus grandis* and *E. urophylla* are better suited to warmer climates and belong to the section *Latoangulatae* (George 1988).

1.1 THE STUDY SPECIES

For the South African forestry industry, the most important hardwood species has historically been *E. grandis* (Schonau 1991). However, an increasing demand for hardwoods, particularly for the pulp and paper industry, has led to expansion into much cooler areas where *E. grandis* does not survive (Pallett and Sale 2004). This has consequently led to the introduction of the cold tolerant eucalypts. Species trials have confirmed the superiority of *E. nitens* on high altitude cold sites on the Highveld plateau, and *E. dunnii* and *E. smithii* as species of good growth potential over a range of sites in the mid altitudes (Shaw 1994). In South Africa, hybrids of *E. grandis* and *E. urophylla* are routinely deployed on the warmer, low altitude sites to alleviate disease (Wingfield *et al.* 1997; Morris 2007).

1.1.1 *Eucalyptus grandis*

Eucalyptus grandis is the most widely used species in plantation forestry worldwide in tropical and subtropical areas, not only as a pure species, but also as a parental species in hybrid breeding (Myburg *et al.* 2006). It displays the fastest growth rates and widest range of adaptability of all other *Eucalyptus* species, most likely due to its extensive natural distribution in Australia. It has a natural occurrence extending from Newcastle in New South Wales to Bundaberg in Queensland, where the altitude varies from 500 – 1100 m above sea level, with overall latitude range from 25 – 33°S. The mean maximum and minimum temperatures of the hottest and coolest months are 30°C and 3°C respectively in the south and 32°C and 10°C respectively in the north (Boland *et al.* 1989). The greatest area of plantations of *E. grandis* and its hybrids are those established in Brazil and several other Central and South American countries. It has been planted extensively in India, South Africa, Zambia, Zimbabwe, Tanzania, Uganda and Sri Lanka (Myburg *et al.* 2006).

1.1.2 *Eucalyptus macarthurii*

The natural occurrence of *E. macarthurii* is restricted to the central and southern tablelands of New South Wales, from the Blue Mountains to Goulburn. The altitude

varies from 500 – 1200 m above sea level, with overall latitude range from 33 – 35°S. The mean maximum and minimum temperatures are 25°C and -1°C respectively, with frosts severe and frequent, and light snowfalls regular. Of the six species included in the study, *E. macarthurii* is the most frost-tolerant (Boland *et al.* 1989). It is mostly grown on low productivity sites for pulp and paper production, although there is presently some controversy about the pulp properties of the species (Swain and Gardner 2003). Within its optimum temperature range, *E. macarthurii* is considered to be the most hardy of the eucalypt species.

1.1.3 *Eucalyptus nitens*

In its natural habitat in Australia, *E. nitens* occurs between 600 and 1200 m above sea level in the Victoria Alps, eastern Victoria and southern New South Wales provinces. Two distinct populations are also found at Barrington Tops and Ebor in northern New South Wales, at altitudes of up to 1600 m, with overall latitude range from 30 to 38°S (Boland *et al.* 1989). The mean maximum and minimum temperatures of the hottest and coolest months are 26°C and -5°C respectively. In South Africa, *E. nitens* is the most snow and cold tolerant of all the eucalypts grown commercially (Swain and Gardner 2003). It was traditionally grown for mining timber in the cold, high altitude areas of the summer rainfall regions of South Africa, but has since been established for pulp and paper production. *Eucalyptus nitens* is classified as frost tolerant, but is not as hardy as *E. macarthurii* (Darrow 1996) and is also very sensitive to fire (Swain and Gardner 2003).

1.1.4 *Eucalyptus dunnii*

Eucalyptus dunnii has a restricted natural occurrence on northeastern New South Wales extending into southeastern Queensland. The distribution covers approximately 250 km from west of Coffs Harbour in New South Wales northwards to the McPherson range. The altitude varies from 300 – 750 m above sea level, with overall latitude range from 28 – 30°S. The mean maximum and minimum temperatures of the hottest and coolest months are 30°C and 0°C respectively, with frosts varying from 20 to 60 every winter (Boland *et al.* 1989). This species grows better than *E. grandis* on cooler sites with more frost and snow tolerance (Swain and Gardner 2003). Of the cold tolerant eucalypt species, *E. dunnii* has the best natural stem form, with very little taper. In South Africa, *E. dunnii* has a growth rate, form

and gum bark similar to that of *E. grandis* and is considered the alternative species choice on sites too dry and/or cold for *E. grandis* (Swain and Gardner 2003).

1.1.5 *Eucalyptus smithii*

The fast growth, high density and exceptional pulp properties of *Eucalyptus smithii* makes it a commercially desirable species, provided it is planted on sites which are well drained and which do not encourage *Phytophthora* root rot (Swain and Gardner 2003). The species occurs naturally along the eastern edge of the tablelands of southeastern New South Wales and adjacent coastal escarpment and lowlands. Scattered populations are also found in the eastern Gippsland district of Victoria. The altitude varies from 50 – 1150 m above sea level, with overall latitude range from 34 to 38°S (Boland *et al.* 1989). The mean maximum and minimum temperatures of the hottest and coolest months are 28°C and -2°C respectively. During the drought which occurred in South Africa from 1991 to 1993, *E. smithii* proved to be one of the most drought hardy species, as tolerant of drought as *E. dunnii* (Darrow 1994). However, the species is not as frost tolerant as *E. macarthurii*, but is again similar to *E. dunnii* in this respect (Darrow 1996).

1.1.6 *Eucalyptus urophylla*

Eucalyptus urophylla is one of two eucalypts that do not originate from Australia (the other is *E. deglupta*). The species naturally occurs on volcanically derived soils on seven islands in eastern Indonesia (Flores, Adonara, Lomblen, Pantar, Alor, Wetar and Timor) at altitudes that range from 180 – 3000 m (Eldridge *et al.* 1993). It is one of the most commercially important exotic forest species in the world, and is often crossed with *E. grandis* to produce hybrids that are well adapted to tropical conditions and which are more disease resistant than the *E. grandis* parent. *Eucalyptus urophylla* grows best in climates that are tropical to warm-temperate and humid, with mean maximum and minimum temperatures between 27 – 30°C and 8 – 12°C, respectively.

1.2 EUCALYPT FLORAL BIOLOGY

In developing an effective controlled pollination system, it is essential to have an understanding of the floral biology of the species of interest (van Wyk 1987; Oddie and McComb 1998; Moncur and Boland 2000). The floral characteristics will

determine the steps to be taken during controlled pollination to ensure that the flower is only fertilised by the applied pollen.

All the species in the present study have flowers that are bisexual, with the style surrounded by numerous anthers (Griffin 1982). During development, these flower buds (Figure 1.1A and B) are covered by two cap-like opercula, which protect the male and female reproductive structures. The presence of opercula is one of the most distinctive features of *Eucalyptus* and is derived from fusion of the petals and sepals (Pryor 1976). In species that have two opercula, the outer operculum is shed early during bud development, while the inner one is shed only at anthesis (when pollen is released from anthers) (Figure 1.1C). There is much variation in the size and shape of eucalypt flower buds, although within a species this tends to be relatively stable (Potts *et al.* 1995). Flowers generally occur in clusters in an inflorescence (referred to as an 'umbel'), in units of 3, 7, 9, 11 and 15 (Potts *et al.* 1995). Single flowers in the axil of a leaf are found in only a few species, for example *E. globulus*. The study species all have 7 flowers per umbel (Figure 1.1D).

Within *Eucalyptus*, stigma and style morphology vary greatly but are related to the taxonomic groupings (Boland and Sedgley 1986). In all species the stigma is wet (Heslop-Harrison and Shivanna 1977), covered by a sticky mucilagenous secretion at receptivity (Anderson 1984). Most *Symphyomyrtus* species (which include the study species) have blunt or pin-head shaped stigmas with a heavily cutinised styler canal, whereas *Monocalyptus* species have blunt stigmas with few papillae and hollow styles (Boland and Sedgley 1986). Stigma morphology may be important in allowing pollen to remain on the stigma for a certain time before germination (Griffin and Hand 1979). Generally, the stigmas of *Symphyomyrtus* species have a larger surface for pollen to contact than the stigmas of *Monocalyptus* species (due to papillae number), and hence the timing of controlled pollination would be more crucial in the latter subgenus. Studies on *E. camaldulensis* (subgenus *Symphyomyrtus*) have shown that pollen was able to remain on the stigma for three days, until stigma receptivity, after which the pollen began to germinate (Oddie and McComb 1998). Hodgson (1976) also demonstrated that pollen grains remain ungerminated on the stigmas of *E. grandis* for several days.

The time from anthesis to the onset of stigma receptivity, and the duration of receptivity, vary from species to species. For example, the stigma is receptive 4 – 6 days after anthesis in *E. grandis* (Hodgson 1976), compared to 6 – 10 days in *E. globulus* (Harbard *et al.* 1999) and 10 – 14 days in *E. regnans* (Eldridge and Griffin 1983). The sequence of anthesis and receptivity is also affected by environmental conditions, proceeding more rapidly at higher temperatures (Hodgson 1976). Individual flowers in an inflorescence and within a tree vary in their timing and so, despite the protandrous nature of the flowers (whereby pollen is shed before the stigma of the same flower is receptive), there is ample opportunity for geitonogamous self-pollination, particularly for the later-opening flowers within the crown of a flowering eucalypt tree (Eldridge *et al.* 1993; Hardner *et al.* 1996; Tibbits 1989). Thus, to produce an effective control-pollination system, it is evident that the development of an appropriate flower-isolation method to control selfing is highly desirable.

After flowering, the stamens wither and fall (Figure 1.1E) and there is some evidence to suggest this senescence of the stamens is triggered by pollination and/or fertilisation of the flower (Savva *et al.* 1988). The resulting eucalypt capsule (Figure 1.1F) is a false fruit (Williams and Woinarski 1997), which on drying will liberate seeds through openings formed by the spreading of valves on the top (Boland *et al.* 1985). The time period from pollination to seed-set varies between species, and for the tropical and subtropical species in the present study (*E. urophylla* and *E. grandis*) is between 8 and 10 months, while the cold-tolerant species (*E. nitens*, *E. smithii*, *E. dunnii* and *E. macarthurii*) take 12 months for seed maturation (personal observation).

The capsule contains fertile seed, aborted seed and chaff, all of which is normally found in a seedlot. The chaff is derived from the non-functional ovulodes towards the top of the ovary as well as abnormal or unpenetrated ovules which have aborted early in their development (Sedgley 1989). The number of viable seed obtained per capsule under open (natural) pollination varies between species, but in *E. nitens* (Tibbits 1989), *E. regnans* (Eldridge and Griffin 1983) and *E. globulus* (Potts *et al.* 1995), ranges from 2 – 4 per capsule. However, following controlled outcrossing, seed set per capsule has been reported to increase in virtually all species examined

(Tibbits 1989; Eldridge and Griffin 1983; Potts *et al.* 1995). This suggests that the amount of outcrossed pollen transferred to the stigma may be a factor limiting seed set in natural pollinations and once again emphasises the importance of controlled pollinations.

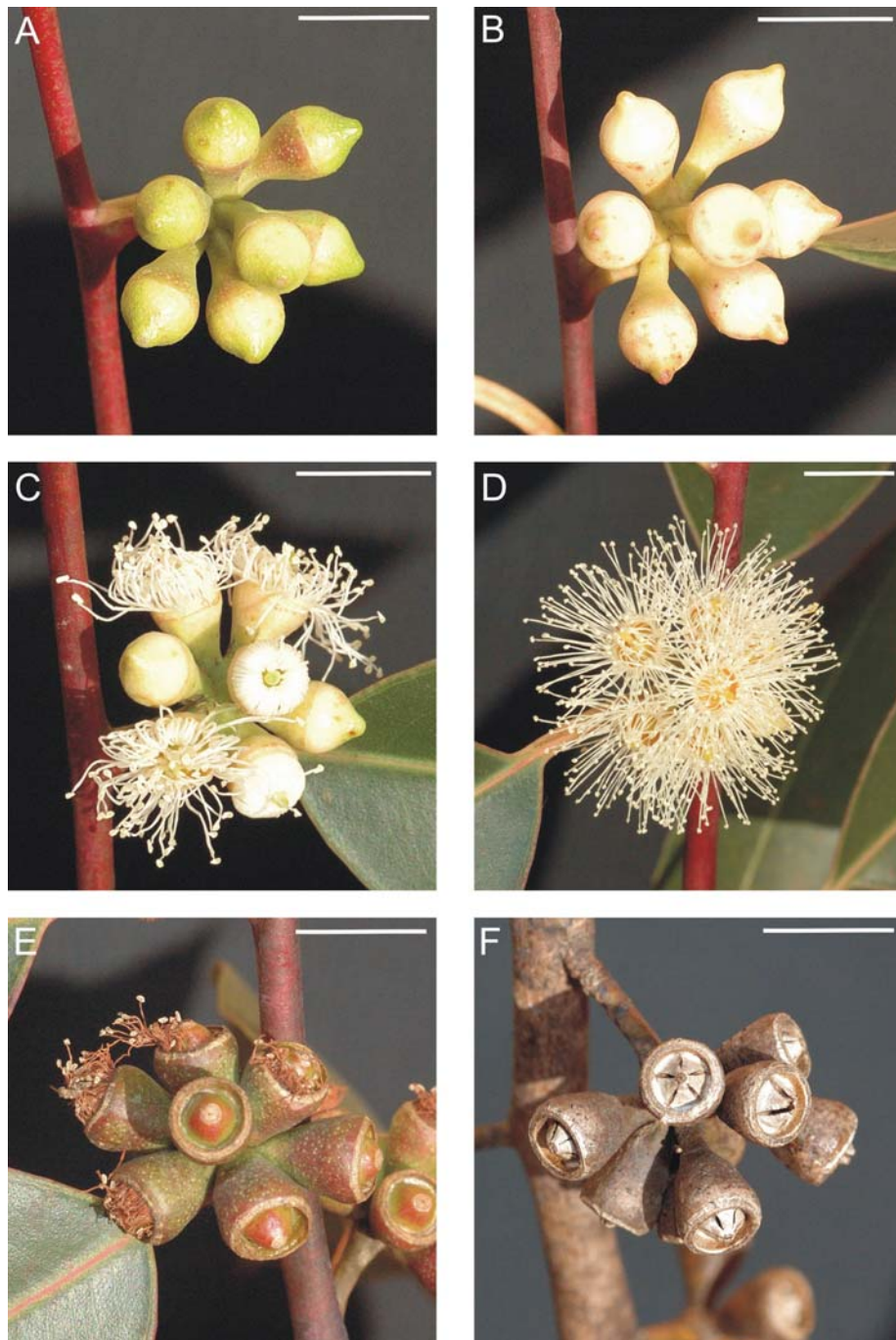


Figure 1.1: *Eucalyptus grandis* floral morphology: A) green flower buds; B) ripe flower buds showing colour change from green to yellow; C) operculum fall and spreading of the stamens; D) open flowers; E) anther senescence after fertilisation; F) woody capsules housing fertile seed, aborted seed and chaff. Bars = 1 cm

1.3 STUDY FOCAL AREAS

In the present study, three areas were identified as key to improving controlled pollination efficiency of the eucalypt study species, viz. pollen handling, breeding systems and controlled pollination technology. Pollen was chosen as the starting point, because availability of viable pollen when trees are flowering is essential for controlled pollinations to be undertaken. The second focal area was the breeding system, since the essence of controlled pollination is the manipulation of the flower's reproductive biology, while the final area targeted controlled pollinations and in particular, trying to find ways to reduce the costs associated with this technique.

1.3.1 Pollen research

Pollen grains embody the male partners in sexual reproduction and develop inside the anther (Shivanna and Johri 1985). Before or just after anther opening, the pollen is desiccated to varying degrees (Heslop-Harrison 1987). It becomes functional and desiccation-tolerant a few days before anthesis, when starch breaks down and sucrose, the primary soluble carbohydrate, doubles in quantity (Hoekstra and van Roekel 1988). At the same time new proteins responsible for desiccation tolerance (known as dehydrins) are synthesised, allowing the desiccated pollen to become more resistant to the abiotic stresses associated with dispersal (Campbell and Cloose 1997).

After dispersal, pollen is affected by natural variations in temperature and relative humidity, which cause the pollen water content and carbohydrate levels to fluctuate, ultimately affecting pollen viability (Pacini and Hesse 2005). Pollen of different species have different sensitivities to environmental changes. For example, grass pollen remains hydrated and viable for an extremely short time and is particularly sensitive to environmental stress (Heslop-Harrison 1979). On the other hand, *Eucalyptus* pollen has a long presentation, during which it is subjected to, and survives, severe environmental stresses, with about 30% of eucalypt pollen grains still remaining viable after exposure to 60°C for 24 h, and a small proportion surviving at 70°C (Heslop-Harrison and Heslop-Harrison 1985). Griffin *et al.* (1982) reported storing pollen from *E. regnans* satisfactorily at room temperature for 36 days. However, when pollen is subjected to cyclic environmental stress, such as repeated hydration and desiccation, it tends to lose its viability (Heslop-Harrison and Heslop-

Harrison 1992). Guarnieri *et al.* (2006) showed that maintenance of pollen viability in *Trachycarpus fortunei* is associated with the maintenance of low water content and high levels of sucrose.

Pollen viability is generally considered to indicate the ability of the pollen grain to perform its function of delivering the sperm cells to the embryo sac following compatible pollination (Shivanna *et al.* 1991). However, assessment of pollen viability on the basis of its function is cumbersome, time-consuming, and not always feasible, and many short-cut methods have been devised. Pollen viability has been evaluated by various staining techniques (e.g., tetrazolium salts to detect dehydrogenase activity, aniline blue to detect callose in pollen walls and pollen tubes, iodine to determine starch content, fluorescein diacetate to determine esterase activity and the intactness of the plasma membrane), by *in vitro* and *in vivo* germination tests, or by analysing final seed set (Adhikari and Campbell 1998; Dafni and Firmage 2000). The choice of method depends on the species and particular application.

Viable pollen is necessary for controlled pollination to be successful, and it becomes important to know the variability in pollen viability when considering a specific mating design (Beineke *et al.* 1977). In addition, sources in the seed orchard with low pollen viability would require compensation for the lack of viable pollen from these sources. A reliable method for testing pollen viability is essential in this regard. Staining techniques for assessing pollen viability are not popular because the researcher has to determine subjectively when a pollen grain is stained darkly enough to be classified as “viable”, often leading to overestimation of pollen viability. The results of staining tests also do not always correlate well with other measures of pollen viability (Dafni 1992). Although the stigma provides a suitable site for pollen germination, *in vivo* studies are not easy because of the complications involving pistillate tissue (Shivanna and Johri 1985). *In vitro* pollen germination is believed to provide the best estimate of pollen viability *in vivo* (Stone *et al.* 1995). In fact, in most of the studies on staining techniques, the effectiveness of staining methods was evaluated based on their correlation with pollen germination *in vitro* (Dafni and Firmage 2000).

A number of culture media and methods have been used for *in vitro* germination and, in general, most media contain boron, combined with sucrose as an osmoticum and nutritional source (Brewbaker and Kwack 1963). However, the optimal medium composition is species-dependent and for many species, ideal testing conditions remain to be determined. For this reason, one of the aims of the present study was to identify a suitable liquid *in vitro* germination medium for the reliable testing of pollen viability of *E. grandis*, *E. dunnii*, *E. smithii*, *E. nitens* and *E. macarthurii*.

Storage of *Eucalyptus* pollen is generally required for controlled hybrid crosses (Harbard *et al.* 1999), since some *Eucalyptus* species flower asynchronously and successful crosses are seldom accomplished with fresh pollen due to non-availability of the seed parent when the pollen parent is in full bloom (Eldridge *et al.* 1993). In this instance, stored pollen, recovered with acceptable viability, is necessary to accomplish the breeding objective (Griffin 1982). Special storage conditions are, however, needed to preserve the viability of pollen for long periods.

For a wide range of species, practical storage procedures, effective for months to several years, have been developed by simple reduction of environmental temperature and humidity (Barnabas and Kovacs 1997). For example, pollen of *Simmondsia* stored at 24°C showed marked reduction in viability during the first week, and the percentage germination came down to zero in two months (Beasley and Yermanos 1976). In the sample stored at 4°C, viability was comparable to fresh pollen up to two months, and germination decreased to 0% by eight months. When stored at -20°C, there was very little reduction in viability up to ten months, and germination came down by only 25% over fresh pollen, even after one year (Beasley and Yermanos 1976). This is understandable because the major cause of loss of viability in long-term storage appears to be the deficiency of metabolites due to continued metabolic activity of the pollen (Shivanna and Johri 1985). Lowering the temperature as well as the humidity drastically reduces the metabolism of the pollen and prolongs viability.

A second aim of the present study was therefore to determine temperatures suitable for the short-, medium- and long-term storage of *E. smithii*, *E. nitens* and *E. grandis* pollen.

1.3.2 *Breeding system research*

One of the unique features of sexual reproduction in flowering plants is the interaction of the pollen grain and microgametophyte with the massive sporophytic tissue of the pistil (the stigma and style). This is referred to as pollen-pistil interaction and when it results in successful fertilisation, allows the two primary functions of sexual reproduction, viz. maintenance of stability of the species and maintenance of a reasonable degree of genetic variability within the species, to be fulfilled (Shivanna and Johri 1985). These functions are fulfilled because all sexually reproducing organisms have the ability to recognise and select suitable gametes for fertilisation. In flowering plants, the pistil has developed mechanisms to recognise pollen grains and to permit the growth of tubes from compatible ones, while incompatible pollen tubes are effectively prevented from reaching the female gamete (Shivanna and Johri 1985).

Incompatibility is an integral part of pollen-pistil interaction and results in the arrest of post-pollination events at various levels. Shivanna and Johri (1985) define it as “the inability of functional male and female gametes to effect fertilisation in particular combinations”. It can operate between species (interspecific), as well as within a species (intraspecific). In the former, fertilisation between gametes which originate from unrelated parents is prevented, while in the latter, fertilisation is prevented between gametes which originate from two individuals of the same species. Thus, interspecific incompatibility maintains the integrity of the species, whereas intraspecific incompatibility promotes genetic variation within the species by encouraging outbreeding (Shivanna and Johri 1985).

Barriers to gene flow between and within species are often categorised according to whether they reduce the likelihood that gametes will combine to form a viable zygote (prezygotic) or whether they reduce the viability or reproductive potential of the progeny (post-zygotic). These barriers can be further divided into pre- and post-pollination mechanisms. Pre-pollination barriers, which are exclusively pre-zygotic, include ecological factors that prevent individuals from different lineages from growing close enough to exchange gametes and reproductive factors that result from genetic differences in flowering times and divergence of floral characters (Levin 1978). Post-pollination isolation, on the other hand, may result from pre-zygotic or

post-zygotic mechanisms (Snow 1994). Pre-zygotic mechanisms include pollen-pistil interactions that manifest themselves as low pollen germination rates, slow pollen tube growth and failure of pollen tubes to penetrate ovules. Post-zygotic mechanisms include offspring inviability and sterility.

Self-incompatibility (SI) is a form of intraspecific incompatibility and can be defined as “the inability of a fertile hermaphrodite seed plant to produce zygotes after self-pollination” (Lundqvist 1964). The significance of self-incompatibility is that it leads to obligate outbreeding and the maintenance of heterozygosity within a species (Stebbins 1950). It also provides a way to hybridise two genotypes without emasculation, nuclear or cytoplasmic sterility, or resorting to gametocides. However, the negation of self-seed is not always 100% and may be dependent upon environmental conditions. Furthermore, not all species possess natural self-incompatibility. For this reason, isolating the eucalypt flower during controlled pollination is highly desirable to prevent geitonogamous self-pollination.

One of the main effects of selfing is inbreeding depression, i.e. the lower success of selfed compared to outcrossed progeny, which is considered as a strong evolutionary force because of its negative effects on different components of individual fitness (Charlesworth and Charlesworth 1987). Inbreeding depression is thought to be a selective agent preventing the evolution of selfing and its effects often increase throughout the life cycle of many plant species (Lande and Schemske 1985; Charlesworth and Charlesworth 1987). This suggests that deleterious alleles contributing to inbreeding depression vary in their effects across the life cycle, with more weakly deleterious alleles expressed later in the life history (Husband and Schemske 1996). For this reason, a perennial woody species such as *Eucalyptus* is more likely to harbour a higher genetic load than an annual species because of the increased number of cell divisions and greater difficulty of purging weakly vs. strongly deleterious alleles from the population (Barrett and Eckert 1990). *Eucalyptus*, being a largely outcrossing group, would thus be expected to express greater inbreeding depression compared to a selfer (Lande and Schemske 1985).

Self-incompatibility is one way of preventing uniparental inbreeding depression, and studies in the early 1950s unraveled two distinct forms, viz. sporophytic and

gametophytic (de Nettancourt, 2001; McCubbin and Kao, 2000; Silva and Goring, 2001). Sporophytic SI generally results in inhibition of pollen germination on the stigma (de Nettancourt, 1977) and features associated with this mechanism are trinucleate pollen, with short storage capacity and poor germination *in vitro* (Brewbaker, 1967), and dry stigmas (Heslop-Harrison and Shivanna, 1977). Gametophytic SI is the most frequently reported SI mechanism in woody species (Sedgley, 1994). It generally results in pollen tubes ceasing growth in the style following self-pollination and is more often associated with species that have wet stigmas (Heslop-Harrison and Shivanna, 1977), and those that produce binucleate pollen, with good storage capacity and the ability to germinate well *in vitro* (Brewbaker, 1967).

The concept of late-acting SI, also termed ovarian SI, was introduced by Seavey and Bawa (1986) to accommodate the increasing number of reports where the self-incompatibility mechanism does not act in the stigma or in the style. In this type of SI, self-pollen germinates and reaches the ovules, but no fruit is set (Seavey and Bawa, 1986; Sage *et al.*, 1994). It can manifest pre-zygotically with deterioration of the embryo sac prior to pollen tube entry, or post-zygotically with malformation of the zygote or embryo (Sage *et al.*, 1994). Reduced ovule penetration by self-pollen tubes compared with cross-pollen tubes has been found in *Eucalyptus woodwardii* (Sedgley and Smith, 1989) and *E. morrisbyi* (Potts and Savva, 1988), suggesting late-acting pre-zygotic control, while in *E. regnans* (Sedgley *et al.*, 1989), *E. cladocalyx*, *E. leptophylla* (Ellis and Sedgley, 1992) and *E. nitens* (Pound *et al.*, 2003) the SI mechanism appears to be post-zygotic, with no difference in the development of selfed- and crossed-seed from 4 to 16 weeks after pollination. Both pre- and post-zygotic SI barriers have been reported to occur in *E. spathulata*, *E. platypus* (Sedgley and Granger, 1996) and *E. globulus* (Pound *et al.*, 2002), as not only were pollen-tube penetration and fertilisation reduced following self-pollination, but there was also a reduction in the number of fertilised ovules achieving zygotic division.

In another type of self-incompatibility, viz. cryptic SI, the simultaneous presence of cross and self pollen on the stigma results in higher seed set from cross pollen relative to self pollen (Bateman, 1956). However, as opposed to complete or

absolute self-incompatibility, in cryptic SI, self-pollination without the presence of competing cross pollen results in successful fertilisation and seed set. In this way reproduction is assured, even in the absence of cross-pollination. Cryptic SI acts at the stage of pollen tube elongation in the style and leads to faster elongation of cross pollen tubes relative to self pollen tubes (Bateman, 1956). To date, this type of self-incompatibility has never been associated with *Eucalyptus*.

Cytological details of rejection following interspecific pollinations have not been subjected to as in-depth an investigation as for self-incompatibility and in most instances, crosses have been classified as compatible or incompatible on the basis of their seed set. From the limited information available, it is apparent that the rejection reaction may occur at any level, depending on the extent of reproductive isolation of the male partner. Closely related species would accomplish more of the post-pollination events compared to distantly related species (Potts *et al.* 1987; Griffin *et al.* 1988).

Breeding systems of different *Eucalyptus* species have been investigated using a variety of methods and found to be one of mixed mating with preferential outcrossing (Moran and Bell 1983; Yeh *et al.* 1983; Fripp *et al.* 1986; Potts and Savva 1988). This has largely been demonstrated in a reduction in capsule production, seed yield and seedling vigour after self pollination compared with cross pollination (Griffin *et al.* 1987; Eldridge *et al.* 1993). Within species, individual trees may vary in their capacity to set self-pollinated seed (Ellis and Sedgley 1992) and outcrossing is promoted by the protandrous nature of the flower, as well as by pollinator behaviour (Williams and Woinarski, 1997). In a study carried out by Jones *et al.* (2008) on *E. grandis*, the outcrossing rate was estimated to be in the range of 0.64 to 1.00, similar to a previous estimate of 0.84 by Moran and Bell (1983). This is also similar to the outcrossing rates estimated in seed orchards and breeding populations of other eucalypt species: 0.75 in *E. nitens* and *E. camaldulensis*, 0.77 in *E. globulus* (Moncur *et al.* 1995); 0.91 for *E. regnans* (Moran *et al.* 1989); and 0.89 – 0.93 for *E. urophylla* (Gaiotto *et al.* 1997). The level of self-pollination in individual *E. grandis* trees was shown to range from 0 – 36%, with an overall observed selfing rate of 14% (Jones *et al.* 2008).

In the literature there is a notable lack of information on comparative growth rates of self- and cross-pollen in the eucalypt pistil, and pollen-pistil interactions have been studied in just eight species to date, viz. *Eucalyptus morrisbyi* (Potts and Savva 1988), *E. regnans* (Sedgley *et al.* 1989), *E. woodwardii* (Sedgley 1989; Sedgley and Smith 1989), *E. spathulata*, *E. cladocalyx*, *E. leptophylla* (Ellis and Sedgley 1992), *E. globulus* (Pound *et al.* 2002) and *E. nitens* (Pound *et al.* 2003). The present study thus also aimed to examine the breeding systems of *E. urophylla* and *E. grandis*, using epifluorescence microscopy to study pollen-pistil interaction after controlled self- and cross-pollinations.

1.3.3 Controlled pollination research

In small populations, such as seed orchards used for long-term breeding programmes, genetic diversity can decrease due to random genetic drift, and the probability of crossings between closely related individuals could be increased, resulting in inbreeding (Paschke *et al.* 2002). Inbreeding can strongly reduce both population and individual viability, and negative effects of small population size on offspring fitness have been found in many species (Kery *et al.* 2001; Keller and Waller 2002). To increase the vigor of plants in such populations, increasing the gene flow between populations to enhance levels of heterozygosity has been suggested (Oostermeijer *et al.* 1995).

Many plants, including *Eucalyptus*, rely on animal pollinators for the transfer of pollen onto their stigmas for ovule fertilisation. However, in small populations plant-pollinator interactions may become disrupted, and reproduction may be reduced because of insufficient pollination (i.e. pollen limitation). In addition to these plants being less attractive to pollinators (and thus visited less frequently), in small populations the local density of plants is often reduced and there is less pollen transfer between individuals (Roll *et al.* 1997; Bosch and Waser 2001). Self-incompatible species (which include most forest tree species) are more likely to be affected by pollen limitation than self-compatible species because they cannot compensate for reduced pollinator services by selfing. Also, because the diversity of pollen genotypes in these small populations is reduced, it is more likely that flowers will receive incompatible pollen (Byers 1995). Pollen limitation may also reduce progeny vigor by reducing the selectivity among gametes (i.e. pollen competition)

before and during fertilisation (Winsor *et al.* 2000). This was seen in *Scorzonera humilis* (Asteraceae), where pollen limitation resulted in a reduction in both the number and quality of offspring produced (Colling *et al.* 2004).

Controlled pollination can be used to alleviate the above problems. In forestry the technique plays an important role in combining the genetic material of selected elite trees to produce high quality, high value seed (Frampton 1997; Eldridge *et al.* 1993). However, the high cost of performing such crosses and the relatively low seed production results in reliance on lower quality, open pollinated seed for the establishment of the majority of commercial forests. Since controlled crossing is integral to rapid genetic improvement, any increase in the efficiency of controlled pollinations would be highly desirable to the forestry industry.

Eucalypt pollen is difficult to collect and handle in any appreciable quantity and thus supplementary pollination techniques which work well with wind-pollinated species, like conifers (Webber 1987), and large-flowered eucalypt species, like *E. globulus* (Potts *et al.* 2008), are unlikely to prove as applicable for the majority of small-flowered eucalypt trees. However, as Griffin (1989) points out, there is considerable scope for innovative developments in the methodology of controlled pollination. The most costly steps are emasculation and bagging, and the question is whether these are essential. Emasculation is carried out to avoid selfing, but application of the desired outcrossed pollen at the optimal time, combined with careful stage-matching of flowers in an isolation bag so that pollen has been shed by the time the stigmas are receptive, may obviate the need for this (Griffin 1989). Isolation bags are used to prevent visits from vectors carrying pollen of unknown origin. They may not be needed if the vectors can be excluded from the vicinity or the attractiveness of flowers is diminished. Some citrus breeders favour pollination on depetalled flowers which are left unbagged (Soost and Cameron 1975). Placing the whole plant in an insect proof environment can also be effective, but will only be practical with a move to a containerised planting system (Sedgley and Alexander 1983).

For *Eucalyptus*, the first-developed CP-method, termed the 'Conventional method', took advantage of the natural protandry of the eucalypt flower (van Wyk 1977). The technique, however, involved three flower visits (emasculation, subsequent

pollination of receptive stigmas and bagging, removal of bags), and was consequently very time consuming. In addition, low seed returns due to cross-incompatibility or poor pollen quality made this a very expensive exercise. A more efficient cross-pollination method was later developed, originally for *Eucalyptus globulus*, requiring only one visit to the flower (emasculating and immediate pollination of stigmas cut to induce receptivity, followed by bagging) and consequently named One Stop Pollination (OSP; Harbard *et al.* 1999). However, the flowers of *E. globulus* are considerably larger than those of the study species, necessitating careful adaptation of OSP to be successful on smaller flowers. Artificially Induced Protogyny (AIP) is an exciting new technique, recently developed in Brazil (Assis *et al.* 2005). It involves cutting off the tip of the operculum of the mature flower bud, just prior to anthesis, to expose the cut surface of the upper style to which the target pollen is applied, without emasculating or isolating the flower. However, due to differences in flower morphology, the method once again needs to be adapted to suit different eucalypt species.

The final part of this PhD study was aimed at establishing an efficient controlled pollination technique that would allow CPs to be performed on a commercial scale on small-flowered eucalypts. An innovative isolation mechanism was developed in order to reduce contamination from self- and foreign-pollen sources during controlled pollinations.

1.4 SCOPE OF THIS THESIS

Of particular importance to efficient controlled pollinations is being able to optimally store pollen for various lengths of time, due to differences in flowering time between the species. However, before this pollen can be used in controlled pollinations, the viability has to be assessed, and thus a reliable *in vitro* testing protocol has to first be established. Chapter 2 concentrated on identification of a suitable liquid *in vitro* germination medium for testing the viability of both freshly collected and stored eucalypt pollen. Suitable temperatures for short-, medium- and long-term pollen storage were then addressed by comparing the viability of pollen stored at room (25°C), refrigerator (4°C), freezer (-10°C) and liquid nitrogen (-196°C) temperatures. Since controlled pollination is in essence the manipulation of the reproductive biology, an attempt was made to learn more about the breeding systems of the study

species. In chapter 3, epifluorescence microscopy was used to study the growth rates of pollen tubes within the style, in addition to comparing seed yields, after self- and cross-pollinations. In order to confirm the type of breeding system, microsatellite markers were then used in chapter 4 to examine the siring ability of self- and cross-pollen after both mixed- and single-donor pollinations. Chapters 5 and 6 focused on establishing an efficient commercial control-pollination technique, specifically applicable to small-flowered eucalypts. In chapter 5, three different CP-techniques were compared, while chapter 6 explored a novel method of isolating the pollinated flower.

1.5 SPECIFIC STUDY AIMS

The specific aims of this PhD study were to:

- i. Maximise the use of pollen by developing a reliable pollen-viability testing protocol.
- ii. Enhance short-, medium- and long-term storage of pollen to facilitate use in controlled pollinations.
- iii. Understand the eucalypt breeding system by studying pollen tube growth in the style following self- and cross-pollinations.
- iv. Identify a commercial controlled pollination technique for use on small-flowered eucalypts.
- v. Develop an efficient method of isolating the flower bud during commercial controlled pollinations.

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CHAPTER 2: OPTIMISING STORAGE AND *IN VITRO* GERMINATION OF *EUCALYPTUS* POLLEN

2.1 ABSTRACT

The best sucrose solution for maximum *in vitro* germination of *Eucalyptus* pollen was investigated in order to evaluate pollen germination rate as an indicator of pollen viability. *In vitro* germination of both freshly collected and one-year old pollen (stored at 4°C) of *Eucalyptus grandis*, *E. smithii*, *E. nitens*, *E. dunnii* and *E. macarthurii* was carried out in 0, 10, 20, 30, 40 and 50% (w/v) sucrose solutions, with (0.15 mg l⁻¹) and without boric acid. Similar trends were obtained for both fresh and one-year old pollen, with all species responding most favourably to 30% (w/v) sucrose and 0.15 mg l⁻¹ boric acid. When an optimal *in vitro* germination medium had been established, the viability (% germination) of *E. smithii*, *E. nitens* and *E. grandis* pollen, stored at room (25°C), refrigerator (4°C), freezer (-10°C) and liquid nitrogen (-196°C) temperatures, were compared. For all tested species, germination declined as storage temperature increased, and by 8 months, the highest survival was obtained with cryostored pollen.

2.2 INTRODUCTION

Due to its fast growth and production of high value timber, *Eucalyptus* has been a major focus of forestry industries world-wide for raw material for pulp, paper and solid wood markets (Eldridge *et al.* 1994). However, the genus is still in its earliest stages of breeding and improvement depends on the ability to combine characteristics from different parents in order to develop new, superior genotypes. One way of achieving this is through controlled pollination (Frampton 1997), for which high quality pollen is a pre-requisite (Matthews and Bramlett 1986).

Storage of *Eucalyptus* pollen is generally required for controlled crosses, either to achieve a desired breeding objective, or to overcome a constraint involved in seed production (Harbard *et al.* 1999). For example, in some breeding programmes, interspecific crosses are attempted to introgress genetic traits into desirable species, from pollen parents known for providing resistance to biotic stresses. However, some *Eucalyptus* species flower asynchronously and successful crosses are seldom accomplished with fresh pollen due to non-availability of the seed parent when the

pollen parent is in full bloom (Eldridge *et al.* 1994). Stored pollen, recovered with acceptable viability, can be used in crosses with the desired female genotype, thereby accomplishing the breeding objective (Griffin *et al.* 1982).

Practical storage procedures, effective for months to several years, have been developed for a wide range of species (Barnabas and Kovacs 1997). The properties required for a successful storage system are: the ability to maintain pollen viability at the highest possible level; maintenance of full developmental and functional potential when pollen is returned to normal physiological conditions; minimal growth and development in storage; and significant savings in labour input and materials (Grout 1995). In addition, pollen grains that survive drying, like *Eucalyptus*, can be stored more easily than those requiring a high moisture content (Buitink *et al.* 1998).

The most ideal method for long-term pollen storage is that of cryopreservation (Engelmann 1997). This refers to storage at ultra-low temperatures (usually that of liquid nitrogen). At these low temperatures all cellular activities are arrested and in this state, genetic material can theoretically be stored without alteration or modification for indefinite periods. Furthermore, the material is protected from contamination and requires very little maintenance. However, the development of cryopreservation methods requires the empirical determination, on a species basis, of the optimal conditions for cryostorage (Watt *et al.* 2000).

The viability of stored pollen samples can be estimated by using vital stains, *in vitro* germination, or *in vivo* assays (Kearns and Inouye 1993). Vital stains are difficult to quantify, requiring the researcher to determine subjectively when a pollen grain is stained darkly enough to be classified as “viable”. Personal experience with vital staining of stored *Eucalyptus* pollen suggests that certain enzymes may remain active, although the pollen grain itself may be dead, leading to overestimates in pollen longevity. The results of vital-stain tests also do not always correlate well with other measures of pollen viability (Dafni 1992). Because the ultimate purpose of pollen storage is successful fertilisation and seed production, it follows that well-designed *in vivo* tests should most accurately reflect the utility of stored samples. An important point to bear in mind, however, is that genetic incompatibility systems can

confound the results of *in vivo* germination and seed-set assays (de Nettancourt 1977). For this reason, *in vitro* tests were used in the present study.

In vitro pollen germination is rapid, reasonably simple and fully quantitative (Kearns and Inouye 1993). In a number of taxa the percentage *in vitro* germination of stored pollen can be correlated with its ability to set fruits and seeds following *in vivo* pollination (Janssen and Hermsen 1980). A major limitation of the test, however, is the difficulty in achieving *in vitro* germination in several taxa. In addition, the medium that gives optimal germination of fresh pollen may not be optimal for stored pollen.

Successful germination of eucalypt pollen has been obtained using either liquid (Griffin *et al.* 1982) or semi-solid agar media (Heslop-Harrison and Heslop-Harrison 1985) with sucrose and boron (usually boric acid) normally the sole constituents. However, the optimal medium composition is species-dependent and for many species, ideal testing conditions remain to be determined. For this reason, the first aim of the present study was to identify a suitable liquid *in vitro* germination medium for the reliable testing of pollen viability of five commercially important *Eucalyptus* species, viz. *E. grandis*, *E. dunnii*, *E. smithii*, *E. nitens* and *E. macarthurii*. The second aim was to determine temperatures suitable for the short-, medium- and long-term pollen storage. This was done by comparing the viability of *E. smithii*, *E. nitens* and *E. grandis* pollen stored at room (25°C), refrigerator (4°C), freezer (−10°C) and liquid nitrogen (−196°C) temperatures.

2.3 MATERIAL AND METHODS

2.3.1 Plant material used in study

Pollen samples of all species were collected from clonal (grafted) orchards planted at the Sappi, Shaw Research Centre (SRC) in KwaZulu-Natal, South Africa. The orchards were situated at 29° 29'S, 30° 11'E at 1100 m above sea level. Breeding populations for all species were made up of open-pollinated families from selections made in the land-race in South Africa and from provenances in the natural range in Australia.

For the first part of the study, viz. developing an optimal *in vitro* germination medium, five eucalypt species were used: *E. grandis*, *E. smithii*, *E. dunnii*, *E. macarthurii* and

E. nitens. Pollen from three ramets was mixed in equal quantities to provide a polymix of three genotypes for each species. In addition, both freshly collected and one-year old pollen (stored at 4°C) were included as separate samples. For the second part of the study, viz. pollen storage, freshly-collected pollen from three eucalypt species, *E. smithii*, *E. nitens* and *E. grandis*, were used. Three genotypes of *E. smithii*, three of *E. nitens* and one of *E. grandis* were collected. Pollen from *E. smithii* and *E. nitens* was mixed separately to give a single representative pollen sample for each species.

2.3.2 Pollen collection and processing

For pollen extraction, branches containing ripe flower buds were collected and kept in 100 ml bottles containing water to prevent drying out of the branch. To ensure that there was no contamination from other pollen, all open flowers were removed from the branches before placing them in the laboratory overnight. When the operculum had shed and the filaments unfolded, the anthers were excised and left in an incubator in the presence of silica gel to dry for approximately 48 hours at room temperature. When the relative humidity (RH) had reached 10% (as measured with an hygrometer), the dried anthers were sieved through a 30 micron mesh to remove debris. The resulting pollen was placed into polypropylene vials and then sealed in glass bottles containing silica gel.

2.3.3 In vitro pollen germination

Before use, pollen samples were left at room temperature (approximately 80% RH) for 8 hours to rehydrate. For the determination of an optimal *in vitro* testing medium, 6 levels of sucrose 0, 10, 20, 30, 40 and 50% (w/v) were tested with (0.15 mg l⁻¹) and without boric acid in a liquid medium. For the pollen storage part of the study, samples were germinated in a standard liquid solution of 30% (w/v) sucrose and 0.15 mg l⁻¹ boric acid.

For pollen germination, polymixes from each species were placed into separate test tubes containing germination medium (three tubes per species per treatment) and left to incubate in a germination chamber in a completely randomised design for 48 hours at 29°C. After the required time period had elapsed, 5 µl was transferred from the test-tube to a glass slide. Percent germination was scored using a light microscope

(x100 magnification) to count the number of pollen grains germinated out of a total of 50 grains. Six glass slides per species (two slides per test tube) were scored for germination (sub-samples), giving a total of 300 pollen grains counted per treatment. Pollen was deemed to have germinated if the pollen tube length was greater than one-half of the diameter of the pollen grain (Potts and Marsden-Smedley 1989).

To obtain consistent results, some precautions had to be observed. Firstly, similar quantities of pollen (by visual inspection) were used for all samples. The reason for maintaining optimal pollen density is that the pollen grains of many species exhibit a population effect (Shivanna and Rangaswamy 1992). In such cases, pollen germination is strictly dependent on pollen density and below a critical threshold no germination occurs (Brewbaker and Kwack 1964). This dependence of germination on pollen density is related to a dependence on calcium leakage from pollen grains (Shivanna and Rangaswamy 1992), the optimum level of which is reached in the presence of a minimum number of germinating grains. Similarly, too large an amount of pollen grains is undesirable, as the nutrients in the culture medium may become limiting.

To avoid erratic results, pollen grains were uniformly distributed in the medium before scoring germination in the present study. In addition, since the pollen of most eucalypts is somewhat sticky, with the grains tending to adhere in clusters of varying size and thus making accurate microscopic counts of an entire field difficult, assessments were generally made of single grains and smaller groups where all grains were readily discernible. Germination counts were thus based on three to four fields until 50 or more pollen grains could be recognised as germinated or ungerminated per microscope slide.

2.3.4 Pollen storage treatments

Pollen samples (in polypropylene vials, sealed in glass bottles containing silica gel) were stored at each of the following temperatures: room (+/- 25°C); refrigerator (4°C); freezer (-10°C) and liquid nitrogen (-196°C) for 12 months. Pollen samples for cryogenic storage were transferred to 2 ml volume cryovials of which 0.2 ml was the maximum volume of the sample. Based on the previous use of a pre-cooling period by several researchers (Ganeshan 1986; Bowes 1990; Lanteri *et al.* 1993), the pollen

was pre-cooled at -10°C for 8 hours before dipping into liquid nitrogen. During storage, the level of liquid nitrogen in the storage vessel was regularly monitored and replenished as appropriate. Every two months, a subsample was thawed by removal of the vial from liquid nitrogen and placement in a freezer for 8 hours (to prevent damage from rapid thawing) before being left at room temperature for 8 hours, and then assessed for viability (% germination).

2.3.5 Statistical Analysis

To normalise the data distribution, values for the proportion of pollen germination were angular transformed prior to analysis. For the optimisation of *in vitro* germination medium, GenStat Release 8.1 was used to perform an analysis of variance (ANOVA), and consequently establish the statistical significance of observed differences between treatments for both fresh and one-year old pollen. The data were analysed as a completely randomised design and pseudo-replication taken into account by using the mean of the two slides scored for each test-tube. For the optimisation of pollen storage, SPSS Version 11.51 was used to calculate ANCOVA and consequently establish the statistical significance of observed differences between treatments in the percentage of pollen that germinated. Fresh pollen was excluded from the analysis and a full factorial model used. Time was treated as the covariate.

2.4 RESULTS

2.4.1 Optimising liquid *in vitro* germination medium

There were highly significant differences in pollen germination between media containing boric acid compared to media without ($p < 0.001$) (Tables 2.1 and 2.2) for all species tested, with the former resulting in higher germination. This effect became more pronounced as the sucrose concentration was increased, reaching a maximum in 30% (w/v) sucrose and then decreasing as the sucrose concentration was further increased to 50% (Figure 2.1). Pollen tube length also tended to be greater in the presence of boric acid, suggesting improved pollen vigour (Figure 2.2). Furthermore, highly significant species differences ($p < 0.001$) were evident for both freshly collected (Table 2.1) and one-year old (Table 2.2) pollen germination. With the exception of *E. macarthurii*, there was also a clear decrease in pollen germination for the older pollen sample (Figure 2.1).

Table 2.1: Analysis of variance for transformed values of percentage germination of fresh pollen, used in the determination of an optimal *in vitro* germination medium.

Source of variation	Degrees of freedom	Mean square	F value	Probability
species	4	993.21	105.66	<0.001
sucrose	5	4997.42	531.65	<0.001
boric acid	1	8904.38	947.30	<0.001
species x sucrose	20	195.84	20.83	<0.001
species x boric acid	4	597.06	63.52	<0.001
sucrose x boric acid	4	776.58	82.62	<0.001
species x sucrose x boric acid	16	103.37	11.00	<0.001
residual	108	9.4		

Table 2.2: Analysis of variance for transformed values of percentage germination of stored pollen, used in the determination of an optimal *in vitro* germination medium.

Source of variation	Degrees of freedom	Mean square	F value	Probability
species	4	2597.05	253.56	<0.001
sucrose	5	2585.20	252.40	<0.001
boric acid	1	4159.44	406.10	<0.001
species x sucrose	20	244.73	23.89	<0.001
species x boric acid	4	363.35	35.48	<0.001
sucrose x boric acid	4	588.70	57.48	<0.001
species x sucrose x boric acid	16	120.29	11.74	<0.001
residual	108	10.24		

In the absence of boric acid, both 30% and 40% (w/v) sucrose proved suitable when germinating stored pollen (Figure 2.1c). For fresh pollen germinated in the absence of boric acid, 30% (w/v) sucrose resulted in higher germination for most species. There was no significant difference between 30% and 40% (w/v) sucrose for fresh *E. nitens* pollen germinated in the absence of boric acid (Figure 2.1d). There were highly significant interactions ($P < 0.001$) between all main effects for both fresh and stored pollen (Tables 2.1 and 2.2, respectively).

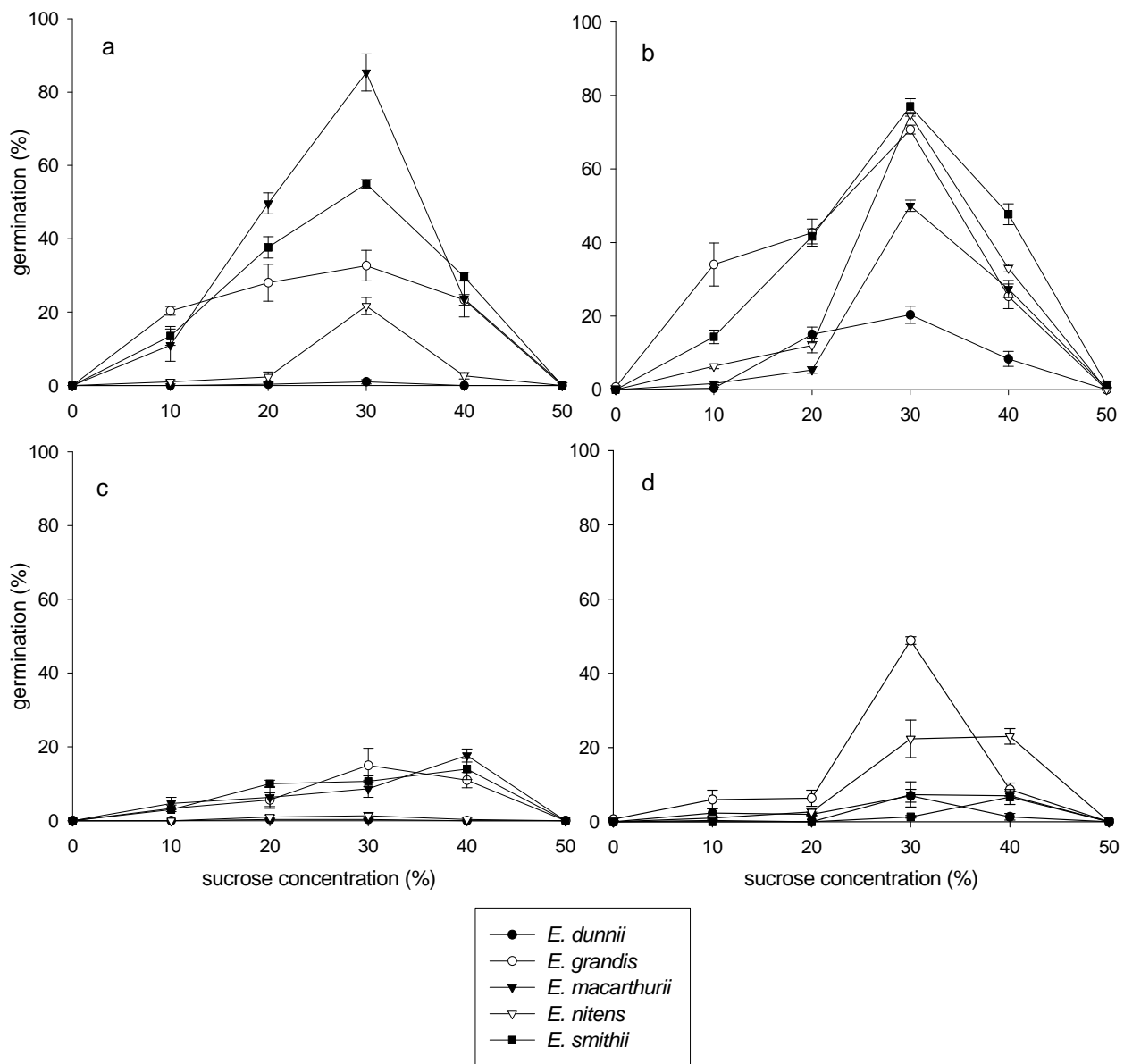


Figure 2.1: Percentage germination of fresh and stored (1-year old) pollen of five *Eucalyptus* species, incubated in media containing various concentrations of sucrose, with or without boric acid: (a) stored pollen with boric acid; (b) fresh pollen with boric acid; (c) stored pollen without boric acid; (d) fresh pollen without boric acid. Symbols indicate means \pm s.e.



Figure 2.2: Pollen tube growth of *E. macarthurii* pollen incubated in *in vitro* germination medium containing 30% (w/v) sucrose and (a) 0.15 mg l⁻¹ boric acid and (b) without boric acid supplementation (x20). This was the typical response to boric acid for all species tested.

2.4.2 Optimising pollen storage regimes

There was no overall effect of storage time on pollen germination ($p = 0.439$), as well as no significant overall effect of species on pollen germination (Table 2.3). There was, however, a significant species-by-storage time interaction ($p = 0.002$) and a highly significant treatment-by-storage time interaction ($p < 0.001$).

Table 2.3: Analysis of covariance for transformed values of percentage germination of pollen used in the determination of temperature regimes for optimal pollen storage.

Source of variation	Degrees of freedom	Mean square	F value	Probability
species	2	279.25	2.83	0.062
treatment	3	195.20	1.98	0.119
time	1	59.39	0.60	0.439
species x time	2	634.16	6.42	0.002
species x treatment	6	262.45	2.66	0.017
treatment x time	3	3522.03	35.65	0.000
species x treatment x time	6	179.17	1.81	0.098
residual	192	98.80		

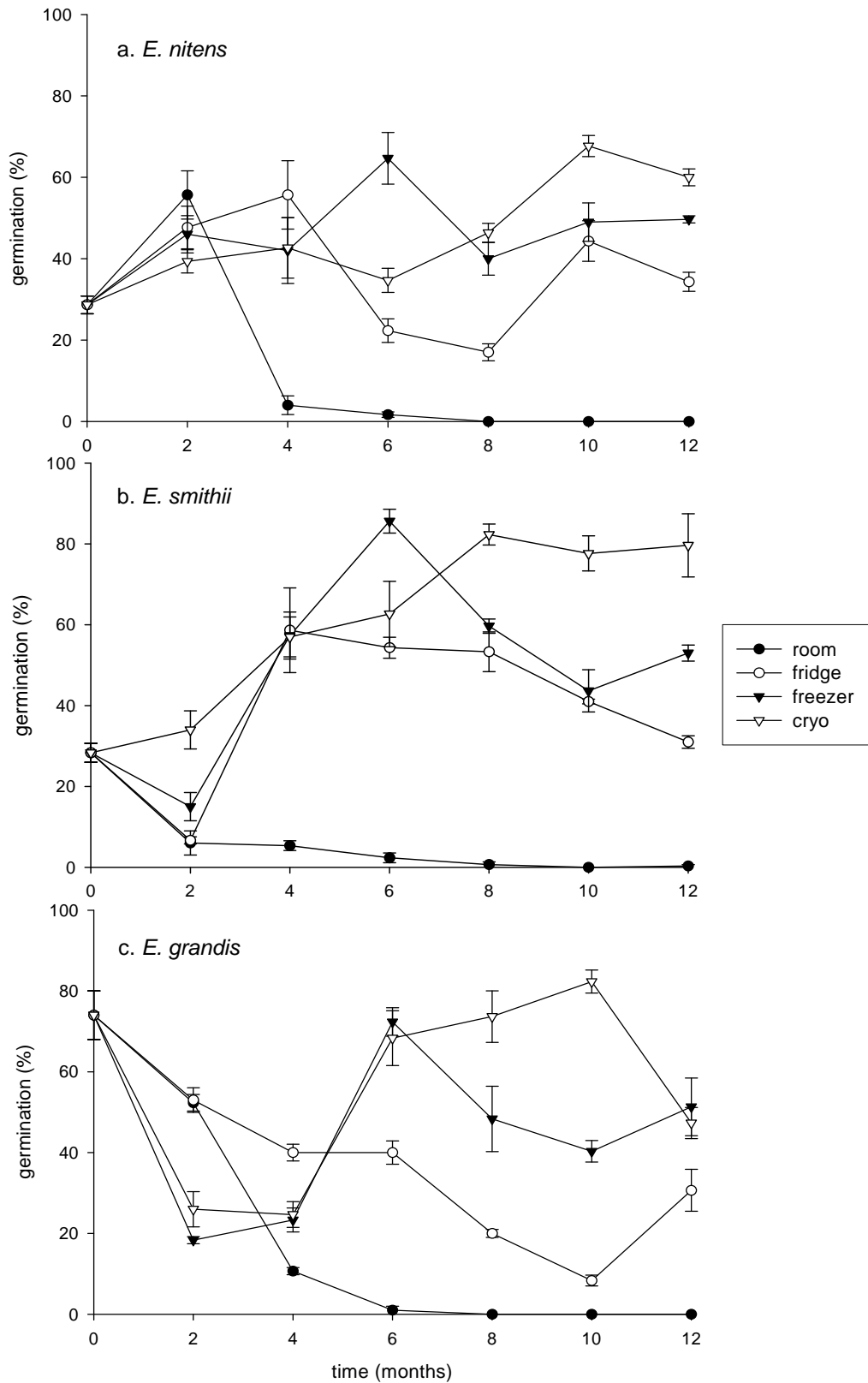


Figure 2.3: Effect of storage temperature over a 12-month period on *in vitro* pollen germination of (a) *Eucalyptus nitens*, (b) *E. smithii* and (c) *E. grandis*. Symbols indicate means \pm s.e.

By 8 months, cryostored pollen from all three species appeared to germinate better than pollen stored at any of the other temperatures (Figure 2.3). On the other hand, germination declined as storage temperature increased, with virtually no germination of room-stored pollen by 8 months. Surprisingly, there was a noticeable reduction in germination of *E. grandis* pollen after 12 months of storage in liquid nitrogen.

2.5 DISCUSSION

A liquid medium consisting of 30% (w/v) sucrose and 0.15 mg l⁻¹ boric acid appeared to be optimal for the *in vitro* germination of both fresh and stored *Eucalyptus* pollen of all species used in the study. However, as the sucrose concentration of the *in vitro* germination medium was decreased, pollen integrity began to deteriorate and grains appeared to burst in 0% (w/v) sucrose (with and without boric acid). Dumont-BeBoux and von Aderkas (1997) also observed this plasmolysis in Douglas-fir (*Pseudotsuga menziesii*) pollen cultured on medium without sucrose. In *Eucalyptus globulus*, *E. morrisbyi*, *E. ovata* and *E. urnigera*, Potts and Marsden-Smedley (1989) found that virtually no pollen germination (<10%) occurred in the absence of sucrose when examining the effect of boric acid (0 – 0.45 mg l⁻¹) and sucrose (0 – 40% w/v) on pollen germination. Similar to the present study, those authors reported 30% (w/v) sucrose to be optimal for maximum *in vitro* pollen germination.

The results clearly indicate the importance of sucrose in the pollen germination medium and Leduc *et al.* (1990) have suggested that sucrose functions as an osmoticum rather than energy source. Osmotic conditions above and below the optimum have been found to reduce pollen germination and pollen-tube lengths (Loguercio 2002). Like in the present study, Loguercio (2002) also found that at low osmotic potential, a high proportion of burst pollen grains were evident and suggested that this was due to the rapid influx of water into the grains, causing excessive leakage of endogenous soluble substances and ions.

The stimulatory effect of boric acid, although not occurring in all species (eg. *Pinus*), is widespread and increasing evidence suggests that boron plays an important role in the growth and development of vascular plants (Stangoulis *et al.* 2001). Although boron seems to be involved in many processes including sugar transport, cell wall synthesis and maintenance, membrane integrity, and RNA metabolism (Loomis and

Durst 1992; Dordas and Brown 2000), its precise role has not yet been elucidated. It is believed to promote pollen germination by affecting H⁺-ATPase activity, which initiates pollen germination and tube growth (Feijo *et al.* 1995; Obermeyer and Blatt 1995). Similar to the present study, Wang *et al.* (2003) found that boron deficiency reduced pollen germination rate, leading to retardation of pollen tube growth. From their study on *Eucalyptus*, Potts and Marsden-Smedley (1989) recommend the inclusion of between 0 – 0.1 mg l⁻¹ boric acid in the *in vitro* germination medium, while Vasil (1964) recommends slightly higher concentrations of 0.1 – 0.15 mg l⁻¹.

Since the success of controlled pollination is often dependent on the quality of stored pollen (Bonnet-Masimbert *et al.* 1998), the second part of the study focused on determining suitable temperature regimes for the short-, medium- and long-term storage of *E. nitens*, *E. smithii* and *E. grandis* pollen. In this part of the study, the optimal *in vitro* medium of 30% (w/v) sucrose and 0.15 mg l⁻¹ boric acid was used to measure the germination of pollen stored at various temperatures over a 12 month period. There was a rapid decline in the germination of pollen that had been stored at room temperature, suggesting that temperatures of around 25°C are not suitable for long-term pollen storage. However, while undertaking operational controlled pollinations within a season, storage of pollen at room temperature, or even at 4°C, is feasible over short periods of up to two months, thereby saving processing time. In the present study, temperatures cooler than 4°C appeared to maintain pollen viability for the duration of the 12-month study.

An interesting phenomenon of increased germination in the first months of storage was observed in *E. nitens* and *E. smithii* samples (Figure 3). The same phenomenon was observed by Lanteri *et al.* (1993), who stored *Picea abies*, *Pinus nigra*, *P. pinea*, *P. strobus*, *P. sylvestris* and *P. uncinata* pollen at –18°C and –196°C for 24 months, and tested for viability every 2 months. They found an increase in pollen germination during the first months of storage at –196°C in *Picea abies*, *P. sylvestris* and *P. uncinata*. The authors suggested that the increase in germination could be due to the freezing process, which causes the release of some necessary nutrients into the medium. Since the increased germination in the present study was also observed in pollen stored at room temperature, in this case it is suggested to either be

attributable to after-ripening processes occurring in the pollen grains after shedding, or perhaps random variation in media quality and testing conditions.

The drastic reduction in germination of *E. grandis* pollen after 12 months of storage at cryogenic temperatures could be due to a different person handling the final *E. grandis* sample (due to logistical problems); there may have been too long a time-lapse between removing the sample from liquid nitrogen and placing it in the freezer, and the consequent rapid thawing could have resulted in damage to the pollen grains. This was corroborated by the appearance of burst pollen grains and abnormal pollen tubes.

The present results are similar to those obtained by Boden (1958) on *E. maculosa*, in which pollen storage was carried out in a deep freeze (-16°C), refrigerator (2°C) and room temperature and germination tested monthly. The authors reported that after 1 month there was no germination in room-stored pollen and the germination of pollen subjected to deep freeze and refrigerator treatments had fallen by 20%. After 3 months storage, pollen stored in the refrigerator had begun to lose viability and by 6 months very few grains were capable of germination. On the other hand, pollen stored in the deep freeze maintained its germination percentage after the initial fall-off in the first month and after 7 months was still approximately 60%. Craddock *et al.* (2000) also arrived at the conclusion that storage temperature has a significant effect on pollen germination, after studies performed on *Cornus florida* revealed that pollen stored at -196°C and at -20°C germinated significantly better than that stored at 5°C .

The gradual freezing of pollen by placement in a freezer for 8 hours before direct immersion in liquid nitrogen, as well as gradual thawing by again placing in a freezer for 8 hours before being left at room temperature overnight, appeared to be favourable for the cryopreservation of eucalypt pollen in the present study. This was suggested by the relatively high *in vitro* germination sustained by cryostored pollen for the duration of storage. Water content is possibly the principle factor to consider in preparation for cryostorage, as it controls the amount of ice crystal formation within the sample. Both the rate of water loss and the water content to which the material is dried are critically important (Pammenter *et al.* 1999). Slow freezing dehydrates the pollen by drawing water to extracellular centres of ice nucleation (Benson 1994).

Consequently, when the material is placed in liquid nitrogen, little or no lethal ice crystal damage should occur.

Although untested, it would appear that cryoprotectants are not necessary for the cryostorage of the *Eucalyptus* species' pollen used in the present study. This is inferred from the relatively good germination and integrity of pollen grains after retrieval from liquid nitrogen. Cryoprotectants are a heterogeneous group of compounds which depress both the freezing and supercooling points of pure water, thereby reducing the volume of water in the cells available to freeze (Finkle *et al.* 1985). Certain cryoprotectants can, however, be toxic to plant tissues (e.g. dimethylsulfoxide), the extent of toxicity varying with the type and concentration of cryoprotectant and plant species (Grout 1995). It is therefore sometimes necessary to remove the cryoprotectant after retrieval from the frozen state, making pollen germination estimates difficult.

2.6 CONCLUSION

Of the combinations tested, an optimal *in vitro* germination medium was found to be 30% (w/v) sucrose and 0.15 mg l⁻¹ boric acid. This medium could be used to test the viability of both fresh and stored pollen of the species used in the present investigation, thereby allowing generalised procedures for pollen viability testing. Although not quantified, boric acid was visually found to stimulate the rate of pollen tube growth. General recommendations for pollen storage are that storage at -10°C can be useful in *Eucalyptus* breeding programmes where the pollen of the male parent must be stored from one season to another. On the other hand, the more elaborate procedure of storage in liquid nitrogen would be advantageous for the preservation of male germplasm in gene banks. Storage at room temperature and at 4°C is only acceptable if the pollen will be utilised within 1 to 2 months.

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CHAPTER 3: IS *EUCALYPTUS* CRYPTICALLY SELF-INCOMPATIBLE?

3.1 ABSTRACT

The probability that seeds will be fertilised from self versus cross pollen depends strongly on whether plants have self-incompatibility systems, and how these systems influence the fate of pollen tubes. In this study of breeding systems in *Eucalyptus urophylla* and *Eucalyptus grandis*, epifluorescence microscopy was used to study pollen tube growth in styles following self- and cross-pollinations. Pollen tubes from self-pollen took significantly longer than those from cross-pollen to grow to the base of the style in both *E. urophylla* (120 h vs 96 h) and *E. grandis* (96 h vs 72 h). In addition, both species exhibited reduced seed yields following self-pollination compared to cross-pollination. The present observations suggest that, in addition to a late-acting self-incompatibility barrier, cryptic self-incompatibility could be a mechanism responsible for the preferential outcrossing system in these two eucalypt species.

3.2 INTRODUCTION

To develop sound breeding strategies it is necessary to understand both the reproductive biology and breeding system of a species (Eldridge and Griffin, 1983). The breeding system includes, in its broadest sense, all aspects of sex expression which affect the relative genetic contribution to the next generation of individuals (Wyatt, 1983). Thus an understanding of the breeding system, and the variables that influence it, is essential for a thorough understanding of the ecology, dynamics and long-term viability of populations, not to mention the opportunity for commercial exploitation of the species (Ellis and Sedgley, 1992).

Breeding systems of different *Eucalyptus* species have been investigated using a variety of methods, including isozyme analysis (Moran and Brown, 1980; Yeh *et al.*, 1983; Fripp *et al.* 1986) and controlled pollinations (Potts and Savva 1988). However, information on comparative growth rates of self- and cross-pollen in the eucalypt pistil is still lacking. Pollen-pistil interactions have been studied in just eight species to date, viz. *Eucalyptus morrisbyi* (Potts and Savva, 1988), *E. regnans* (Sedgley *et al.*, 1989), *E. woodwardii* (Sedgley, 1989; Sedgley and Smith, 1989), *E.*

spathulata, *E. cladocalyx*, *E. leptophylla* (Ellis and Sedgley, 1992), *E. globulus* (Pound *et al.*, 2002) and *E. nitens* (Pound *et al.*, 2003).

Eucalyptus is considered to have a breeding system that is preferentially outcrossing, although selfing is not uncommon (Griffin *et al.*, 1987; Eldridge *et al.*, 1994). High outcrossing rates, of between 0.69 and 0.84, have been found in the genus (Moran and Bell, 1983) and are aided by protandry (Pryor, 1976) and reinforced by selection against the products of self-fertilisation in later stages of the life cycle (Potts *et al.*, 1987). Most species exhibit a marked reduction in seed yield following self-pollination compared to outcrossing (Potts and Savva, 1988; Ellis and Sedgley, 1992). In fact, there have been reports of more than one self-incompatibility (SI) mechanism operating in a species, which may act at both the pre- and post-zygotic levels (Sedgley and Griffin, 1989). The situation is further complicated by the fact that even in the natural situation, few species are completely selfing or outcrossing, and there are many reports of partial or variable self-incompatibility (Sedgley *et al.*, 1990).

One possible explanation for the variability in outcrossing rates in *Eucalyptus* is the existence of a system of cryptic self-incompatibility. Cryptic SI usually acts at the stage of pollen tube elongation in the style and leads to faster elongation of cross pollen tubes relative to self pollen tubes (Bateman, 1956). As opposed to complete or absolute self-incompatibility, self-pollination without the presence of competing cross pollen in plants with cryptic SI results in successful fertilisation and seed set (Bateman, 1956). To date, this type of self-incompatibility has never been associated with *Eucalyptus*.

The aim of the present study was to examine the breeding systems of *Eucalyptus urophylla* and *E. grandis*, by using epifluorescence microscopy to study pollen tube growth in the style following controlled self- and cross-pollinations.

3.3 MATERIAL AND METHODS

3.3.1 Plant material used in study

The experiments were conducted on mature trees located in clonal (grafted) orchards planted at the Sappi, Shaw Research Centre in KwaZulu-Natal, South Africa. The

orchards were situated at 29° 29'S, 30° 11'E at 1100 m above sea level. Breeding populations for all species were made up of open-pollinated families from selections made in the land-race in South Africa and from provenances in the natural range in Australia. Trees were chosen on the basis of floral abundance and accessibility for hand-pollinations. All trees had previously produced seed crops for several years. Two genotypes from each species, viz. M1401 and M1413 from *E. urophylla*, and P1362 and P1369 from *E. grandis*, were used as maternal parents in the study. Pollen was also collected from these genotypes for use in self- and intraspecific cross-pollinations.

3.3.2 *Pollen collection and processing*

For pollen extraction, branches containing ripe flower buds were collected and kept in 100 ml bottles containing water to prevent drying out of the branch. To ensure that there was no contamination from other pollen, all open flowers were removed from the branches before placing them in the laboratory overnight. When the operculum had shed and the filaments unfolded, the anthers were excised and left in a desiccator in the presence of silica gel to dry for approximately 48 h at room temperature. When the relative humidity (RH) in the desiccator had reached 10%, the dried anthers were sieved through a 30 micron mesh to remove debris. The resulting pollen was placed into polypropylene vials, sealed in glass bottles containing silica gel and stored in a freezer at -10°C until needed.

3.3.3 *In vitro pollen germination*

Pollen viability was tested under laboratory conditions before use in controlled pollinations. Pollen was left at room temperature and RH for 8 hours to rehydrate. *In vitro* germination was carried out using 30% (w/v) sucrose, supplemented with 0.15 mg l⁻¹ boric acid in a liquid medium (Horsley *et al.*, 2007). Pollen from each genotype was placed into glass vials containing the *in vitro* medium (three replications per genotype) and left to incubate in a germination chamber in a completely randomised design for 48 hours at 29°C. After the required time period had elapsed, 5 ul was transferred from the test-tube to a glass slide. Percent germination was scored using a light microscope (x100 magnification) to count the number of pollen grains germinated out of a total of 50 grains. Six glass slides per genotype (two slides per test tube) were scored for germination (sub-samples), giving a total of 300 pollen

grains counted per treatment. Pollen was deemed to have germinated if the pollen tube length was greater than one-half of the diameter of the pollen grain (Potts and Marsden-Smedley, 1989).

3.3.4 *Controlled pollination*

Pollinations were carried out using two ramets per genotype of each species. The number of flowers suitable for pollination (in terms of accessibility) determined the number of pistil samples that could be fixed for microscopic analysis. This consequently led to differences in sampling time between the different species. Ripe flower buds were emasculated and isolated at anthesis. Each isolation bag enclosed three umbels, with seven flowers per umbel. Treatments were separately isolated, with only one treatment occurring in an isolation bag. Pollen was applied seven days later, when the stigmas were receptive, and then re-isolated. For *E. urophylla*, 1600 flowers were pollinated. Pistil samples were then taken at 24, 30, 48, 96, 120, 144 and 216 hours after pollination and immediately fixed in formalin-acetic acid-alcohol (FAA) solution. For *E. grandis*, 400 flowers were pollinated and pistil samples taken at 24, 48, 72 and 96 hours after pollination. Controls consisted of flowers that were not hand-pollinated. Fixed samples of both species were stored at room temperature until needed. Capsules were also left on the tree for estimation of seed yield.

3.3.5 *Seed set*

All capsules remaining at maturity (12 months after pollination) 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 viable if they were rounded, solid and dark in colour as opposed to flat and possessing a light-brown colour (Pound *et al.*, 2002). Seed-set data were used to determine the level of self-incompatibility in each species from the following formula:

$$ISI = [(V_c - V_s) / V_c] \times 100$$

where ISI = index of self-incompatibility, V_c = viable seed per flower cross-pollinated and V_s = viable seed per flower self-pollinated (Pound *et al.*, 2002).

3.3.6 *Sample preparation for epifluorescence microscopy*

For each species, eight pistils from each treatment and time interval were studied. Fixative was removed from the pistil samples by rinsing with tap water. The styles

were then excised from the buds and left to soften in 4N NaOH for either 48 h (*E. grandis*) or 72 h (*E. urophylla*). After the required time period had elapsed, NaOH was replaced with tap water and samples left for 60 min to rinse. Samples were then placed in analine blue-0.1N K₃PO₄ to stain overnight. The next day, samples were mounted with a drop of glycerol onto glass slides and viewed under UV light. Pollen tubes were studied at five levels in the pistil, viz. stigma surface (0% style penetration), upper style (25% style penetration), middle style (50% style penetration), lower style (75% style penetration) and base of style (100% style penetration). The data were summarised as the number of samples per treatment in which pollen tubes had successfully penetrated to the five different regions of the pistil.

3.3.7 Statistical Analysis

To test for differences in self- and cross-pollen tube growth in the pistil, Analysis of Covariance using SPSS Version 13.0 was used to establish the statistical significance of observed differences between treatments within each species, with “number of samples with pollen tubes at the base of the style” as the dependent variable and time as a covariate. A count of pollen tubes at various regions of the style was not possible as pollen tubes were too close to each other to be identified individually. As it was common for more than one capsule to be harvested from within a pollination bag, the mean number of seeds set per flower pollinated was calculated for each bag, and these values were analysed using T-tests. Observed differences between the percentage of capsules set after self- vs cross-pollinations were compared using G-tests. Pollen viability data were angular transformed prior to Analysis of Variance and Tukey Tests.

3.4 RESULTS

3.4.1 In vitro pollen germination

Significant genotypic differences with respect to *in vitro* pollen tube growth were displayed by both species. Of all the genotypes, *E. grandis* P1369 had the highest pollen germination ($76 \pm 2.52\%$), while *E. grandis* P1362 had the lowest ($19 \pm 2.96\%$). For *E. urophylla*, genotype M1413 pollen had higher *in vitro* germination (69 ± 3.18) than M1401 pollen (35 ± 0.33).

3.4.2 Seed set following controlled pollinations

Capsule retention following cross-pollination was greater than that following self-pollination in *E. urophylla* (64 vs 37% capsule set), while the opposite occurred in *E. grandis* (6 vs 11% capsule set). However, in both species cross-pollination produced more seeds per flower pollinated compared to self-pollination (Table 3.1). These differences in average number of seeds per flower were significant in *E. urophylla*. However, seed yields for *E. grandis* could not be analysed statistically due to insufficient replication of individual plants. Of the two species, *E. urophylla* was slightly more self-incompatible (62.5%) compared to *E. grandis* (46%).

Table 3.1: Mean number of seeds per flower following self- and cross-pollination in *Eucalyptus urophylla* and *E. grandis*, and calculated levels of self-incompatibility. SP self-pollinated, CP cross-pollinated, N number of replicates, ISI index of self-incompatibility, * $P < 0.05$.

Species	Flowers pollinated			% Capsules set		Sig.	Mean seed set per flower		Sig.	ISI (%)
	SP	CP	N	SP	CP		SP	CP		
<i>E. urophylla</i>	96	75	6	37	64	*	3.1	8.3	*	62.5
<i>E. grandis</i>	37	28	3	11	6	*	10.7	19.8	-	46.0

- *E. grandis* seed set could not be statistically analysed due to insufficient data

3.4.3 In vivo pollen tube growth

Significant differences in the growth rate of self- and cross pollen tubes were observed in *Eucalyptus urophylla* (Table 3.2), with self pollen tubes taking approximately 120 h to penetrate 100% of the style, compared to 96 h taken by cross pollen tubes (Figure 3.1a). Self pollen tubes of *E. grandis* also showed a slower rate of growth in the style (Table 3.3), taking 96 h to reach 100% of the style, compared to the 72 h taken by cross pollen tubes (Figure 3.1b). In addition to the reduced rate of growth, observed pollen tube abnormalities (such as twisting) were increased following selfing in both species (Figure 3.2a), although this was not quantified. The majority of the controls exhibited pollen grains germinating on the stigma surface, even though these buds had not been hand-pollinated.

Table 3.2: Analysis of covariance of *in vivo* pollen-tube growth after controlled self- and cross-pollinations in *Eucalyptus urophylla*.

Source of variation	d.f.	Mean square	F-value	P-value
treatment	1	1811.07	8.80	0.003
genotype	1	151.26	0.74	0.392
time	1	112915.67	548.70	0.000
treatment x genotype	1	396.81	1.93	0.166
treatment x time	1	924.17	4.49	0.035
genotype x time	1	74561.95	362.33	0.000
treatment x genotype x time	1	0.03	0.00	0.990
residual	381	205.79		

$R^2 = 0.794$ (adjusted $R^2 = 0.790$)

Table 3.3: Analysis of covariance of *in vivo* pollen-tube growth after controlled self- and cross-pollinations in *Eucalyptus grandis*.

Source of variation	d.f.	Mean square	F-value	P-value
treatment	1	1589.34	7.32	0.008
genotype	1	503.11	2.32	0.130
time	1	205555.60	947.21	0.000
treatment x genotype	1	184.47	0.85	0.358
treatment x time	1	1017.01	4.69	0.032
genotype x time	1	1418.31	6.54	0.012
treatment x genotype x time	1	134.67	0.62	0.432
residual	149	217.01		

$R^2 = 0.875$ (adjusted $R^2 = 0.869$)

3.5 DISCUSSION

For the species in the present study, no evidence of self-incompatibility was found at the stage of pollen adhesion and germination in the stigmatic exudate. The expression of SI occurred as pollen tubes grew down the style and resulted in a reduction in the pollen tube growth rate following self-pollinations, relative to those following cross-pollinations. This self-pollen tube growth retardation is suggested to be a form of cryptic SI and is in agreement with many reports of SI in the Solanaceae (McGuire and Rick, 1954; Hardon, 1967; Ascher, 1976). While this phenomenon has been investigated in several wild plant species (Bateman, 1956; Waser *et al.*, 1987;

Aizen *et al.*, 1990), no evidence of selective stylar inhibition of pollen tube growth has been presented in any of the eucalypt species studied to date. To the authors' knowledge, this is the first study to suggest cryptic SI in *Eucalyptus*.

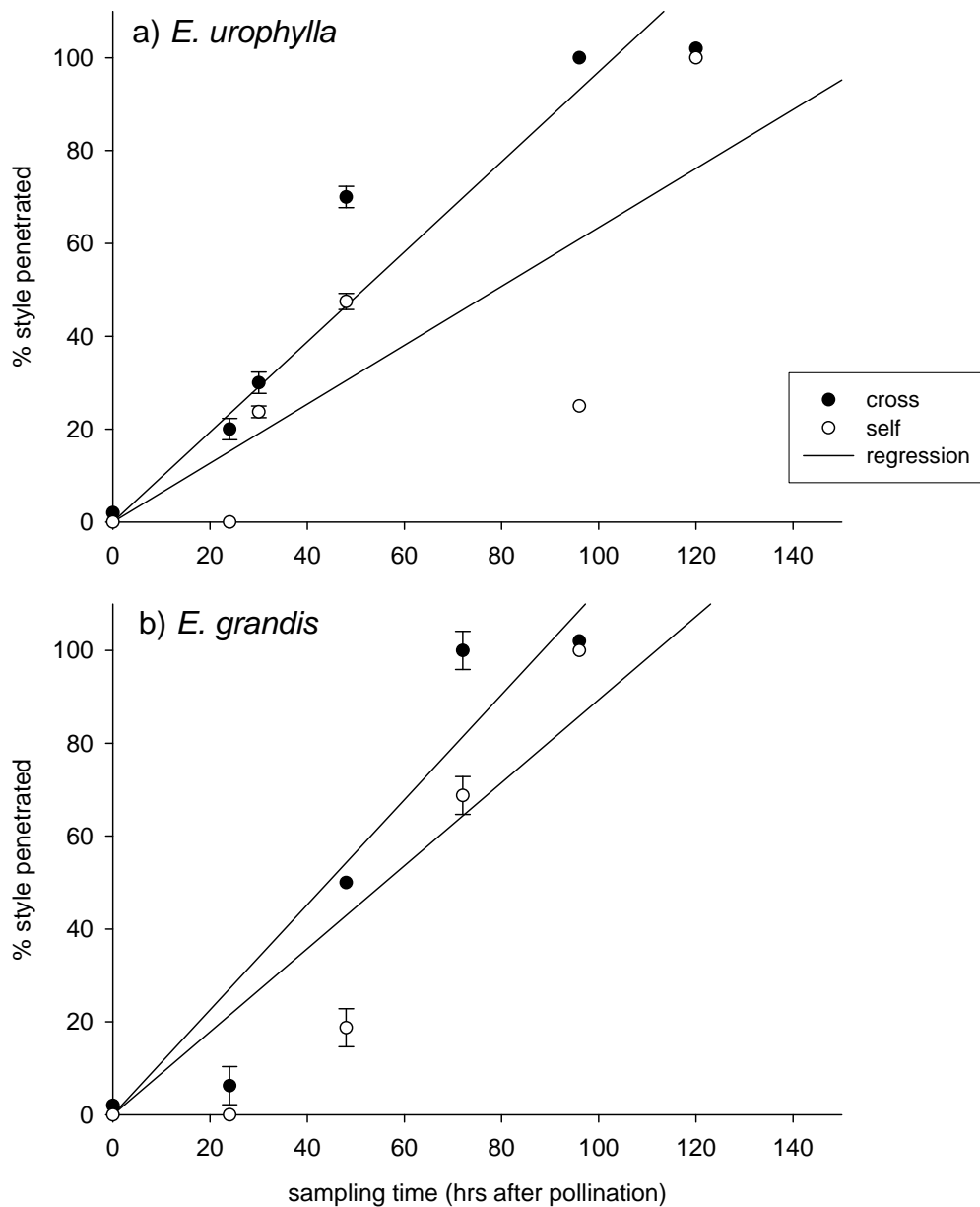


Figure 3.1: Comparison of *in vivo* pollen tube growth after controlled self- and cross-pollinations in (a) *Eucalyptus urophylla* and (b) *Eucalyptus grandis*. Cross-pollinations were performed within each species (intraspecific).

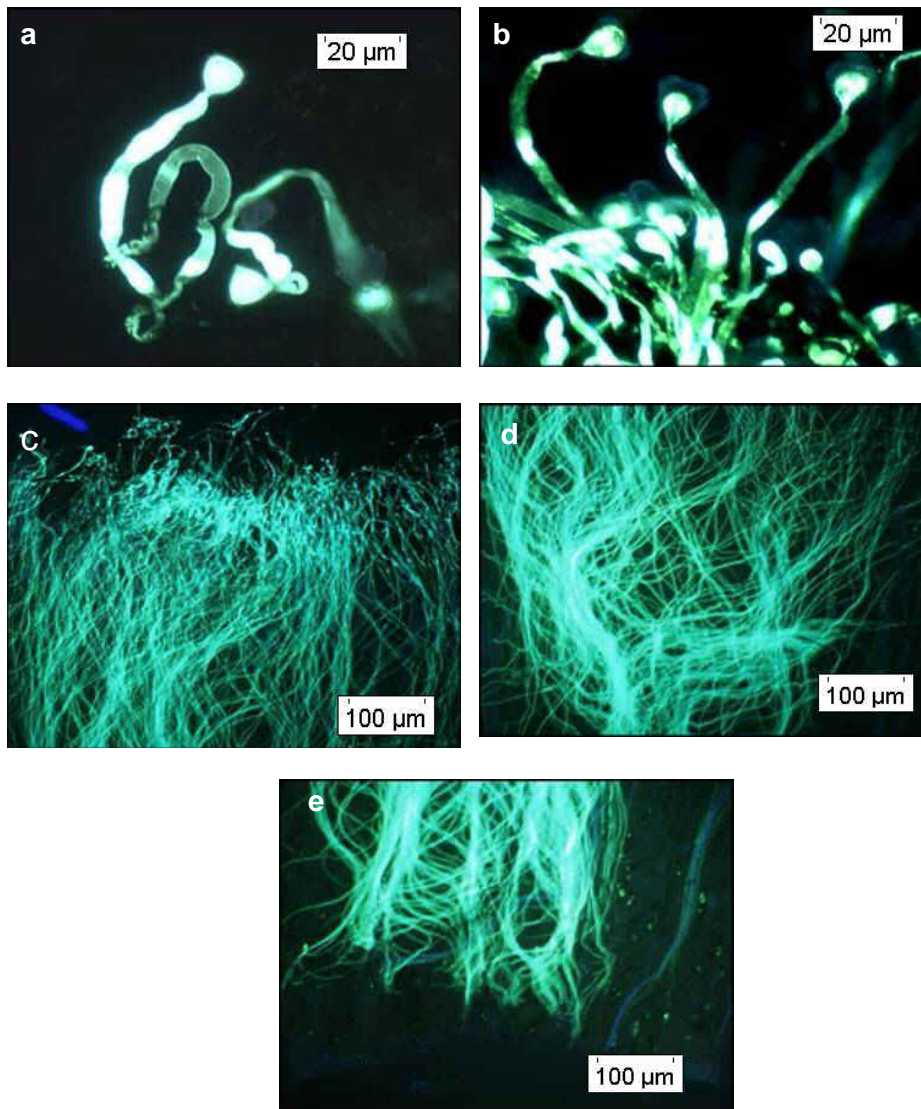


Figure 3.2: Fluorescence micrographs of squashed *Eucalyptus* styles harvested after different pollination treatments and stained with aniline blue. (a) Twisted pollen tubes after self-pollination in *E. urophylla*; (b) Straight pollen tubes after cross-pollination in *E. urophylla*; (c), (d) & (e) Pollen tube pathway in an *E. grandis* cross 72 h after pollination where (c) shows pollen grains germinating on the stigma, (d) shows pollen tubes growing in the middle style and (e) shows pollen tubes at the cut end of the style (at the style base).

Direct measurement of pollen tube growth in *Amsinckia grandiflora* (Weller and Ornduff, 1989), *Erythronium grandiflorum* (Cruzan, 1989), and *Delphinium nelsonii* (Waser *et al.*, 1987) have also shown differences in pollen tube growth rate between self and cross-pollen. As appears to be the case for *E. urophylla* and *E. grandis*, these species can be considered cryptically self-incompatible, because growth of incompatible pollen tubes was slower than that of compatible ones, rather than being

completely inhibited. Recent studies have shown that there may be more plasticity in the growth of self-pollen tubes than has previously been appreciated (Stephenson *et al.*, 2003; Travers *et al.*, 2004). In the study by Stephenson *et al.* (2003), self-pollen tube growth in *Solanum carolinense* was arrested when cross-pollen was available, but when cross-pollen was scarce, the growth of self-pollen tubes (and hence the strength of SI) became a quantitative trait that varied among individuals. Stephenson *et al.* (2003) and Travers *et al.* (2004) have subsequently suggested that the plasticity in SI systems be viewed as a mechanism that promotes outcrossing by modulating the intensity with which it handicaps the growth of self pollen.

Recorded effects of selfing in eucalypts include reduced seed set (Potts and Savva, 1988; Sedgley and Smith, 1989; Tibbits, 1989), decreased germination percentage (Eldridge and Griffin, 1983), increased frequency of abnormal phenotypes (Potts *et al.*, 1987), depressed field growth and decreased nursery and field survival (van Wyk, 1981; Eldridge and Griffin, 1983; Potts *et al.*, 1987). In the present study, in addition to the reduced self-pollen tube growth rates in *E. urophylla* and *E. grandis*, there was a reduction in seed yields following self-pollinations, as well as an observed increase (not quantified) in the number of pollen-tube abnormalities at various locations in the pistil and at different times after self-pollination (Figure 3.2a). Similar pollen-tube abnormalities have been reported in *Rhododendron* L., a woody plant genus that shows some similarities with *Eucalyptus*, including a wet stigma and mixed mating system (Williams *et al.*, 1982).

Eucalyptus grandis exhibited particularly low fruit set overall, retaining only 6 – 11% of the pollinated capsules, compared to 37 – 64% capsule retention in *E. urophylla*. This is similar to levels reported in *E. regnans*, where an average fruit set of only 9% was reported by Sedgley *et al.* (1989). The reason for the differences in capsule retention between *E. grandis* and *E. urophylla* is unclear. Both species had near-identical floral morphology and experienced similar temperatures during the course of the experiment (both were pollinated in summer). As in *E. woodwardii* (Sedgley and Smith, 1989), it is possible that some *E. grandis* flowers started to shed their opercula prematurely and so were pollinated at an immature stage.

Late-acting self-incompatibility appears to be an additional SI mechanism operating in *E. urophylla* and *E. grandis* on account of the low number of seeds set following self-pollination relative to the number of self-pollen tubes in the style (pollen tubes were so abundant that they could not be quantified). Pound *et al.* (2003) came to the same conclusion in their study on *E. nitens*, where, like in the present study, seed yields were reduced following self-pollinations even though both self- and cross-pollen tubes had grown down the style. Since most self-fertilised *E. nitens* ovules had begun to degenerate within the first few weeks following pollination instead of being spread over the entire seed development time, Pound *et al.* (2003) suggested that ovule breakdown was a self-incompatibility response. At present it is difficult to experimentally determine which system is operating within a species. Seavey and Bawa (1986) suggest that 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. However, Waser and Price (1991) question whether inbreeding depression could account for very high levels of ovule abortion.

The observed pollen grains on the stigma of most control treatments emphasises the importance of isolating the flower after hand-pollinations. There are several possible sources of contamination during the controlled pollination process, one of these being the presence of pollen on the anthers of mature buds, which could result in self-pollination during the emasculation process (Hodgson, 1975). Another source of self-pollen could be from flowers higher up in the canopy of the tree, with contamination of lower flowers through the action of gravity and wind movement (Eldridge and Griffin, 1983).

A potential drawback of the present study is that single-donor pollinations, as opposed to mixed-pollinations, were used to study differences in pollen tube growth rate. The reason for utilising single-donor pollinations was to avoid the difficulty in distinguishing respective self- and cross-pollen tubes in the style after mixed-pollinations. The sources of pollen included in mixtures could additionally confound breeding system observations, where differences in pollen tube growth rate could be due to differing pollen viability and pollen-pollen interactions (Waser *et al.*, 1987). In a follow-up study (Chapter 4), microsatellite markers were used to distinguish the

contribution of self- and cross-pollen to seed set after both single- and mixed-donor pollinations were performed on *Eucalyptus grandis*.

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CHAPTER 4: RELATIVE SUCCESS OF SELF AND OUTCROSS POLLEN AFTER MIXED- AND SINGLE-DONOR POLLINATIONS IN *EUCALYPTUS GRANDIS*

4.1 ABSTRACT

A previous observation that self-pollen tubes traversed the style at a slower rate than cross-pollen tubes in *Eucalyptus grandis* and *E. urophylla*, suggested the presence of cryptic self-incompatibility (CSI) in these species. The aim of the present study was, with the help of molecular markers, to examine the siring ability of self- and cross-pollen in *E. grandis*, after both mixed- and single-donor pollinations, in order to confirm the presence of CSI. Single-donor cross-pollinations set a significantly higher number of seeds per flower pollinated compared to those performed with self-pollen, while there were no significant differences between the open control and single-donor self-pollinations. Molecular markers revealed that 100% of the progeny from mixed-donor pollinations were outcrossed, confirming the competitive advantage of cross-pollen. In addition, there was a significant change in the self:outcross seed ratio between single- and mixed-donor pollinations, suggesting that the observed deficit of selfed seeds in mixed-donor fruits could be the result of differential pollen tube growth. From the extremely low seed yields following single-donor self-pollinations, it is clear that an additional incompatibility mechanism is operating in *E. grandis*, and this is suggested to be late-acting self-incompatibility, acting before fertilisation.

4.2 INTRODUCTION

Many plants have mechanisms to avoid deposition of self-pollen, such as the separation of male and female functions in time and space. However, as pollen deposition in animal-pollinated species, such as *Eucalyptus*, depends on the activities of the pollinators, which in turn depend on variable factors, such as the weather, the actual proportion of self- vs outcross-pollen on stigmas is quite unpredictable (McCall and Primack 1992). Also, paternity success often does not reflect the composition of pollen loads, as maternal plants may exert some control over which pollen grains successfully fertilise ovules and/or which zygotes are matured into viable seeds (Marshall and Folsom 1992). In order to compare the post-pollination performance of pollen from different individuals, hand pollinations are frequently made using pollen from different donors to pollinate different flowers on

one or more recipient plants. This technique has the advantage that resultant seeds are readily assigned to particular donors (Bertin 1990). However, the above assessments of post-pollination male performance will be erroneous if paternal success in mixed-pollen loads differs from that in single-donor loads.

Single-donor pollen loads are undoubtedly rare on plant stigmas in nature. Furthermore, during natural pollination it is likely that the fraction of pollen on a stigma contributed by a particular donor will vary greatly in time and space (Bertin 1990). In the absence of data, it is usually assumed that a plant's male success is directly related to pollen production or pollen export (Lloyd 1984). This assumption requires that pollen is deposited on stigmas in proportion to its availability on or removal from anthers, and that its success in siring offspring is proportional to its abundance on a stigma. However, several studies have found that mixtures of self- and outcross-pollen yield a lower proportion of selfed offspring than expected based on the proportion of self-pollen on stigmas (Bateman 1956; Weller and Ornduff 1977). This selective filtering of pollen or zygotes can occur in a variety of forms, including the rejection of self-pollen or selfed ovules due to self-incompatibility (de Nettancourt 1997; Seavey and Bawa 1986), cryptic self-incompatibility caused by differential pollen tube growth (Bateman 1956), differential provisioning of outcrossed versus selfed embryos or fruits (Rigney 1995), and death of selfed embryos expressing lethal recessive alleles (Husband and Schemske 1996). It is often difficult to identify the actual mechanism of post-pollination selection. For example, some forms of late-acting self-incompatibility are difficult to distinguish from early-acting inbreeding depression (Seavey and Bawa 1986) and many post-pollination mechanisms can be environmentally and developmentally plastic (Goodwillie *et al.* 2004).

In mass-flowering species in the genus *Eucalyptus*, the ranges in time of anthesis within inflorescences and within branches ensure high levels of geitonogamous self-pollination, even though individual flowers are protandrous (Griffin 1980). Estimates of the outcrossing rate, however, have been found to be consistently high in these species ($t_m = 0.79 - 0.96$), suggesting an outbreeding mechanism operating between the stages of pollination and seed maturation (Moran and Bell 1983; Moran 1992). Horsley and Johnson (2007) observed that self-pollen tubes traversed the style at a

slower rate compared to cross-pollen tubes in *E. grandis* and *E. urophylla*, and therefore suggested the presence of cryptic self-incompatibility (CSI) in these species. In species with CSI, self-pollination without the presence of cross pollen results in successful fertilisation and seed set (Bateman 1956). The advantage of CSI, according to Bateman (1956), is that it can enforce outcrossing when foreign pollen is available, yet still allow for seed-set should foreign pollen not arrive at the stigma.

There are two principle types of evidence for CSI, both of which are usually required for understanding its potential impact on mating patterns in natural populations. First, the siring success of self- and cross-pollen can be compared after mixed pollinations using marker genes (Eckert and Allen 1997). This approach can demonstrate siring differences among pollen types but usually provides little information on the mechanism causing the observed siring differences. The second line of evidence for CSI comes from direct observations of pollen tube growth, and although this approach can identify possible mechanisms through which cross-pollen might outcompete self-pollen, it does not provide direct evidence for siring differences (Montalvo 1992). Despite the complementarity of these two methods for detecting CSI, most investigations have used only one or the other approach. Both types of data are available for only a handful of taxa, viz. *Amsinckia grandiflora* (Weller and Ornduff 1989), *Erythronium grandiflorum* (Rigney 1995), *Hibiscus moscheutos* (Snow and Spira 1991), *Aquilegia caerulea* (Montalvo 1992) and *Eichhornia paniculata* (Cruzan and Barrett 1993).

The aim of the present study was, with the help of molecular markers, to examine the siring ability of self- and cross-pollen after both mixed- and single-donor pollinations in *E. grandis*, in order to confirm the presence of CSI in this eucalypt species.

4.3 MATERIAL AND METHODS

4.3.1 Plant material used in study

Conventional controlled pollinations were conducted on a mature *E. grandis* tree located in a clonal (grafted) orchard planted at the Sappi, Shaw Research Centre in KwaZulu-Natal, South Africa. The orchard was situated at 29° 29'S, 30° 11'E at 1100 m above sea level. The breeding population for this species was made up of open-

pollinated families from selections made in land-races in South Africa and from provenances in the natural range in Australia. The maternal tree was chosen on the basis of floral abundance and accessibility for hand-pollinations. To take into account different micro-climates, replications were evenly distributed around the tree (viz. north, south, east and west).

4.3.2 *Pollen collection and processing*

For pollen extraction, branches containing ripe flower buds were collected and kept in 100 ml bottles containing water to prevent drying out of the branch. To ensure that there was no contamination from other pollen, all open flowers were removed from the branches before placing them in the laboratory overnight. The following morning, when the opercula of unopened flowers had shed and the filaments unfolded, the anthers were excised and left in a desiccator in the presence of silica gel to dry for approximately 48 h at room temperature. When the relative humidity (RH) in the desiccator had reached 10%, the dried anthers were sieved through a 30 micron mesh to remove debris. The resulting pollen was placed into polypropylene vials, sealed in glass bottles containing silica gel and stored in a freezer at -10°C until needed. For the mixed-donor pollen treatment, pollen from self- and outcross-genotypes were mixed according to their pollen viability to give a representative pollenlot.

4.3.3. *In vitro pollen germination*

Pollen viability was tested under laboratory conditions before use in controlled pollinations. Pollen was left at room temperature and RH for 8 hours to rehydrate. *In vitro* germination was carried out using 30% (w/v) sucrose, supplemented with 0.15 mg l⁻¹ boric acid in a liquid medium (Horsley *et al.* 2007). Pollen from each genotype was placed into glass vials containing the *in vitro* medium (three replications per genotype) and left to incubate in a germination chamber in a completely randomised design for 48 hours at 29°C. After the required time period had elapsed, 5 µl was transferred from the test-tube to a glass slide. Percent germination was scored using a light microscope (x100 magnification) to count the number of pollen grains germinated out of a total of 50 grains. Six glass slides per genotype (two slides per test tube) were scored for germination (sub-samples), giving a total of 300 pollen grains counted per treatment. Pollen was deemed to have germinated if the pollen

tube length was greater than one-half of the diameter of the pollen grain (Potts and Marsden-Smedley 1989).

4.3.4 Controlled pollination

Conventional controlled pollinations were performed on *E. grandis* flowers, maternal genotype T1144, using one of the following pollen treatments: (1) single-donor self-pollen (paternal genotype T1144), (2) single-donor outcross-pollen (paternal genotype T1087) and (3) mixed-donor self- and outcross-pollen (paternal genotypes T1144 + T1087). On average, 50 flowers were pollinated per treatment (Table 4.1). During controlled pollinations, the operculum was removed from ripe flower buds and the anthers excised to prevent self-pollination. An isolation bag (Quick-dry nappy-liners, manufactured by Unsgaard Packaging Ltd, South Africa) was placed over the emasculated flowers to prevent access by insect pollinators. A week later, when the stigma had ripened, the isolation bag was opened and the relevant pollen applied. The pollinated flowers were then re-isolated. Isolation bags were only removed when the stigma had fully oxidised, which occurred two weeks after pollination. The final step involved collecting ripe capsules 10 months after pollination. Open controls consisted of buds that had not been artificially pollinated nor isolated and were included to give an indication of natural pollination success.

Table 4.1: Mating design showing number of *Eucalyptus grandis* flowers pollinated per treatment. N refers to the number of isolation bags (or replications).

CROSS	POLLEN TREATMENT	FLOWERS POLLINATED	N
T1144 x T1144	Single-donor self	53	5
T1144 x T1087	Single-donor outcross	55	4
T1144 x (T1087 + T1144)	Mix-donor	56	4
T1144 open control	Open control	131	3*

*For the open control, N refers to the number of pollination events (i.e. number of branches containing pollinated flowers). Open-pollinated controls were not manually isolated.

4.3.5 Seed set

All capsules remaining at maturity (10 months after pollination) were harvested and allowed to dry out in the laboratory and release their seed. The number of viable seeds in each capsule were counted. Seeds were considered viable if they were

rounded, solid and dark in colour as opposed to flat and possessing a light-brown colour (Pound *et al.* 2002).

4.3.6 Molecular marker analysis of progeny

Molecular marker analysis was performed on leaf samples from progeny of seed parent T1144 in order to distinguish progeny derived from cross-pollinations to those derived from self-pollinations. DNA was extracted using the Qiagen DNeasy Plant Kit (QIAGEN, Valencia, CA, USA) and samples fingerprinted using the eight markers shown in Table 4.2. Fourteen samples were analysed from the single-donor self-pollinations, 20 samples from the open control and 150 samples from mixed-donor pollinations. Sample sizes were dependent on viable seedlings obtained after germinating seed in the nursery.

Table 4.2: Polymorphic microsatellite markers used to analyse T1144 seed parent and seedlings obtained from controlled pollinations.

MARKER	COLOUR	SIZE RANGE (base pairs)
Embra 28	6-FAM	170 – 230
Eg 126	6-FAM	325 – 350
En 16	VIC	145 – 180
Eg 65	VIC	230 – 280
Embra 168	NED	70 – 80
Embra 27	NED	110 – 150
Embra 186	PET	130 – 190
Embra 227	PET	302 – 320

4.3.7 Statistical analysis

SPSS Version 15.0 was used for all statistical analyses. Pollen viability data were angular transformed prior to Analysis of Variance (ANOVA) and Duncan Multiple Range tests. Percentage capsule retention and seed set per flower were analysed by ANOVA, followed by Duncan Multiple Range tests. As it was common for more than one capsule to be harvested from within a pollination bag, the mean number of seeds set per flower pollinated was calculated for each bag. Percentage capsule retention was angular transformed prior to analysis. A Chi-square Contingency test was performed to compare the proportion of selfs in single-self and mixed-donor pollinations. To determine if mixed-donor results were additive and predictable from

results of single-donor pollinations, a Chi-Square Goodness of Fit test was performed, examining differences between observed and expected proportions of selfed- and outcrossed seeds.

4.4 RESULTS

4.4.1 *In vitro pollen germination*

There were no significant differences in pollen viability between paternal samples used in the controlled pollination experiments ($F = 0.640$; $P = 0.560$), with germination ranging between 61.7 and 64.0% (Table 4.3).

Table 4.3: *In vitro* germination of *Eucalyptus grandis* pollen used in controlled pollinations. There were no statistically significant ($P < 0.05$) differences between treatments.

PATERNAL GENOTYPE	N	MEAN \pm STD ERROR (%)
T1087	3	64.0 \pm 1.7
T1144	3	64.0 \pm 1.5
T1087 + T1144 mix	3	61.7 \pm 1.8

4.4.2 *Controlled pollination*

There were no significant differences for capsule retention ($F = 1.095$; $P = 0.389$), with 95 – 100% of the pollinated buds setting seed. Seed yields, on the other hand, differed significantly between treatments ($F = 4.548$; $P = 0.024$). Single-donor pollinations performed with outcross-pollen set a significantly higher number of seeds per flower pollinated (23.63 ± 8.4) compared to those performed with self-pollen (2.47 ± 0.9), while there were no significant differences between the open control (1.67 ± 0.3) and single-donor self-pollinations (Figure 4.1). Seed yields from mixed-donor pollinations (16.20 ± 5.4 seeds per flower pollinated) were not significantly different from single-donor outcross pollinations.

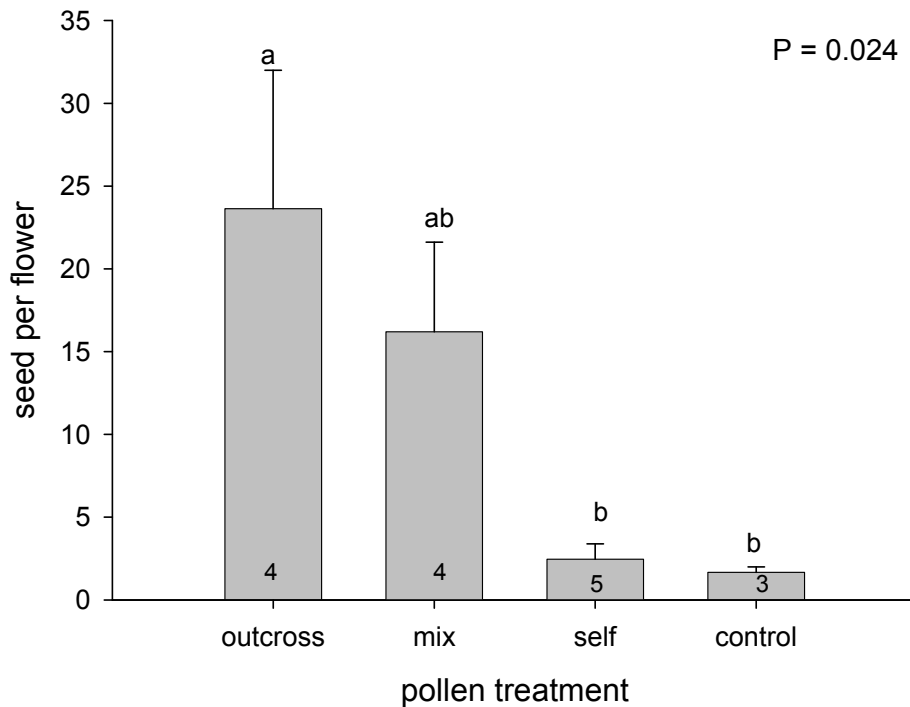


Figure 4.1: Seed per flower after different pollen treatments were used in control-pollinations on *Eucalyptus grandis*. Letters (a) and (b) indicate statistical significance, where treatments indicated by the same letter are not significantly different. Error bars represent standard error of the mean. Sample sizes at the base of each bar refer to number of replications (isolation bags) per treatment.

4.4.3 Molecular marker analysis of progeny

Maternal genotype T1144 was confirmed in all seedlings. For the mixed-donor pollinations, the presence of alleles unique to the outcross-pollen parent T1087 were detected in all progeny, with none from the self-pollen parent T1144 (i.e. progeny were 100% outcrossed). Of the 14 progeny analysed from the single-donor self-pollinations (the total germinants produced from this cross), 8 were confirmed selfs and 6 were outcrossed with paternal parent T1087 (which was a neighbouring tree in the orchard). All 20 open control samples were found to be outcrossed (Table 4.4). Chi-square analysis revealed significant differences between the proportion of selfs in single-self and mixed-donor pollinations [χ^2 (1df) = 55.1; $P < 0.001$]. In addition, the self:outcross ratio of 0:150, obtained from molecular analysis of progeny from the mixed-donor pollinations, was found to differ significantly from the expected self:outcross ratio of 120:1208 generated from single-donor seed yields [χ^2 (1df) = 13.566; $P < 0.001$].

Table 4.4: Molecular marker analysis of *Eucalyptus grandis* progeny after mixed- and single-donor pollinations were performed on seed parent T1144.

POLLEN TREATMENT	N	PATERNAL CONTRIBUTION TO PROGENY (%)		
		T1144	T1087	Foreign
Mixed-donor	150	0	100	0
Single-donor self	14	57	43	0
Open control	20	0	0	100

4.5 DISCUSSION

A previous study (Chapter 3) showed that self-pollen tube growth in *E. grandis* and *E. urophylla* styles was slower than cross-pollen tube growth, leading to the suggestion of cryptic self-incompatibility in these species (Horsley and Johnson 2007). From those observations it was expected that, following mixed pollinations, the relatively faster-growing cross-pollen tubes would be the first to arrive at the ovary and thus penetrate the majority of the ovules before the arrival of self-tubes. Using molecular markers, the present study on *E. grandis* confirmed the competitive advantage of cross-pollen, showing that 100% of the progeny was sired from outcrossed donors following mixed pollination with self- and outcross-pollen. This is in accordance with other studies which have found that in species that exhibit CSI, outcross-donors sire an average of 76 – 92% of the seeds per fruit following pollinations with equal amounts of self- and outcross-pollen (Bateman 1956; Weller and Ornduff 1977; Bowman 1987).

The results of single-donor self-pollinations would suggest that *E. grandis* is self-compatible to some extent since self-seed was set, although at a much reduced rate compared to outcross seed-set (Figure 4.1). However, these results differ from other CSI studies on species such as *Cheiranthus cheiri* (Bateman 1956), *Amsinckia grandiflora* (Weller and Ornduff 1977), *Decodon verticillatus* (Eckert and Barrett 1994) and *Clarkia gracilis* (Jones 1994), which have found no differences in seed set when self- and outcross-pollen were applied to separate flowers. Waser (1992) used the term “pseudo incompatibility” to describe responses that occur even after single-donor pollinations but which are partial, and Montalvo (1992) has warned that this phenomenon may obscure the detection of CSI. Despite these difficulties, the fact that there were significant differences in the proportion of selfed seeds from mixed-

donor fruits compared to expected values based on averages for single-donor self- and outcross fruits ($\chi^2 = 13.566$; $P < 0.001$), the case for CSI is strengthened in *E. grandis*. This significant change in the self:outcross seed ratio suggests that the observed deficit of selfed seeds in mixed-donor fruits could be the result of differential pollen tube growth.

From the results of single-donor self-pollinations (2.47 ± 0.9 seeds per flower pollinated), it is clear that an additional incompatibility mechanism is operating in *E. grandis*, and this is suggested to be late-acting self-incompatibility (LSI). Generally, in this type of self-incompatibility, self-pollen germinates and reaches the ovules, but no fruit is set (Seavey and Bawa 1986; Sage *et al.* 1994). It can manifest pre-zygotically, with deterioration of the embryo sac prior to pollen tube entry (Sage *et al.* 1999), or post-zygotically, with malformation of the zygote or embryo (Sage and Williams 1991). The concept of LSI, also termed ovarian self-incompatibility, was introduced by Seavey and Bawa (1986) to accommodate the increasing number of reports where the self-incompatibility mechanism does not act in the stigma or in the style.

The suggestion of LSI in the study species is strengthened by the observation that seed yields from mixed-donor (16.20 ± 5.4 seeds per flower pollinated) and single-donor outcross pollinations (23.63 ± 8.4 seeds per flower pollinated) were not significantly different (Figure 4.1), suggesting that the incompatibility reaction likely occurred before fertilisation. If the absence of selfed progeny from mixed-donor pollinations were due to the abortion of selfed embryos, we would expect seed yields to be more markedly reduced following mixed pollinations, due to ovules being discounted by self-pollen. A more likely scenario is the combination of slower self-pollen tube growth (CSI), together with LSI (occurring before fertilisation), resulting in outcross-pollen having the competitive advantage during mixed pollinations. Similar to the present study, Horsley and Johnson (2007) also suggested the presence of LSI in *E. grandis*. In that study, a much lower number of seeds were set following self-pollination relative to the number of self-pollen tubes observed in the style (Horsley and Johnson 2007).

In contrast to mixed pollinations, open controls showed a marked reduction in seed yield, setting an average of 1.66 ± 0.3 seeds per flower pollinated. This could be as a result of the time delay in pollen deposition during open pollination, as opposed to simultaneous placement of both self- and outcross-pollen during mixed pollinations. In addition, during mixed pollinations, equal quantities of the two pollen donors were placed on the stigma, whereas unknown quantities of self- and outcross-pollen were involved in the open controls. If self-pollen was deposited in sufficient quantity and well ahead of outcross-pollen, self-pollen tubes would be expected to traverse the style ahead of outcross-pollen tubes. By the time outcross-pollen landed on the stigma, it is possible that oxidation could have already occurred and thus the stigma would no longer have been receptive to pollen germination. An alternative hypothesis is that self-pollen was indeed able to fertilise the ovules, which subsequently aborted due to early-acting inbreeding depression, resulting in the observed low seed yields from the open controls.

The difference in male performance in single and mixed pollen loads is not surprising because of the different levels at which interactions are possible in the two cases (Bertin 1990). In single-donor pollinations, any seeds sired in a fruit are sired by the single-donor, and while seed number may vary among fruits sired by different donors, a maximum is set by the number of ovules and a minimum by the recipient's tendency to abort few-seeded fruits (Bertin 1982). The major determinant of success is the production or non-production of a fruit. In mixed pollinations, however, success of a donor's pollen will be determined firstly by its ability to fertilise ovules in competition with other pollen, secondly by the relative success of its fertilised ovules and thirdly by the likelihood of fruit maturation (Bertin 1990). Slower growth of pollen tubes from poorer donors would have little detrimental effect in single-donor pollinations but would be detrimental in mixed pollinations because of the presence of competing pollen tubes. Similar to the present study, a comparison of male performance in single- and mixed-donor pollinations in *Raphanus sativus* showed that the poorest donor in the single-donor pollinations tended to do even worse in the mixed-donor loads, suggesting the presence of differential pollen tube growth (Marshall and Ellstrand 1986).

Eckert and Allen (1997) have suggested that CSI might be favoured over more stringent forms of SI in species that: (1) exhibit strong inbreeding depression, (2) experience wide spatial or temporal variation in the availability of outcross pollen, and (3) experience selection for maximum fecundity during any given reproductive period. The first of these criteria simply provides the selective force maintaining outcrossing over self-fertilisation, while the second follows the logic that plasticity of any form is most likely selected when the environment is variable (Eckert and Allen 1997). The final criterion recognises that while the default option of selfing when cross pollen is limited might be advantageous in annual species, its value would be eroded in perennial species if the maturation of selfed ovules reduces residual reproductive value. Because *E. grandis* is a long-lived perennial, it is possible that maturing selfed ovules reduces future survival and/or reproductive output, much like it does in *Decodon verticillatus* (Eckert and Allen 1997). This could be the reason for the evolution of the additional LSI mechanism in *E. grandis*.

It should be noted that the genetic base for this experiment was extremely narrow. Travers and Mazer (2000) warn against generalising about CSI from the results of a single population, after their study on *Clarkia unguiculata* showed much lower outcross siring success compared to previous studies by Bowman (1987) and Jones (1994). Travers and Mazer (2000) have suggested that the advantage of outcross-pollen relative to self-pollen may vary among populations that differ in the amount of outcross-pollen typically deposited on stigmas. Alternatively, populations that differ in their genetic load will differ in their strength of selection favouring processes (such as CSI) that lower the rate of inbreeding. In the case of *Eucalyptus*, a more general case could be made by repeating this study using additional genotypes. Future investigations of the effect of environmentally induced stress on inbreeding depression, pseudo and cryptic SI, and maternal control over seed provisioning are warranted.

4.6 CONCLUSION

The combination of pseudo incompatibility and late-acting self-incompatibility makes it difficult to confirm the presence of CSI in *E. grandis*. However, due to the absence of selfed progeny after mixed pollinations (a significant deviation from the expected self:outcross ratio based on seed set in single-donor experiments), combined with

the slower self-pollen tube growth compared to cross-pollen tubes in a previous study (Chapter 3), CSI is still strongly suspected in this eucalypt species.

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CHAPTER 5: COMPARISON OF DIFFERENT CONTROL-POLLINATION TECHNIQUES FOR SMALL-FLOWERED EUCALYPTS

5.1 ABSTRACT

Controlled pollination (CP) is a labour-intensive, but useful procedure applied in tree improvement programmes. However, the high costs involved and relatively low seed yields often obtained has, in most cases, necessitated the use of lower quality, open pollinated seed. The aim of the present study was to compare control-pollination methods for combinations among small-flowered eucalypt species. By making crosses within and among *Eucalyptus grandis*, *E. smithii* and *E. macarthurii*, we compared effectiveness, in terms of seed production and level of genetic contamination, of three CP techniques, namely emasculation of bagged flowers and subsequent pollination of receptive stigmas (Conventional method), emasculation and immediate pollination of stigmas with induced receptivity followed by bagging (One Stop Pollination), and pollination of cut styles without emasculation and bagging (Artificially Induced Protogyny). Although Artificially Induced Protogyny using ripe and semi-ripe buds produced the highest seeds/flower pollinated in the majority of crosses carried out in this study, the technique, when performed on green buds, resulted in the highest capsule abortion. Molecular analysis using microsatellite markers also revealed that progeny from the Artificially Induced Protogyny method, when using green and semi-ripe buds, were highly contaminated by self- and external pollen. Of the three CP-techniques tested, One Stop Pollination had the lowest genetic contamination. However, this technique also had one of the lowest seed yields, while the Conventional method was intermediate in performance.

5.2 INTRODUCTION

In forestry, controlled pollination (CP) allows the combining of genetic material of selected elite trees to produce high quality, and consequently high value, seed (Eldridge *et al.* 1993). The technique has been used to improve seed yields, control the level of outcrossing in seed orchards, improve breeding through knowledge of both female and male parents, achieve interspecific hybridisation, and study self-incompatibility levels in *Eucalyptus* species (Harbard *et al.* 1999; Moncur 1995). However, the high cost of performing controlled crosses and the relatively low seed

production often obtained has necessitated the use of lower quality, open pollinated seed for the establishment of the majority of commercial tree plantations.

Moran and Bell (1983) have warned that the use of open-pollinated seed in plantations, as opposed to controlled outcrossed seed, may result in a significant loss of productivity. Potts *et al.* (1987) suggest that this effect is the result of inbreeding depression and that at least 10 – 30% of open-pollinated seed arises from self-fertilisation. In addition to inbreeding, genetic gains from open-pollinated orchards may be reduced by high contamination from external pollen sources, particularly when they are established from thinned open-pollinated progeny trials planted near plantations or native forests (Potts *et al.* 2008). For example, contamination of open-pollinated orchards of *Eucalyptus grandis* have been reported to be as high as 39% (Chaix *et al.* 2003) and 46% (Jones *et al.* 2007). For these reasons, it is highly desirable for the forestry industry to deploy control-pollinated elite seed.

For *Eucalyptus*, the first-developed CP-method (termed the 'Conventional method' throughout this paper) took advantage of the natural protandry of the eucalypt flower (van Wyk 1977). The technique, however, involved three flower visits (emasculation and bagging, subsequent pollination of receptive stigmas and re-bagging, removal of bags), and was consequently very time consuming. A more efficient cross-pollination method was later developed, originally for *Eucalyptus globulus*, requiring only one visit to the flower (emasculation and immediate pollination of stigmas cut to induce receptivity, followed by bagging) and consequently named One Stop Pollination (OSP; Harbard *et al.* 1999). The yield of *E. globulus* seeds per flower pollinated by OSP were reportedly as high as those from conventional CP (Williams *et al.* 1999), and because OSP involved less labour, the cost per seed produced was also reduced. For *E. globulus*, with its large, easily-pollinated flowers and capsules bearing up to 20 – 30 seeds each, OSP is now used to mass-produce seeds of elite crosses for operational deployment in plantations in Chile, Portugal and Australia (Assis *et al.* 2005; Potts *et al.* 2008). Although OSP has been used with some success on a range of eucalypt species, including *E. grandis* (Harbard *et al.* 2000) and *E. dunnii* (Barbour and Spencer 2000), small-flowered species such as *E. nitens* have displayed unacceptably low seed set with this CP-technique (Williams *et al.* 1999).

Artificially Induced Protogyny (AIP) is a new technique for the controlled pollination of eucalypt trees (Assis *et al.* 2005). It involves cutting off the tip of the operculum of the mature flower bud just prior to anthesis (release of pollen from anthers), with the cut positioned so as to remove the stigma and expose the cut surface of the upper style to which the target pollen is applied, without emasculating or isolating the flower. Trials in Australia, using *E. grandis* mother plants and *E. camaldulensis* pollen, showed that it was possible to produce 528 seeds per hour with the AIP method, compared to 240 seeds per hour with OSP and 98 seeds per hour with the Conventional method (Assis *et al.* 2005). However, despite this high productivity, Assis *et al.* (2005) warn that contamination, especially of highly self-compatible mothers by self pollination, could present a problem when using AIP. Those authors suggest the examination of isolated and non-isolated flowers following AIP under field conditions, since their study was conducted in a climate-controlled greenhouse (Assis *et al.* 2005).

The aim of the present study was to compare the Conventional, OSP and AIP pollination methods, in terms of seed yield and degree of genetic contamination, for the controlled crossing of small-flowered eucalypt species.

5.3 MATERIAL AND METHODS

5.3.1 Plant material used in study

The experiments were conducted on mature trees located in three separate clonal (grafted) orchards planted at the Sappi, Shaw Research Centre in KwaZulu-Natal, South Africa. All three orchards were situated at 29° 29'S, 30° 11'E at 1100 m above sea level. Species included in the study were *Eucalyptus grandis* Hill ex Maiden, *E. smithii* R. T. Baker and *E. macarthurii* Deane & Maiden, all belonging to the family *Myrtaceae* in the subgenus *Symphyomyrtus* (Brooker 2000). *Eucalyptus grandis* belongs to the section *Latoangulatae* (George 1988), while *E. smithii* and *E. macarthurii* fall under *Maidenaria* (Pryor and Johnson 1981). Breeding populations for all species were made up of open-pollinated families from selections made in land-races in South Africa and from provenances in the natural range in Australia. Genotypes occurring in the *E. grandis* orchard were seven years old, those in the *E. smithii* orchard were four years old, while those in the *E. macarthurii* orchard were three years old. All three orchards had previously been treated with paclobutrazol to

stimulate flowering (two years after orchard establishment). Pollinations were carried out during peak flowering (approximately 80% of genotypes flowering in the orchard), with trees chosen on the basis of floral abundance and accessibility for hand-pollinations. In general, flower bud sizes were approximately 0.8 x 0.5 cm for *E. grandis*, 0.7 x 0.4 cm for *E. smithii* and 0.5 x 0.3 cm for *E. macarthurii* (Brooker and Kleinig 1983).

5.3.2 *Pollen collection and processing*

For pollen extraction, branches containing ripe flower buds were collected and kept in 100 ml bottles containing water to prevent drying out of the branch. To ensure that there was no contamination from other pollen, all open flowers were removed from the branches before placing them in the laboratory overnight. The following morning, when the opercula of unopened flowers had shed and the filaments unfolded, the anthers were excised and left in a desiccator in the presence of silica gel to dry for approximately 48 h at room temperature. When the relative humidity (RH) in the desiccator had reached 10%, the dried anthers were sieved through a 30 micron mesh to remove debris. The resulting pollen was placed into polypropylene vials, sealed in glass bottles containing silica gel and stored in a freezer at -10°C until needed. For the polymix crosses, pollen from three unrelated genotypes were mixed in equal quantities (by weight) to give a representative polymix pollen-lot per species.

5.3.3 *In vitro pollen germination*

Pollen viability was tested under laboratory conditions before use in controlled pollinations. Pollen was left at room temperature and RH for 8 hours to rehydrate. *In vitro* germination was carried out using 30% (w/v) sucrose, supplemented with 0.15 mg l⁻¹ boric acid in a liquid medium (Horsley *et al.* 2007). Pollen samples were placed into glass vials containing the *in vitro* medium (three replications per genotype) and left to incubate in a germination chamber in a completely randomised design for 48 hours at 29°C. After the required time period had elapsed, 5 µl was transferred from the test-tube to a glass slide. Percent germination was scored using a light microscope (x100 magnification) to count the number of pollen grains germinated out of a total of 50 grains. Six glass slides per genotype (two slides per test tube) were scored for germination (sub-samples), giving a total of 300 pollen grains counted per treatment. Pollen was deemed to have germinated if the pollen

tube length was greater than one-half of the diameter of the pollen grain (Potts and Marsden-Smedley 1989).

5.3.4 *Controlled pollination*

Three different CP techniques were performed, namely the Conventional method (van Wyk 1977), One Stop Pollination (OSP, Harbard *et al.* 1999) and Artificially Induced Protogyny (AIP, Assis *et al.* 2005). Figures 5.1 A – F illustrate the steps taken when performing the Conventional and OSP techniques, while G – H depict AIP.

For the Conventional method, the operculum was removed from ripe flower buds and the anthers excised to prevent self-pollination. An isolation bag was placed over the emasculated flowers to prevent access by insect pollinators. A week later, when the stigma had ripened, the isolation bag was opened and the relevant pollen applied. The pollinated flowers were then re-isolated. Isolation bags were only removed when the stigma was thought to be fully oxidised, at about two weeks after pollination. The final step involved collecting ripe capsules 10 months after pollination of *E. grandis*, and 12 months after pollination of *E. smithii* and *E. macarthurii*.

For the OSP method, a horizontal cut was made through the stigma after the flower had been emasculated, and pollen immediately applied to the cut surface. The pollinated flowers were then isolated. As with the Conventional method, these isolation bags were removed two weeks after pollination. The final step involved collection of ripe capsules.

The AIP method involved cutting off the tip of the operculum prior to anthesis to expose the cut surface of the upper style, to which the target pollen was applied. AIP-pollinated flowers were not isolated. Three types of buds were tested using this method, viz. green (up to 20 – 10 days before operculum lift), semi-ripe (10 – 3 days before operculum lift) and ripe (2 – 0 days before operculum lift) (Assis *et al.* 2005).

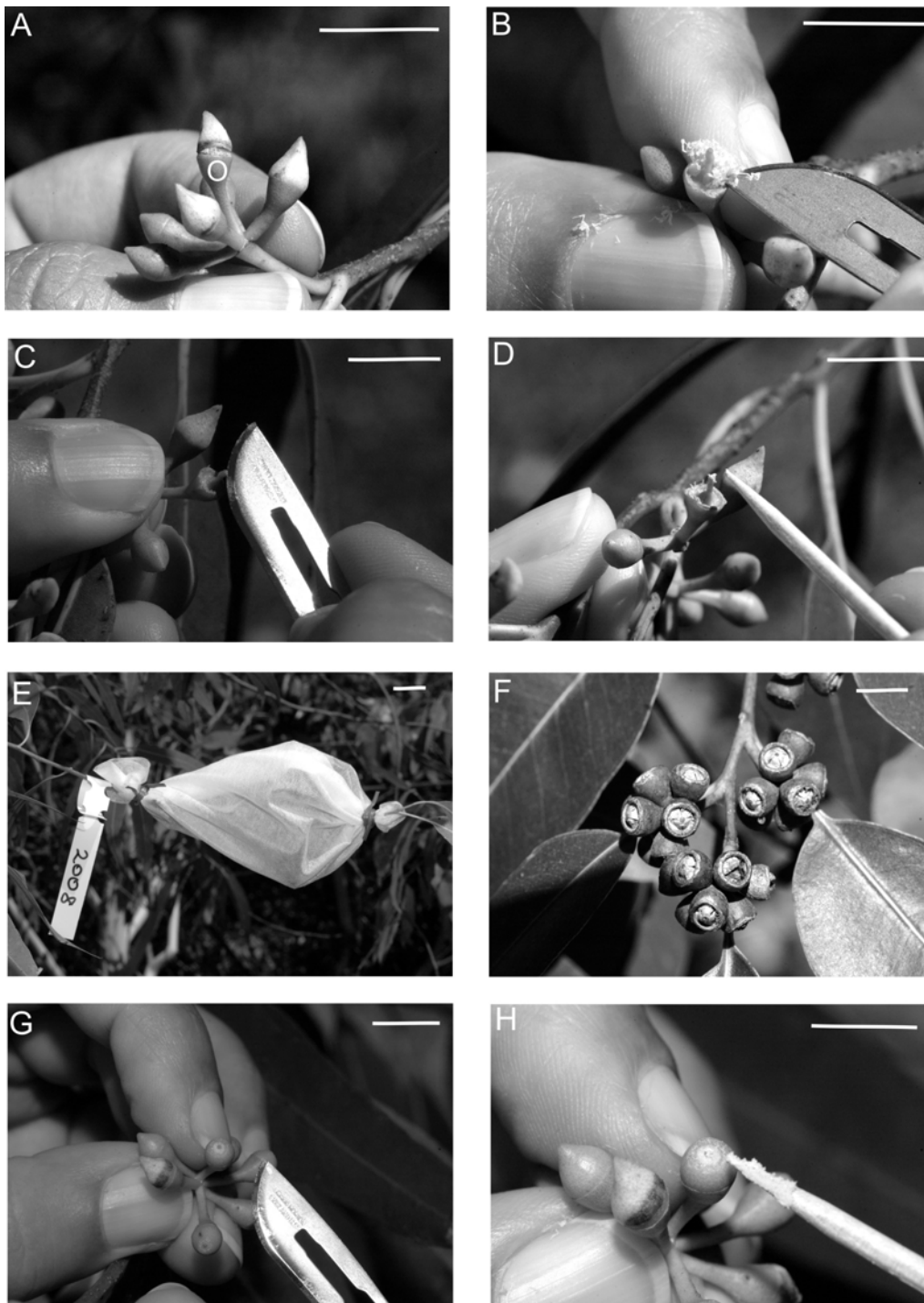


Figure 5.1: A – F show steps taken when performing the Conventional and One Stop Pollination (OSP) techniques, while G – H depict Artificially Induced Protogyny (AIP). (A) Flower buds at the operculum-lift stage of development (O) are chosen for both the Conventional and OSP methods; (B) buds are emasculated by removing all anthers; (C) a horizontal cut is made to the stigma during OSP to induce stigma receptivity; (D) pollen is applied to the receptive stigma; (E) pollinated buds are isolated; (F) ripe capsules are collected 6 – 12 months after pollination, depending on the species; (G) for AIP, a horizontal cut is made through flower buds prior to the operculum-lift stage of development, without removing the operculum; (H) pollen is applied immediately to the cut surface. Ripe capsules would also be collected 6 – 12 months after pollination. Bars = 1 cm

The maternal parents used in these CP-experiments were *E. smithii* (genotype S12) and *E. macarthurii* (genotype M196), pollinated with a polymix of three *E. grandis* paternal genotypes (EG45, T902 & P1376), and *E. grandis* (genotype P1369), pollinated with a polymix of three *E. smithii* paternal genotypes (S12, S20 & S8) and a polymix of three *E. macarthurii* paternal genotypes (M198, M199, M212). *Eucalyptus grandis* was also pollinated with self-pollen (P1369), as well as with intraspecific cross-pollen (T902). Open controls consisted of buds that had not been artificially pollinated nor isolated and were included to give an indication of natural pollination success.

5.3.5 Seed set

All capsules remaining at maturity (10 – 12 months after pollination) were harvested and allowed to dry out in the laboratory and release their seed. The number of viable seeds in each capsule were counted. Seeds were considered viable if they were rounded, solid and dark in colour as opposed to flat and possessing a light-brown colour (Pound *et al.* 2002).

5.3.6 Molecular marker analysis of pollen contamination

Leaf samples from the five CP-treatments (AIP-ripe buds; AIP-semi-ripe buds; AIP-green buds; OSP and Conventional method) were collected for molecular marker analysis, with twenty randomly selected progeny sampled from each of the following crosses: *E. grandis* x *E. smithii* interspecific cross; *E. grandis* x *E. grandis* intraspecific cross; *E. grandis* self-pollination. DNA extraction was performed using the Qiagen DNeasy Plant Kit (QIAGEN, Valencia, CA, USA). The contamination rate of each of the treatments was determined using microsatellite markers (Brondani *et al.* 1998) to test for non-parental (contaminant) alleles in each progeny set. Eight highly informative microsatellite markers (viz. EMBRA 37, EMBRA 45, EMBRA 48, EMBRA 56, EMBRA 94, EMBRA 98, EMBRA 219, EMBRA 227) were used to ensure adequate power to discriminate closely related pollen contaminants from pollen used in the CP trials.

5.3.7 Statistical analysis

SPSS Version 15.0 was used for all statistical analyses. Species were treated as replicates of the pollination methods since pollination occurred in the same way in all three maternal genotypes. As it was common for more than one capsule to be harvested from within a pollination bag in the Conventional and OSP pollination methods, the mean number of seeds set per flower pollinated was calculated for each bag. In the case of AIP pollinations, mean number of seeds set per pollination event was calculated, with a pollination event consisting of three umbels. Seed set per flower pollinated was analysed using the General Linear Model (GLM) Univariate procedure (with Type III sum of squares). Due to the unbalanced nature of the experimental design, the CP-data had to be split and two separate analyses performed. The first analysis compared the performance of the CP-techniques across three maternal parents (*E. grandis*, *E. macarthurii* and *E. smithii*) when pollinations were done using *E. grandis* outcross pollen, while the second analysis looked at CP-performance when pollinations were done on a common maternal parent (*E. grandis*) using four different pollen parents (*E. grandis* self, *E. grandis* outcross, *E. macarthurii* and *E. smithii*). The independent variables 'tree', 'pollen' and 'CP' were specified as fixed effects, while 'seed per flower pollinated' was the dependent variable. A full factorial model was used to analyse all main effects and their interactions. Pollen viability data were angular transformed prior to Analysis of Variance (ANOVA) and Duncan multiple range tests.

5.4 RESULTS

5.4.1 In vitro pollen germination

In vitro germination rates varied among pollen samples, ranging from 35.3 to 66.0%, with significant differences between treatments ($P < 0.001$; Table 5.1).

Table 5.1: *In vitro* germination of *Eucalyptus* pollen used in controlled pollinations. Letters (a) and (b) indicate statistical significance ($P < 0.05$), where treatments indicated by the same letter are not significantly different.

Species	Genotype	N	Mean \pm std error (%)
<i>E. grandis</i> (polymix)	EG45; T902; P1376	3	66.0 \pm 0.6 ^a
<i>E. grandis</i>	P1369	3	64.7 \pm 0.3 ^a
<i>E. smithii</i> (polymix)	S12; S20; S8	3	57.7 \pm 3.5 ^a
<i>E. grandis</i>	T902	3	42.3 \pm 2.3 ^b
<i>E. macarthurii</i> (polymix)	M198; M199; M212	3	35.3 \pm 5.9 ^b

5.4.2 Controlled pollination

There were statistically significant differences in seed/flower pollinated between the different maternal parents ($P < 0.001$) and between the different CP-techniques ($P < 0.001$), as well as a significant interaction between these two fixed effects (Table 5.2).

Table 5.2: Univariate analysis of variance of seed per flower following controlled pollinations of three maternal parents crossed with *E. grandis* pollen. In the table, 'Tree' refers to maternal parents *E. grandis*, *E. macarthurii* and *E. smithii*, while 'CP' refers to the following six pollination treatments: Conventional, OSP, AIP with green buds, AIP with semi-ripe buds, AIP with ripe buds and Open controls.

Source of variation	d.f.	Mean Square	F-value	P-value
Tree	2	2490.81	26.12	0.000
CP	5	480.57	5.04	0.000
Tree x CP	10	441.51	4.63	0.000
Error	77	95.37		

After pollinations, a higher percentage of capsules were retained on the *E. grandis* maternal parent, compared to *E. macarthurii* and *E. smithii* parents (Table 5.3). For all species, high capsule abortion occurred when AIP was performed on green buds, compared to AIP on semi-ripe and ripe buds. OSP also tended to result in abortion of capsules. The highest seed yields were obtained from the *E. grandis* intraspecific outcross, particularly when using the AIP technique on semi-ripe buds (44 \pm 10.4 seeds/flower pollinated), while AIP performed on ripe buds yielded a similar amount of seed to the open-pollinated control in this type of cross (Table 5.3).

Table 5.3: Seed yields obtained after different control-pollination techniques were performed on three maternal parents, viz. *Eucalyptus grandis* (genotype P1369), *E. macarthurii* (genotype M196) and *E. smithii* (genotype S12) using different pollen donors.

Pollination treatments	Flowers pollinated	N	Capsules set (%)	Seed/flower \pm std error	Flowers pollinated	N	Capsules set (%)	Seed/flower \pm std error	Flowers pollinated	N	Capsules set (%)	Seed/flower \pm std error
<i>E. grandis</i> x <i>E. grandis</i> (single-donor) intraspecific outcross				<i>E. macarthurii</i> x <i>E. grandis</i> (polymix) interspecific outcross				<i>E. smithii</i> x <i>E. grandis</i> (polymix) interspecific outcross				
Conventional	60	3	71	9.8 \pm 0.6	81	6	5	0.4 \pm 0.3	58	5	15	0.5 \pm 0.2
OSP	55	4	31	0.0 \pm 0.0	66	5	12	0.8 \pm 0.7	56	5	1	0.1 \pm 0.1
AIP ripe	60	7	82	27.9 \pm 7.7	52	4	26	0.4 \pm 0.3	54	15	19	0.3 \pm 0.2
AIP semi-ripe	75	7	80	44.0 \pm 10.4	84	6	18	0.8 \pm 0.3	52	6	22	1.0 \pm 0.6
AIP green	55	2	32	5.8 \pm 3.3	87	6	20	1.0 \pm 0.7	93	3	2	0.0 \pm 0.0
Open control	70	2	83	26.0 \pm 10.5	98	5	16	0.3 \pm 0.2	66	4	9	0.1 \pm 0.1
<i>E. grandis</i> x <i>E. grandis</i> (single-donor) intraspecific self-cross				<i>E. grandis</i> x <i>E. macarthurii</i> (polymix) interspecific outcross				<i>E. grandis</i> x <i>E. smithii</i> (polymix) interspecific outcross				
Conventional	57	4	24	1.1 \pm 0.4	60	4	19	10.5 \pm 10.1	58	5	49	18.3 \pm 16.6
OSP	60	4	59	1.7 \pm 0.7	58	7	46	2.0 \pm 0.5	50	5	60	7.7 \pm 2.3
AIP ripe	55	7	71	7.2 \pm 2.0	54	10	75	32.8 \pm 13.9	78	6	71	11.3 \pm 3.0
AIP semi-ripe	63	7	79	7.1 \pm 3.0	62	3	73	14.8 \pm 10.1	52	2	70	8.2 \pm 6.8
AIP green	63	3	30	8.8 \pm 8.1	86	5	19	0.4 \pm 0.2	51	2	29	2.9 \pm 2.9

N refers to number of replications (i.e. isolation bags / pollination events), OSP to One Stop Pollination and AIP to Artificially Induced Protogyny. The *E. grandis* polymix was made up of EG45, T902 and P1376 paternal genotypes, *E. smithii* polymix consisted of S12, S20 and S8, and *E. macarthurii* consisted of M198, M199 and M212. The single-donor pollen used in the *E. grandis* intraspecific outcross was genotype T902.

In contrast to *E. grandis*, the remaining maternal parents (*E. macarthurii* and *E. smithii*) exhibited extremely low seed yields (less than 1 seed/flower pollinated on average) across all pollination treatments, including the open-pollinated controls. Although there were no statistically significant differences between pollen donors (*E. grandis* self, *E. grandis* outcross, *E. smithii* and *E. macarthurii*) when pollinations were performed on a common *E. grandis* maternal parent ($P = 0.309$), there were significant differences between the CP-techniques ($P = 0.039$; Table 5.4). On this maternal parent, the lowest seed yields were generally obtained from OSP and AIP green-bud treatments. Conventional pollinations produced higher seed yields on average, across all species, compared to OSP (Table 5.3).

Table 5.4: Univariate analysis of variance of seed per flower for an *E. grandis* maternal parent control-pollinated with four different pollen parents, viz. *E. grandis* self, *E. grandis* outcross, *E. macarthurii* and *E. smithii*. In the table, ‘Pollen’ refers to the paternal parents, while ‘CP’ refers to the following five pollination treatments: Conventional, OSP, AIP with green buds, AIP with semi-ripe buds and AIP with ripe buds.

Source of variation	d.f.	Mean Square	F-value	P-value
Pollen	3	527.25	1.22	0.309
CP	4	1150.99	2.66	0.039
Pollen x CP	12	511.50	1.18	0.311
Error	77	432.54		

Indicative costs, based on mean seed yields obtained from *E. grandis* (viz. 10 seeds per flower pollinated), showed that the AIP method could lead to a substantial reduction in total costs, with approximately 50% reduction in labour costs alone. This was due to the higher number of flowers that could be pollinated in one hour by one labour unit when using AIP (viz. 300 flowers), compared to the Conventional method and OSP (56 and 75 flowers, respectively). However, in the case of *E. smithii* and *E. macarthurii*, there were no cost reductions when using AIP compared to the other CP-techniques, due to the low number of seed per flower pollinated in the genotypes tested.

5.4.3 Molecular marker analysis of pollen contamination

Molecular analysis of progeny from the *E. grandis* x *E. smithii* cross revealed that controlled pollinations using the AIP method on green buds resulted in high contamination by self (65%) and external (foreign) pollen (35%), with none of the applied pollen contributing to the progeny (Table 5.5). A similar trend was observed when semi-ripe buds were used in the *E. grandis* x *E. smithii* cross, with progeny showing 80% selfing and 15% external contamination. On the other hand, molecular analysis of the *E. grandis* self and intraspecific outcross (Table 5.5) performed on ripe buds using the AIP method, showed acceptable levels of external pollen contamination (5%). In the samples tested, OSP had the lowest self-pollen contamination (0%), followed by the Conventional method (5%).

Table 5.5: Molecular analysis of pollen contamination, where ‘gran’ and ‘smit’ refer to *E. grandis* and *E. smithii*, respectively. A total of 20 progeny from each treatment were fingerprinted using microsatellite markers.

CP method	Cross	Bud stage	Contribution to progeny (%)		
			Applied pollen	Self pollen	Foreign pollen
AIP	gran x smit	green	0	65	35
AIP	gran x smit	semi-ripe	5	80	15
AIP	gran self	ripe	95	*	5
AIP	gran x gran	ripe	60	35	5
OSP	gran x smit	ripe	95	0	5
Conventional	gran x smit	ripe	70	5	25

*Self-pollen was manually applied for this treatment and therefore occurs in the applied pollen column

5.5 DISCUSSION

Of the three CP-techniques, the AIP method not only produced the highest seeds/flower pollinated when ripe and semi-ripe buds were used (Table 5.3), but also reduced the time to perform pollinations since flowers were not emasculated. However, determining the exact levels of contamination became a key issue, as flowers were also not isolated from non-intended sources of pollen when using this technique. Microsatellite markers revealed contamination present for all three CP methods. This is of concern, especially in the case of Conventional and OSP (both being well-established CP-techniques) and a possible cause could be the use of

inadequate isolation material when bagging flowers. Harbard *et al.* (2000) have suggested a contamination rate of around 10% as acceptable for commercial CP-seed production. In the present study, self-pollen contamination ranged between 5 – 80% in the samples tested, while outcross-pollen contamination was found to be between 5 – 35%, across all techniques (Table 5.5).

For the *E. grandis* maternal parent, there was a significant reduction in seed set following self-pollination (5 seeds/flower on average), compared to the open-pollinated control (26 seeds/flower) and cross-pollinations (10 –18 seeds/flower). Similar results were obtained in Chapters 3 and 4, where both *E. grandis* and *E. urophylla* set fewer seeds following single-donor self-pollinations compared to cross-pollinations. Reduced self-seed yields have also been demonstrated in *E. globulus* (Potts and Savva 1988), *E. nitens* (Tibbits 1989) and *E. gunnii* (Potts *et al.* 1987). Furthermore, Griffin *et al.* (1987) found that preferential outcrossing took place in *E. regnans* following pollination with mixed self- and outcross-pollen.

The present results support Pryor's (1961) observations that selfing can occur in *Eucalyptus*, but less readily than outcrossing and frequently less readily than interspecific hybridisation. Barriers to self-fertilisation have been noted in other *Eucalyptus* species (Pryor 1961; Hodgson 1976; van Wyk 1981; Eldridge and Griffin 1983; Potts and Savva 1988; Sedgley *et al.* 1989; Sedgley and Smith 1989; Ellis and Sedgley 1992; Pound *et al.* 2002; Pound *et al.* 2003) and Pryor (1961) indicates there is some evidence for a gene-controlled self-incompatibility system in some species. Horsley and Johnson (2007) suggest the presence of cryptic self-incompatibility in *E. grandis* and *E. urophylla* since, in addition to reduced self-seed yields, the growth of self (incompatible) pollen tubes was slower than that of outcrossed (compatible) ones, rather than being completely inhibited. Studies by Stephenson *et al.* (2003) and Travers *et al.* (2004) have shown that there may be more plasticity in the growth of self-pollen tubes than has previously been appreciated, and those authors have subsequently suggested that the plasticity in self-incompatibility systems be viewed as a mechanism that promotes outcrossing by modulating the intensity with which it handicaps the growth of self pollen.

The very high rate of selfing obtained with the AIP method was unexpected, raising a number of questions: Firstly, is an internal barrier to self-fertilisation overcome by this method? Secondly, where did the self pollen come from? Mislabelling does not appear to be the case, since no selfing was detected for OSP even though the same pollen was used as for AIP. Brazilian and Australian researchers reported acceptable levels of contamination, viz. less than 5% foreign-pollen contamination and 0% selfing, when using the AIP method (Assis *et al.* 2005). However, those authors used potted trees in an enclosed environment in a greenhouse, compared to field trees which were used in the present study.

In addition to natural movement of pollen within a flower, another possible source of the self pollen contamination in the present study could be from flowers within the canopy of the same tree, and there is evidence that this occurs in most eucalypt species (Eldridge *et al.* 1993; Potts and Cauvin 1988; Pryor 1976). Wind plays a major role under field conditions and can result in increased self-pollination by vibrating the branches of the tree, causing pollen to fall from the anthers of flowers higher in the canopy onto the receptive stigmas of branches lower down (Eldridge and Griffin 1983). In the study by Assis *et al.* (2005), wind was not a factor since the trees were protected from the elements, and this could explain the absence of selfing in that study. Those authors also conceded that there was a possibility that the *E. grandis* genotype subjected to molecular marker analysis in their study could have been self-incompatible, since no seed was set following controlled self-pollinations (Assis *et al.* 2005).

Controlled pollination efficiency is to a large extent dependent on the synchronous timing of pollen application with stigma receptivity (Potts and Potts 1986; Tibbits 1989). A possible reason for the high self- and external contamination when using green buds in the present study could be that since the buds were immature, the applied pollen could not adhere to the non-receptive stigma and was thus easily removed by insects or wind. When the buds later became ripe, self and external pollen were able to contribute to pollinations. Assis *et al.* (2005) have shown that contamination potential in *E. grandis* may exist for at least 7 – 10 days after the style is cut, and in studies on *E. nitens* (Tibbits 1989) and *E. globulus* (Williams *et al.* 1999), there have also been reports that applied pollen easily fell off stigmas which

were not sticky. The natural duration of stigma receptivity can vary between clones and is affected by environmental conditions (Hodgson 1976), and similar variability may be anticipated for the receptivity of cut style surfaces. Differences in self-compatibility, flower morphology or flowering phenology after AIP are possible causes (Assis *et al.* 2005).

There is evidence to suggest that both pre-zygotic (Ellis *et al.* 1991) and post-zygotic (Potts *et al.* 1987; Griffin *et al.* 1988) barriers to crossing within subgenera of *Eucalyptus* increase with increasing taxonomic distance between species. The work of Ellis *et al.* (1991) has shown that if flower size is kept constant, then the severity of pollen tube abnormalities and the probability of pollen tube arrest in the pistil increases with increasing taxonomic distance between the parents. In the present study, the higher seed yields obtained from the intraspecific outcross compared to the interspecific ones could be related to taxonomic distance. Crosses between *E. grandis* and *E. smithii* and between *E. grandis* and *E. macarthurii* are potentially difficult, since even though the species are in the same subgenus (*Symphyomyrtus*), *E. grandis* belongs to the section *Latoangulatae* (George 1988), while *E. smithii* and *E. macarthurii* belong to section *Maidenaria* (Prior and Johnson 1981). Assis (2000) considers that abnormal phenotypes are more often encountered when species from the section *Maidenaria* are involved in intra- or inter-sectional crosses. This emphasises the need for considering potential crosses not only for their combination of desirable characters, but also for taxonomic affinities and possible structural and physiological barriers (Delaporte *et al.* 2001).

The fact that the *E. grandis* maternal parent produced higher seed yields compared to *E. smithii* and *E. macarthurii* could be related to flower size, since the latter species generally have smaller flowers compared to *E. grandis* (Brooker and Kleinig 1983), that are possibly more easily damaged during controlled pollinations. It is also feasible that the smaller-flowered species could possess fewer ovules compared to *E. grandis*, resulting in fewer seeds being formed. This differential pollination-success between large and small flowered eucalypt species has been previously reported (Pryor 1956; Tibbits 1986, 1989; Gore *et al.* 1990). Such an effect can have important implications in breeding programmes, as plants with limited reproductive capabilities are difficult to use, and are therefore often excluded from mating designs,

even if they exhibit other, more desirable, characters (Delaporte *et al.* 2001). A better understanding of the flower anatomy and associated pollen behaviour of the species to be included in the mating design may improve the success rate of controlled pollinations (Williams *et al.* 1999).

Eucalypts are largely insect-pollinated and successful pollinations are influenced by a number of factors, including diversity of flowering times, diversity in flowering intensity and number of pollen vectors present at flowering (Eldridge *et al.* 1993). The lower seed yields obtained from the *E. smithii* and *E. macarthurii* open-pollinated controls in comparison to the *E. grandis* open-pollinated control (Table 5.3) could be attributed to limited insect activity in the *E. smithii* and *E. macarthurii* orchards. *Eucalyptus grandis* has larger flowers with more prominent anthers, compared to *E. smithii* and *E. macarthurii*, and is therefore presumed to be more attractive to bees (the main insect pollinator of these species; Eldridge *et al.* 1993). The former species also flowers more abundantly than the latter two, further contributing to attractiveness to insect pollinators (House 1997; Hayes *et al.* 2005). In addition, genotypes occurring in the *E. grandis* orchard (7 years old) were more mature than the *E. smithii* (4 years old) and *E. macarthurii* (3 years old) genotypes, possibly adding to the differences in flowering intensity.

A potential weakness of the present experimental design is that only one maternal genotype was tested per species. Both Callister (2007) and McGowen (2007) have warned that the choice of female genotype can have a significant effect on the costs of seed production, due to genetic based differences in reproductive success. Delaporte *et al.* (2001) showed that the mean number of seeds produced per flower pollinated and the mean seed weight generally varied more between female plants than between crosses in *E. macrocarpa*, *E. pyriformis* and *E. youngiana*. Harbard *et al.* (1999) have also shown large genotypic variation in seed yields of *E. globulus*, both in terms of harvest percentage and viable seed per capsule. The present results should therefore be used with caution when extrapolating to the species level, as different genotypes might behave differently.

5.6 CONCLUSION

In Australia and Brazil, AIP is a technique showing great promise for the mass controlled pollination of small-flowered eucalypts, and subsequent commercial deployment of elite seed. From the present study, however, it is evident that high levels of pollen contamination and high selfing can result when this CP-method is conducted on open-air field trees, in addition to high capsule abortion when the technique is performed on green buds. Of the three CP-techniques tested, OSP had the lowest genetic contamination. However, this CP-method also produced one of the lowest seed yields. The Conventional method was intermediate in performance, producing, on average, both higher seed yields, as well as higher genetic contamination, compared to OSP. These results should, however, be regarded with caution, due to the limited number of genotypes that were available for this study.

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CHAPTER 6: A NOVEL GEL-BASED METHOD FOR ISOLATION OF STIGMAS DURING CONTROLLED POLLINATION EXPERIMENTS

6.1 ABSTRACT

In forestry, controlled pollination (CP) allows the combining of genetic material of selected elite trees to produce high quality, and consequently high value, seed. The aim of the present study was to develop a novel isolation method that would allow the technique to be conducted without expensive and time-consuming bagging, making CPs on small-flowered eucalypts commercially viable. We compared the current method of isolating inflorescences using exclusion bags to a novel method which uses sodium alginate gel. Sodium alginate was effective in keeping external pollen away from the stigma, since no seed was produced in those treatments that were not manually pollinated but isolated in this way. In addition, flowers hand-pollinated and isolated with sodium alginate produced progeny that were 100% outcrossed with the applied pollen. The exclusion bags, on the other hand, were not as effective in protecting the stigma as seed was produced in those treatments that were isolated with an exclusion bag without being hand-pollinated. Sodium alginate isolation also increased the efficiency of control-pollinations as the gel was naturally shed, removing the need for operators to return to the tree to remove the isolation material.

6.2 INTRODUCTION

Most flowering plants rely on pollinators to deposit compatible pollen onto stigmas for ovule fertilisation and seed set (Ramsey and Vaughton, 2000). However, pollen quantity may be limiting if pollinators are rare, or if plants compete for the services of pollinators (Groom, 1998). Pollen quality may also be limiting, despite adequate pollination, if pollinators deposit self- or incompatible pollen onto stigmas, or deposit closely-related pollen which may lead to early-acting inbreeding depression lowering seed set (Pound *et al.*, 2003). These limitations of natural pollination can largely be overcome with controlled pollination (CP), which allows the quality and quantity of pollen deposited on the stigma to be optimised. In one study, CPs resulted in increased survival, size and reproduction of the progeny in subsequent years (Lehtila and Syrjanen, 1995). In forestry, the technique has been used to improve seed yields, control the level of outcrossing in seed orchards, improve breeding through

knowledge of both female and male parents, achieve interspecific hybridisation, and study self-incompatibility levels (Harbard *et al.*, 1999; Moncur, 1995).

For *Eucalyptus*, the first-developed CP-method, termed the Conventional method, took advantage of the natural protandry (where pollen is released before the stigma becomes receptive) of the eucalypt flower (van Wyk, 1977). The technique involved three flower visits (emasculation and bagging, subsequent pollination of receptive stigmas and re-bagging, removal of bags), and was consequently very time consuming. A more efficient cross-pollination method was later developed, originally for *E. globulus*, requiring only one visit to the flower (emasculation and immediate pollination of stigmas cut to induce receptivity, followed by bagging) and consequently named One Stop Pollination (OSP; Harbard *et al.*, 1999). However, although OSP has been used with some success on a range of eucalypt species (Harbard *et al.*, 2000; Barbour and Spencer, 2000), small-flowered species have displayed unacceptably low seed set (Williams *et al.*, 1999).

Artificially Induced Protogyny (AIP; Assis *et al.*, 2005) is a relatively new technique for the controlled pollination of eucalypt trees. It involves cutting off the tip of the operculum of the mature flower bud just prior to anthesis (release of pollen from anthers), with the cut positioned so as to remove the stigma and expose the cut surface of the upper style to which the target pollen is applied, without emasculating or isolating flowers (Figure 1A and B). A recent study (Chapter 5) has, however, identified the need for self- and external-pollen exclusion in order for the AIP technique to be effective under field conditions (Horsley *et al.*, submitted).

Effective flower isolation is highly desirable during controlled pollinations, since it can enhance the accuracy of breeding through full pedigree control (Dutkowski *et al.*, 2006). The main sources of pollen contamination during eucalypt CPs are foreign pollen from nearby trees and self pollen transferred geitonogamously from other flowers within the canopy of the tree (Snow *et al.*, 1996). Self-pollination within a eucalypt flower is generally prohibited by the protandrous nature of the flower (Eldridge *et al.*, 1993). To prevent unwanted pollen transfer during controlled pollinations, flowers must be physically isolated, with the method of isolation dependant on the flower characteristics, sexual compatibility between genotypes,

pollen quantity and viability, and mode of pollen dissemination (Sundstrom *et al.*, 2002).

For *Eucalyptus* pollinations, bagging is the simplest method of isolation and involves covering the flowers with breathable material, such as a nappy-liner, paper bag, glassine or fine cloth (Moncur, 1995). Individual styles may also be isolated with a small piece of plastic tubing, sealed at one end (Harbard *et al.*, 1999). After fertilisation, the stigma abscises taking the tube with it. Use of this method allows all available flowers to be pollinated, unlike bag isolation where flowers that are pre- or post-anthesis, and likely to be enclosed in the bag, must be removed to prevent contamination (Williams *et al.*, 1999). The plastic-tube isolation method is, however, restricted to large-flowered eucalypt species, such as *E. globulus*, which have single flowers. In small multi-flowered inflorescences, such as in *E. grandis*, it becomes expensive and logistically difficult to isolate individual flowers in this way (Barbour, 1997; Harbard *et al.*, 2000).

Isolation efforts may need to be increased depending on the type of pollinations being carried out (Bradford, 2006). For example, with interspecific or hybrid crosses, in which contamination can be readily observed in the progeny (phenotypic observations), isolation is not imperative. However, when controlled pollinations are performed intraspecifically, it is important that a reliable isolation method be employed since contaminants are not as easily phenotypically observed and the more expensive route of molecular marker analysis may have to be employed.

The aim of the present study was to develop a practical isolation method for application when using the AIP technique to perform controlled pollinations on small-flowered eucalypts.

6.3 MATERIAL AND METHODS

6.3.1 Plant material used in study

The experiments were conducted on mature trees located in two separate clonal (grafted) orchards planted at the Sappi, Shaw Research Centre in KwaZulu-Natal, South Africa. Both orchards were situated at 29° 29'S, 30° 11'E at 1100 m above sea level. *Eucalyptus grandis* was the study species in which intraspecific crosses were

performed. The breeding population for this species was made up of open-pollinated families from selections made in land-races in South Africa and from provenances in the natural range in Australia.

Maternal genotypes included in the study were B0133 (in orchard 1), T1099 and T1144 (in orchard 2). Paternal genotypes included T1074, B0133 and T1087. Pollinations in orchard 1 were carried out during peak flowering (approximately 80% of genotypes flowering in the orchard), while those in orchard 2 took place at the end of the flowering season (approximately 20% of genotypes flowering in the orchard). Trees were chosen on the basis of floral abundance and accessibility for hand-pollinations, leading to one ramet from each genotype being pollinated. To take into account different micro-climates, replications were evenly distributed around each tree (viz. north, south, east and west).

6.3.2 *Pollen collection and processing*

For pollen extraction, branches containing ripe flower buds were collected and kept in 100 ml bottles containing water to prevent drying out of the branch. To ensure that there was no contamination from other pollen, all open flowers were removed from the branches before placing them in the laboratory overnight. The following morning, when the opercula of unopened flowers had shed and the filaments unfolded, the anthers were excised and left in a desiccator in the presence of silica gel to dry for approximately 48 h at room temperature. When the relative humidity (RH) in the desiccator had reached 10%, the dried anthers were sieved through a 30 micron mesh to remove debris. The resulting pollen was placed into polypropylene vials, sealed in glass bottles containing silica gel and stored in a freezer at -10°C until needed.

6.3.3 *In vitro pollen germination*

Pollen viability was tested under laboratory conditions before use in controlled pollinations. Pollen was left at room temperature and RH for 8 hours to rehydrate. *In vitro* germination was carried out using 30% (w/v) sucrose, supplemented with 0.15 mg l⁻¹ boric acid in a liquid medium (Horsley *et al.*, 2007). Pollen from each genotype was placed into glass vials containing the *in vitro* medium (three replications per genotype) and left to incubate in a germination chamber in a completely randomised

design for 48 hours at 29°C. After the required time period had elapsed, 5 µl was transferred from the test-tube to a glass slide. Percent germination was scored using a light microscope (x100 magnification) to count the number of pollen grains germinated out of a total of 50 grains. Six glass slides per genotype (two slides per test tube) were scored for germination (sub-samples), giving a total of 300 pollen grains counted per treatment. Pollen was deemed to have germinated if the pollen tube length was greater than one-half of the diameter of the pollen grain (Potts and Marsden-Smedley, 1989).

6.3.4 *Controlled pollination*

Two controlled pollination experiments were performed, one in 2005 and the other in 2007, both employing the AIP method of controlled pollination (Assis *et al.*, 2005). In the 2005 study, B0133 x T1074 crosses were carried out in orchard 1 to test the effect of sodium alginate on pollen tube growth (i.e. to see if sodium alginate would interfere with pollen germination and tube growth). A follow-up study was conducted in 2007 in orchard 2, where T1099 x B0133, T1099 x T1087, T1144 x B0133 and T1144 x T1087 crosses were performed. This was to confirm the 2005 results, as well as test an additional treatment, viz. sodium alginate isolation of non-pollinated buds, to determine if sodium alginate would be effective in keeping extraneous pollen away from the stigma. A secondary aim was to examine the effect of flowering intensity on open-pollinated (OP) seed production in order to determine whether CPs would increase quantity and quality of seed yields towards the end of the flowering season. Appendix A shows the number of flowers pollinated per treatment.

Artificially Induced Protogyny involved cutting off the tip of the operculum of a mature flower bud prior to anthesis to expose the cut surface of the upper style, to which the target pollen was applied, without emasculating the flower. Pollinated flowers were then subjected to either nappy-liner isolation (also referred to as 'bagging'), sodium alginate isolation or non-isolation treatments. During bagging, a nappy-liner (Quick-dry nappy-liners, manufactured by Unsgaard Packaging Ltd, South Africa) was placed over three umbels (maximum of 21 flowers) and secured at each end using twist wires. These bags were removed when the stigma had fully oxidised, which occurred two weeks after pollination. For sodium alginate isolation, pollinated buds were first sprayed with 100 mM calcium nitrate solution for 5 seconds and then

immediately sprayed with 2.2% sodium alginate solution for 5 seconds, allowing a gel to form around the buds (Figure 6.1). The sodium alginate gel was shed naturally upon operculum-fall. Open controls consisted of buds that had been neither artificially pollinated nor isolated and were included to give an indication of natural pollination success.

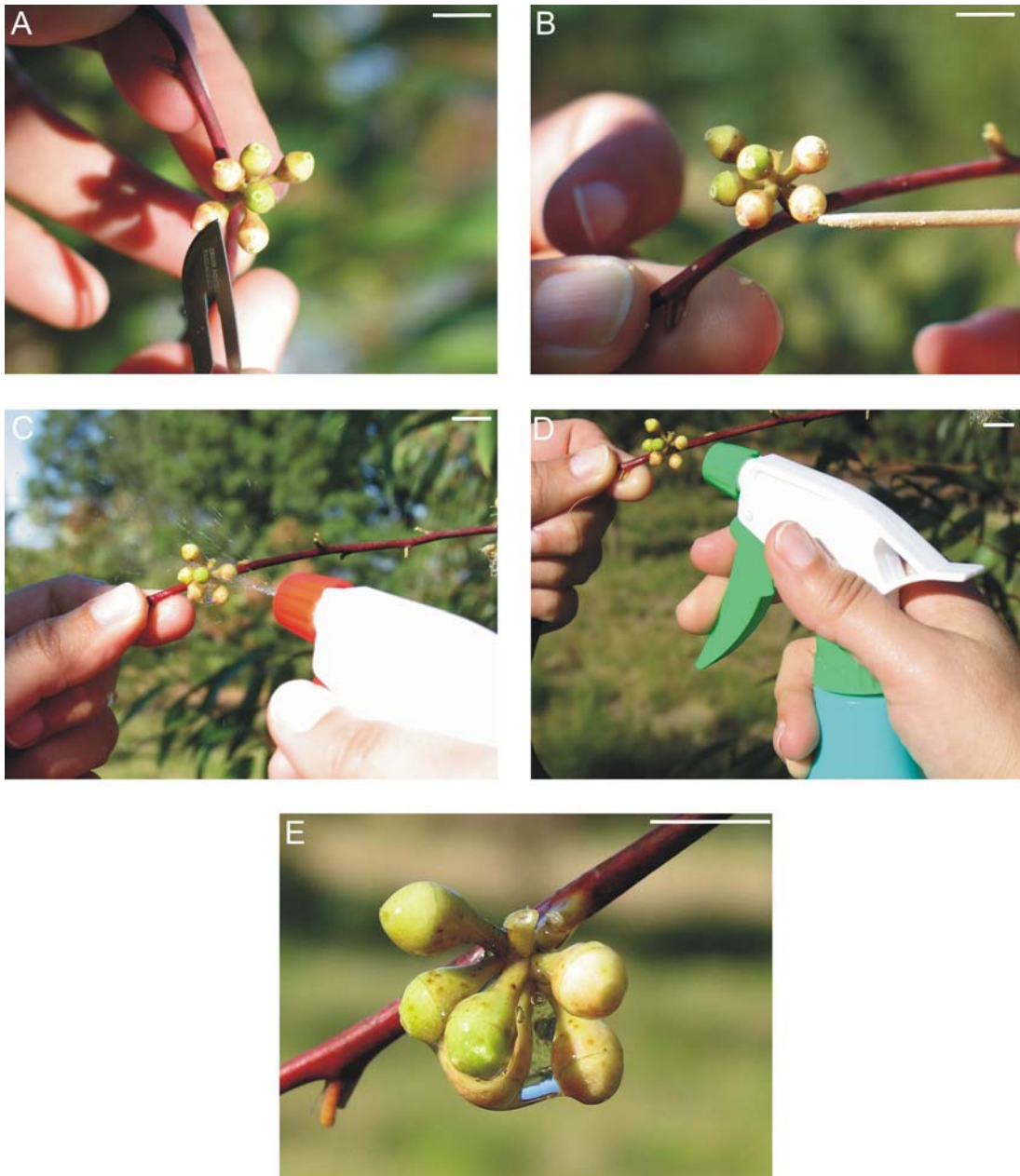


Figure 6.1: Sodium alginate isolation of AIP-pollinated *Eucalyptus grandis* buds. (a) A horizontal cut was made through the top quarter of a ripe flower bud without removing the operculum, (b) pollen was applied directly to the cut surface; (c) pollinated buds were sprayed with 100 mM calcium nitrate solution for 5 sec; (d) buds were then immediately sprayed with 2.2% sodium alginate solution for 5 sec; (e) a protective gel formed around pollinated buds. Bars = 1 cm

6.3.5 Seed set

All capsules remaining at maturity (10 months after pollination) were harvested and allowed to dry out in the laboratory and release their seed. The number of viable seeds in each capsule were counted. Seeds were considered viable if they were rounded, solid and dark in colour as opposed to flat and possessing a light-brown colour (Pound *et al.*, 2002).

6.3.6 Molecular marker analysis of pollen contamination

Molecular marker analysis was performed on leaf samples from progeny of seed parents B0133 and T1144, with 20 individuals per treatment chosen for parentage analysis. DNA was extracted using the Qiagen DNeasy Plant Kit (QIAGEN, Valencia, CA, USA). The contamination rate of each of the treatments was determined using microsatellite markers (Brondani *et al.*, 1998) to test for non-parental (contaminant) alleles in each progeny set. Eight highly informative microsatellite markers (*viz.* EMBRA 37, EMBRA 45, EMBRA 48, EMBRA 56, EMBRA 94, EMBRA 98, EMBRA 219, EMBRA 227) were used to ensure adequate power to discriminate closely related pollen contaminants from pollen used in the CP trials.

6.3.7 Statistical analysis

SPSS Version 15.0 was used for all statistical analyses. Pollen viability and seed set per flower pollinated data were subjected to Analysis of Variance (ANOVA) and Duncan multiple range tests. Percentage pollen viability was angular transformed prior to the analysis.

6.4 RESULTS

6.4.1 *In vitro* pollen germination

There were significant differences between pollen batches used in the controlled pollination experiments ($F = 11.492$; $P = 0.009$). Genotype T1087 exhibited the highest *in vitro* pollen germination ($64.0 \pm 1.7\%$) and genotype T1074 the lowest ($38.7 \pm 6.1\%$; Table 6.1).

Table 6.1: *In vitro* germination of *Eucalyptus grandis* pollen used in controlled pollinations. Letters (a) and (b) indicate statistical significance ($P < 0.05$), where treatments indicated by the same letter are not significantly different.

Species	Genotype	N	Mean \pm std error (%)
<i>E. grandis</i>	T1087	3	64.0 \pm 1.7 ^a
<i>E. grandis</i>	B0133	3	58.0 \pm 2.1 ^a
<i>E. grandis</i>	T1074	3	38.7 \pm 6.1 ^b

P = 0.009

6.4.2 Controlled pollination

In terms of seeds per flower pollinated, there were significant treatment effects in genotype T1099 ($F = 3.872$; $P = 0.006$), with exclusion bag isolation achieving the highest seed yields in both T1099 x T1087 and T1099 x B0133 crosses (Figure 6.2).

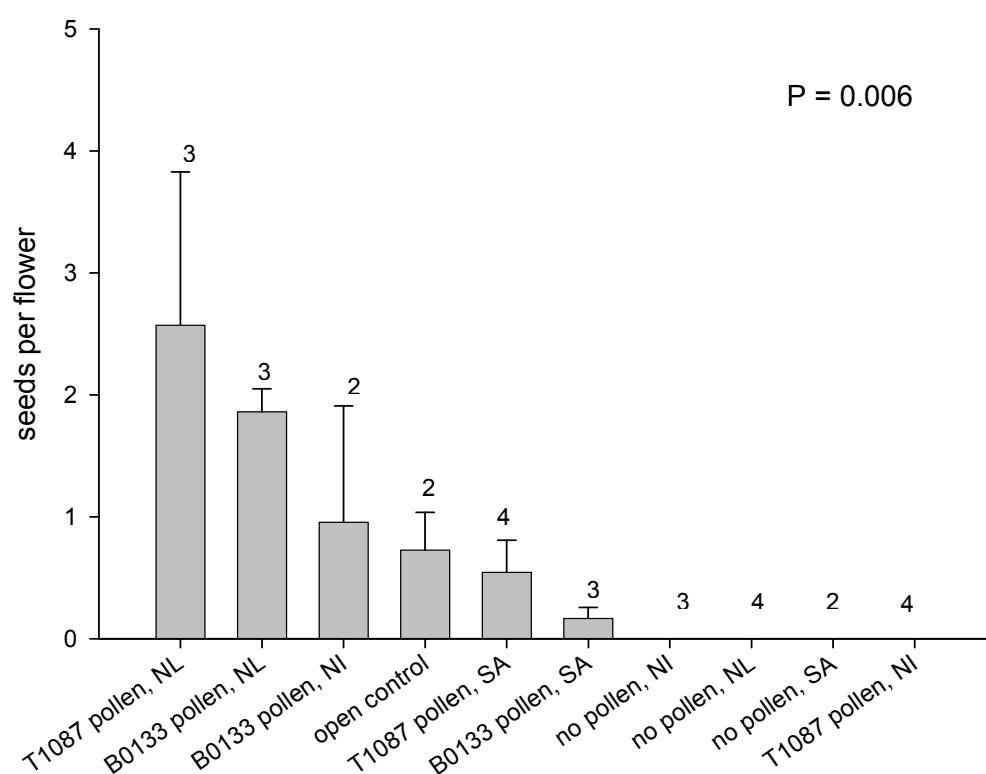


Figure 6.2: Seeds per flower pollinated observed in *Eucalyptus grandis* maternal genotype T1099, after performing AIP-controlled pollinations in combination with different methods of floral bud isolation. NI refers to non-isolation, NL to exclusion bag isolation and SA to sodium alginate isolation. Error bars represent standard error of the mean, with numbers above the error bars showing the number of replications per treatment. On average, each replicate consisted of three umbels, with seven flowers per umbel.

For all three isolation treatments conducted on T1099, no seed was obtained when AIP was performed without manual application of pollen. Genotype T1144 also displayed significant differences ($F = 5.225$; $P < 0.001$) for seeds per flower pollinated, with the non-pollinated sodium alginate isolation the only treatment not producing any seed (Figure 6.3).

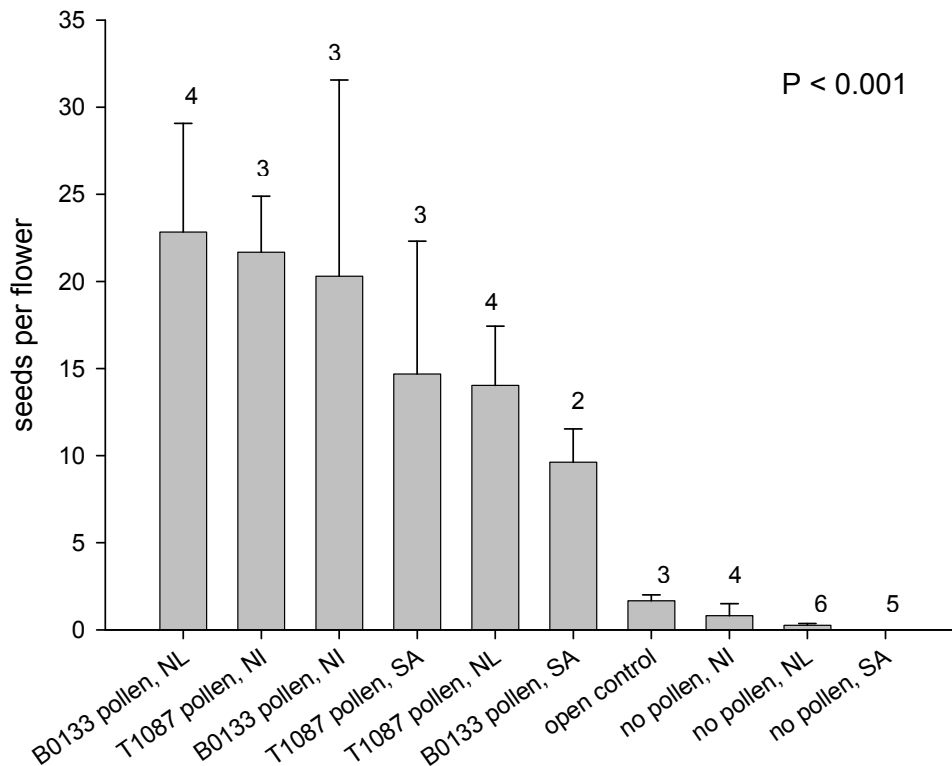


Figure 6.3: Seeds per flower pollinated observed in *Eucalyptus grandis* maternal genotype T1144, after performing AIP-controlled pollinations in combination with different methods of floral bud isolation. NI refers to non-isolation, NL to exclusion bag isolation and SA to sodium alginate isolation. Error bars represent standard error of the mean, with numbers above the error bars showing the number of replications per treatment. On average, each replicate consisted of three umbels, with seven flowers per umbel.

There were no significant differences ($F = 0.743$; $P = 0.596$) between isolation treatments in genotype B0133. In all three maternal genotypes, controlled pollination generally led to an increase in seed yields compared to natural pollination (open controls).

6.4.3 Molecular marker analysis of pollen contamination

Sodium alginate isolation was effective in excluding both self- and foreign-pollen from the stigma, and progeny derived from this isolation method were found to be 100% outcrossed with the applied pollen (Table 6.2). The exclusion bag, on the other hand, was not as effective, with the no-pollen treatment of seed parent B0133 being particularly contaminated with high amounts of self-pollen (67% selfing). Non-isolated treatments had lower contamination than the exclusion bag treatments, although selfing was still evident (11% selfing in the no-pollen treatment of seed parent B0133). There was an unexpectedly high amount of selfing in the B0133 open control (45%), compared to an absence of selfing in the T1144 open control (Table 6.2).

Table 6.2: Molecular marker analysis of pollen contamination in *Eucalyptus grandis* progeny created by the AIP method of controlled pollination in combination with different methods of flower isolation. A total of 20 progeny from each treatment were fingerprinted.

Isolation method	Maternal parent	Pollen applied	Contribution to progeny (%)		
			Applied pollen	Self pollen	Foreign pollen
sodium alginate	B0133	T1074	100	0	0
sodium alginate	T1144	B0133	100	0	0
sodium alginate	T1144	T1087	100	0	0
exclusion bag	B0133	T1074	95	0	5
exclusion bag	B0133	none	*	67	33
exclusion bag	T1144	B0133	95	5	0
exclusion bag	T1144	T1087	100	0	0
exclusion bag	T1144	none	*	0	100
non-isolated	B0133	T1074	100	0	0
non-isolated	B0133	none	*	11	89
non-isolated	T1144	B0133	100	0	0
non-isolated	T1144	T1087	100	0	0
non-isolated	T1144	none	*	0	100
OP control	B0133	none	*	45	55
OP control	T1144	none	*	0	100

* no pollen manually applied

6.5 DISCUSSION

Of the two CP-isolation methods tested here, sodium alginate isolation appears to be the most promising for application in eucalypt commercial controlled pollinations when using the AIP technique. Sodium alginate was effective in keeping external pollen away from the stigma, since no seed was produced in those treatments that were not manually pollinated but isolated in this way. In addition, flowers hand-pollinated and isolated with sodium alginate produced progeny that were 100% outcrossed with the applied pollen. The exclusion bag, on the other hand, was not as effective in protecting the stigma. Seed was produced in those treatments that were isolated with an exclusion bag without being hand-pollinated.

There is no doubt that the floral biology of eucalypts needs to be considered when developing a controlled pollination system (Moncur and Boland, 2000). Although there is a time separation between pollen-shed and stigmatic receptivity in a single flower, it is still possible for a high degree of selfing to occur in the crown of a single tree. Moran and Bell (1983) have predicted this to be in the region of 30% in natural eucalypt populations, but the present study suggests that the selfing rate in orchards can go as high as 45% (Table 6.2).

Prior deposition of self-pollen on the stigma may interfere with the flower's ability to use available cross-pollen, resulting in reduced seed set (Ramsey and Vaughton, 2000). Potential mechanisms of interference include clogging or blocking of stigma surfaces, stilar tissues or ovular micropyles and fertilising ovules that are later aborted due to late-acting self-incompatibility (Seavey and Bawa, 1986) or inbreeding depression (Waser and Price, 1991). In addition to late-acting SI, a previous study identified *Eucalyptus grandis* as also being cryptically self-incompatible (Horsley and Johnson, 2007). In species with cryptic SI, plants are able to set self-seed in the absence of competing cross-pollen (Bateman, 1956). Thus to produce a useful CP-system, it is evident that we need to develop methods to control selfing. Sodium alginate isolation appears to be useful in this regard, since there was no selfing in both 2005 and 2007 studies when using this method of isolation.

Eucalypts are largely insect-pollinated and successful pollinations are influenced by a number of factors, including diversity of flowering times, diversity in flowering

intensity and number of pollen vectors present at flowering (Eldridge *et al.*, 1993). The lower fruit set of naturally pollinated flowers (open controls) in 2007 compared to 2005 could be attributed to limited insect activity in the orchard. The 2005 study was carried out during peak flowering and seed yields from the B0133 open control were relatively high (10.72 seeds per flower), which is suggested to be the result of high insect activity. On the other hand, the 2007 study was carried out at the end of the flowering season and this could have resulted in less insect pollinators being present in the orchard (due to low numbers of flowers). This is reflected in the seed yields obtained from genotypes T1099 and T1144 open controls (0.77 and 1.56 seeds per flower, respectively).

Differences in flowering intensity between the 2005 and 2007 studies could also explain the differences observed in selfing rate between B0133 and T1144 open controls (45 vs 0%). In the 2005 study, individual trees had a higher flower density, which might have caused insect pollinators to remain within the canopy of the tree, thereby increasing selfing (Snow *et al.* 1996). In contrast, trees had a lower volume of flowers in 2007 (being at the end of the flowering season) and there were also fewer trees flowering in the orchard, making insect pollinators travel further distances and more often between trees, and thereby increasing outcrossing (Griffin and Ohmart, 1986; House, 1997). Levri (1998) noticed a similar effect of flowering intensity on selfing rate in *Kalmia latifolia* (Ericaceae). In that study, flowers receiving a mixed pollen load early in the flowering season exhibited a higher selfing rate, compared to flowers of the same age that received pollen later in the season (Levri, 1998).

Apart from physiological and biochemical factors, pollination is undoubtedly affected by weather conditions, such as wind and rain (Ortega *et al.*, 2007), making isolation of the control-pollinated flower imperative under field conditions. The major effect of wind is that it increases self-pollination by vibrating the branches of the tree, causing pollen to fall from the anthers of flowers higher in the canopy onto the receptive stigmas of branches lower down (Eldridge and Griffin, 1983). Rain reduces or inhibits pollinator activity and delays flower opening and anther dehiscence (Eisikowitch *et al.*, 1991). With respect to controlled pollinations, rain could also wash pollen off the stigma (Ortega *et al.*, 2007). All pollinations in the present study

were carried out during summer, which in KwaZulu-Natal, South Africa, is the rainy season, and thus could be a contributing factor to some of the low seed yields obtained. Genotype B0133 occurred in an orchard that consisted of more closely spaced trees than genotypes T1099 and T1144, and therefore B0133 may have been better shielded from rain and wind, giving rise to generally higher seed yields from both open- and control-pollinations. In addition to these environmental effects, the observed genotypic differences could also be attributed to genetic based differences in reproductive success (Callister, 2007; McGowen, 2007). Patterson *et al.* (2004) have shown that the proportion of capsules set following controlled pollination in *E. globulus* can range from 10 – 90% between female trees.

By reducing overlap between male and female reproductive functions, protandry is thought to reduce autogamous self-pollination (i.e. pollination of a flower by its own pollen) and self-pollen interference (Bertin and Newman, 1993). However, the *E. grandis* flowers in the present study were made artificially protogynous (stigma made receptive before anthesis) during the AIP method of controlled pollination, and were thus only partially effective in reducing within-flower selfing. This was confirmed by the high selfing (67%) obtained in the no-pollen exclusion bag isolated treatment from maternal genotype B0133 (Table 6.2). Since the stigma is made receptive before the flower opens during AIP, contamination may occur just after flower opening, when the cut style is still receptive and self-pollen is at its maximum viability (Assis *et al.*, 2005). It is therefore extremely important in the AIP method of controlled pollination that the isolation technique employed be highly efficient in excluding self-pollen.

6.6 CONCLUSION

From these results it is recommended that AIP-pollinated flowers be isolated to exclude foreign- and self-pollen when pollinations are performed under field conditions. Sodium alginate appears to be the isolation method of choice as, in addition to providing maximum protection to the stigma, it can be left on the tree to be disposed of by the ripening flower. Upon flower opening, the sodium alginate gel is shed naturally, increasing labour productivity as operators do not need to return to the tree to remove the isolation material, and hence reducing the cost of producing control-pollinated seed. The risk of physical damage to flower buds is also reduced

when using sodium alginate isolation, as flowers are not exposed to the stresses resulting from a hot and humid atmosphere as they would be within the bag during exclusion bag isolation.

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Appendix A: Mating design showing number of *Eucalyptus grandis* flowers pollinated per treatment

CROSS	ISOLATION TREATMENT		
	None	Exclusion bag	Sodium alginate
B0133 x T1074	102	106	110
B0133 x no pollen	116	102	*
T1099 x B0133	50	41	42
T1099 x T1087	40	39	43
T1099 x no pollen	81	95	73
T1144 x B0133	50	45	47
T1144 x T1087	42	50	48
T1144 x no pollen	80	91	78

*treatment not included

CHAPTER 7: SUMMARY AND CONCLUSIONS

In this study, I set out to develop techniques to improve the efficiency of eucalypt controlled pollinations in order to make it more cost effective for forestry companies to perform them on a commercial scale. Small-flowered species such as *E. grandis*, *E. urophylla*, *E. dunnii*, *E. smithii*, *E. nitens* and *E. macarthurii* were targeted, since I saw this as the gap in the application of eucalypt controlled pollination technology. I identified three study areas as key to the achievement of my aim, viz. pollen handling (Chapter 2), breeding systems (Chapters 3 and 4) and controlled pollination (CP) technique (Chapters 5 and 6).

At the very beginning, I thought it imperative to estimate pollen germination capacity before use in controlled pollinations, in order to avoid the costly use of non-viable pollen. Thus, one of my first aims was to determine an optimal medium for *in vitro* pollen germination, which could be used to reliably test the pollen viability of all six study species. I was able to identify a generalised liquid *in vitro* medium, consisting of 30% sucrose (w/v) and 0.15 mg l⁻¹ boric acid, for testing both fresh and 1-year old eucalypt pollen (Chapter 2). This made the task of pollen viability testing a lot simpler, since the same medium could be used to not only test different species, but to also test both fresh and stored pollen.

In addition to viability testing, storage of eucalypt pollen is generally required for controlled crosses, due to the asynchronous flowering of some species. My next aim was therefore to identify optimal temperatures for short-, medium- and long-term pollen storage. This led me to test the following four storage temperatures: 25°C (room), 4°C (refrigerator), -10°C (freezer) and -196°C (liquid nitrogen). Pollen samples of *E. grandis*, *E. smithii* and *E. nitens* were stored at these four temperatures for a 12-month period, with pollen viability tested every two months. I quickly ascertained that temperatures of around 25°C were not suitable for pollen storage, since there was a rapid decline in the germination of pollen that had been stored at room temperature (Chapter 2). On the other hand, temperatures cooler than 4°C appeared to maintain pollen viability for the duration of the 12-month study.

In light of these results, my recommendations were the following: while undertaking operational controlled pollinations within a season, storage of pollen at 4°C would be feasible over short periods of up to two months, -10°C would be suitable for medium-term storage (up to eight months), while cryopreservation would be ideal for storing pollen in gene banks over the longer term. In addition, an efficient cryopreservation protocol was also recommended for the study species, viz. gradual freezing of pollen by placement in a freezer for 8 hours before direct immersion in liquid nitrogen. Before use in controlled pollinations, the recommendation was to gradually thaw pollen by again placing in a freezer for 8 hours before leaving at room temperature overnight. Cryoprotectants were deemed unnecessary after I achieved relatively good germination and integrity of pollen grains upon retrieval from liquid nitrogen, adding to the simplicity and cost-effectiveness of the cryopreservation protocol.

Since the breeding system can have a major impact on seed set (Byers 1995), I thought it wise to first study the breeding systems of at least two of the study species before turning my attention to the development of a commercial CP-technique. During my literature search, I found a notable lack of information on comparative growth rates of self- and cross-pollen in the eucalypt pistil, with pollen-pistil interactions having only been studied in eight species to date, viz. *E. morrisbyi* (Potts and Savva 1988), *E. regnans* (Sedgley *et al.* 1989), *E. woodwardii* (Sedgley 1989; Sedgley and Smith 1989), *E. spathulata*, *E. cladocalyx*, *E. leptophylla* (Ellis and Sedgley 1992), *E. globulus* (Pound *et al.* 2002) and *E. nitens* (Pound *et al.* 2003). From this the third aim of the study was borne, namely to examine the growth rates of self- and cross-pollen tubes in the style following single-donor pollinations in *E. urophylla* and *E. grandis*. These two species were chosen since according to the literature, they had not been investigated before.

No evidence of self-incompatibility (SI) was found at the stage of pollen adhesion and germination in the stigmatic exudate, ruling out sporophytic self-incompatibility in the study species. The expression of SI occurred as pollen tubes grew down the style, resulting in a reduction in growth rate of self-pollen tubes relative to that of cross-pollen tubes (Chapter 3). This led me to suspect the presence of cryptic self-incompatibility (CSI), since the growth of self-pollen tubes was slower than compatible ones rather than completely inhibited, which according to Bateman (1956)

is a classic sign of CSI. Like Pound *et al.* (2003), I suggested late-acting self-incompatibility (LSI) as an additional SI mechanism on account of the low number of seeds set following self-pollinations relative to the abundant self-pollen tubes in the style. Generally in species with LSI, self-pollen germinates and reaches the ovules, but no fruit is set (Seavey and Bawa 1986). This is in contrast to species that exhibit CSI, where self-seed is set in the absence of competing cross-pollen. Another difference between CSI and LSI is that the latter can manifest both pre-zygotically with deterioration of the embryo sac prior to pollen tube entry or post-zygotically with malformation of the zygote (Sage *et al.* 1994), while CSI only manifests prezygotically by reducing pollen-tube growth rate (Bateman 1956). In the study species, the presence of both CSI and LSI contributes to the complexity of the breeding system.

Early-acting inbreeding depression could also be responsible for the reduced self-seed yields in the present study. However, this phenomenon is experimentally difficult to distinguish from LSI in a species (de Nettancourt 1977). In early-acting inbreeding depression, selfed pistil abortion is triggered by embryonic and/or endospermic lethal recessives (Klekowski 1988), while in LSI abortion of selfed pistils is a consequence of genetically controlled self-pollen tube recognition and rejection (Lipow and Wyatt 2000). These two types of self-sterility systems manifest themselves similarly, i.e. by low or no self-seed set, despite the apparently normal pollen tube growth into the ovary in self-pollinated pistils, and thus the confusion in distinguishing between them. Seavey and Bawa (1986) suggest that uniform ovule abortions may indicate an SI-response, whereas ovule abortions occurring at various stages of embryo development would be indicative of inbreeding depression. I tend to agree with Waser and Price (1991) who question whether inbreeding depression could account for very high levels of ovule abortion – hence my suggestion of LSI as being responsible for the observed low self-seed yields in *E. urophylla* and *E. grandis*.

In a follow-up study, molecular markers were employed to examine the siring ability of self- and cross-pollen after both mixed- and single-donor pollinations were performed on *E. grandis*. According to Bateman (1956), the simultaneous presence of cross- and self-pollen on the stigma of species exhibiting CSI should result in

higher seed-set from cross-pollen relative to self-pollen, while self-pollination without the presence of competing cross-pollen should result in successful self-fertilisation and seed set. I discovered that not only were progeny resulting from the mixed (self + outcross) pollinations 100% outcrossed, but that 57% of the progeny resulting from single-donor self-pollinations were selfs (Chapter 4). In addition, there was a significant change in the self:outcross seed ratio between single- and mixed-donor pollinations ($\chi^2 = 13.566$; $P < 0.001$). This suggested that the observed deficit of selfed seeds in mixed-donor fruits could be the result of differential pollen tube growth, strengthening the case for CSI in *E. grandis*. Seed yields from mixed-donor pollinations were not significantly different from those of single-donor outcross-pollinations, once again suggesting LSI. These results also suggested that the occurrence of LSI was likely pre-zygotic. If the absence of selfed progeny from mixed-donor pollinations were due to the abortion of selfed embryos (i.e. post-zygotic), we would expect seed yields to be more markedly reduced following mixed-donor pollinations compared to single-donor outcross-pollinations, due to ovules being discounted by self-pollen. A more likely scenario is the combination of slower self-pollen tube growth (CSI), together with LSI (occurring before fertilisation), resulting in outcross-pollen having the competitive advantage during mixed-donor pollinations.

After gaining some insight into the breeding system, I turned my attention to the final area of research, viz. controlled pollination technique. In seed orchards, pollen limitation and availability of compatible mates may interact to decrease seed set. If plants receive little pollen, and most of it is not compatible, the resulting seed set would be expected to be especially low. Besides limited seed set, additional consequences of limited pollen quantity are a decrease in the quality of seed (due to lack of competition among pollen-donors) or an increase in self-pollination (Karoly 1992; Richardson and Stephenson 1992). Self-incompatible species receiving mostly self-pollen will have reduced seed set due to either interference by self-pollen or reception of inadequate compatible pollen (Whistler and Snow 1992). Controlled pollination can alleviate these limitations by controlling both the quantity and quality of pollen available to the flower.

In an effort to identify a technique that could be used for commercial CP-seed production of small-flowered eucalypts, I compared the efficiency of three CP-methods, viz. the Conventional method (van Wyk 1977), One Stop Pollination (OSP, Harbard *et al.* 1999) and Artificially Induced Protogyny (AIP, Assis *et al.* 2005). Three types of buds were tested while carrying out AIP, viz. green (20 – 10 days before operculum lift), semi-ripe (10 – 3 days before operculum lift) and ripe (2 – 0 days before operculum lift). Species included as maternal parents were *E. grandis*, *E. smithii* and *E. macarthurii*, and even though only one genotype was tested per species, the fact that pollination occurred in the same way in all three species allowed me to treat the genotypes as replicates of the pollination methods. I used seed yield and degree of genetic contamination as criteria for comparing CP-techniques.

Of the three techniques, the AIP method not only produced the highest seeds/flower pollinated when ripe and semi-ripe buds were used, but also reduced the time to perform pollinations since flowers were not emasculated, nor isolated when performing this technique (Chapter 5). Indicative costs based on mean seed yields obtained from *E. grandis* (viz. 10 seeds/flower pollinated) showed that this technique could lead to a substantial reduction in total costs, with approximately 50% reduction in labour costs alone. This was due to the higher number of flowers that could be pollinated in one hour by one labour unit when using AIP (300 flowers), compared to OSP and Conventional methods (75 and 56 flowers, respectively). However, molecular marker analysis revealed extremely high levels of genetic contamination in resulting progeny, with 5 – 80% self-pollen contamination and 5 – 35% outcross-pollen contamination across all three CP-methods. In the case of OSP and Conventional CPs, I suspected that inadequate isolation material (Quick-dry nappy-liners, manufactured by Unsgaard Packaging Ltd, South Africa) could have been the cause of such high contamination, given the fact that these were well established CP-techniques. It was also obvious that flowers needed to be isolated when performing AIP under field conditions, especially when using self-compatible genotypes.

In addition to high capsule abortion, none of the applied pollen contributed to the progeny when AIP was performed on green buds, possibly due to the inability of

pollen to adhere to the stigma. I would therefore recommend that green buds be avoided when carrying out CPs. A better understanding of flower anatomy and associated pollen behaviour of the species may improve CP success rate, as pointed out by Williams *et al.* (1999). In the present study, *E. grandis* produced higher seed yields (10 – 18 seeds/flower) following cross-pollinations, compared to *E. smithii* and *E. macarthurii* (both exhibiting less than 1 seed/flower) and this could be related to flower size. Flowers are generally larger in *E. grandis* (0.8 x 0.5 cm), followed by *E. smithii* (0.7 x 0.4 cm), with *E. macarthurii* (0.5 x 0.3 cm) being the smallest (Brooker and Kleinig 1983). As a result, *E. smithii* and *E. macarthurii* flowers were possibly more easily damaged during controlled pollinations compared to *E. grandis* flowers. It is also feasible that the smaller-flowered species could possess fewer ovules compared to *E. grandis*, resulting in fewer seeds being formed. This differential pollination-success between large and small flowered eucalypts has been previously reported (Pryor 1956; Tibbits 1986, 1989; Gore *et al.* 1990).

The combination of small flower size and seven-flower umbel arrangement in the study species made emasculating of flowers during the OSP and Conventional methods quite tedious. In addition, I felt that these CP-techniques could work out to be prohibitively expensive when carried out on a large scale, due to labour costs. The fact that flowers were not emasculated during AIP, in addition to the high seed yields obtained, made this technique a very attractive option. However, the high rates of pollen contamination suggested that an effective flower-isolation method was needed to complement this technique. Apart from physiological and biochemical factors, pollination is undoubtedly affected by weather conditions, such as wind and rain, making isolation of the control-pollinated flower imperative under field conditions. The major effect of wind is that it could increase self-pollination by vibrating the branches of the tree, causing pollen to fall from the anthers of flowers higher in the canopy onto the receptive stigmas of branches lower down (Eldridge and Griffin 1983), while rain could possibly wash pollen off the stigma (Ortega *et al.* 2007). An additional point to bear in mind is that since the style is cut before the flower opens during AIP, contamination may occur just after flower opening, when the cut style is still receptive and self-pollen is at its maximum viability (Assis *et al.* 2005). I therefore thought it extremely important that the isolation technique employed be highly efficient in excluding self-pollen.

I compared the current method of flower isolation using exclusion bags to a novel method which used sodium alginate gel. The idea of sodium alginate isolation was borne after a discussion with a colleague about 'artificial seed' technology. Artificial seeds are created during somatic embryogenesis, where somatic embryos are coated with sodium alginate in order to protect them. Since the sodium alginate gel allowed embryos to successfully germinate, I hoped that it would have a similar effect on pollen tube germination. Molecular markers revealed that progeny were 100% outcrossed with the applied pollen, indicating that the gel did not inhibit pollen germination and pollen tube growth. Results also showed that the sodium alginate gel was naturally shed from around the flower upon operculum-fall, therefore still keeping the cost of AIP-pollinations low (Chapter 6). The risk of physical damage to flower buds was also reduced during sodium alginate isolation, as flowers were not exposed to the stresses resulting from a hot and humid atmosphere as they were within the exclusion bag. In addition, sodium alginate was effective in keeping external pollen away from the stigma, since no seed was produced in those treatments that were not manually pollinated but isolated in this way. On the other hand, the exclusion bag proved inferior for flower isolation, since seed was produced in those treatments that were isolated with the bag without being hand-pollinated. This is in accordance with previous results (Chapter 5), where I suspected that inadequate isolation material could have contributed to the high pollen-contamination observed after OSP and Conventional pollinations.

This is the first study to provide recommendations for efficient pollen handling of species such as *E. nitens*, *E. grandis*, *E. dunnii*, *E. smithii*, *E. macarthurii* and *E. urophylla*. The very few pollen studies in the literature have concentrated on *E. maculosa* (Boden 1958), *E. globulus*, *E. morrisbyi*, *E. ovata* and *E. urnigera* (Potts and Marsden-Smedley 1989). This is also the first study to identify cryptic self-incompatibility as a possible incompatibility barrier in the *Eucalyptus* genus. The difference in *in vivo* pollen tube growth rate observed between cross- and self-pollen tubes is noteworthy. No other study on *Eucalyptus* has looked at pollen tube growth rate *per se*. Previous work has concentrated on numbers of pollen tubes in the style, percentage of ovules penetrated, and amount of seed produced (Potts and Savva 1988; Sedgley *et al.* 1989; Ellis and Sedgley 1992; Pound *et al.* 2002, 2003). During the controlled pollination studies, a novel flower-isolation method using sodium

alginate was developed, which in conjunction with AIP, could make commercial CPs on small-flowered eucalypts a practical reality.

As a final point, it should be noted that the genetic base for the breeding system and controlled pollination experiments were extremely narrow. The results should therefore be used with caution when extrapolating to the species level, as different genotypes may behave differently. A more general case could be made by repeating this study using additional genotypes. A limitation of the statistical analysis for the CP-studies is that the mean number of seeds set per flower pollinated was calculated for each isolation bag / pollination event (i.e. data were pooled). To provide more statistical power, future studies should keep seed per capsule data separate. Investigations on the effect of environmentally induced stress on inbreeding depression, pseudo- and cryptic-SI, and maternal control over seed provisioning are also warranted.

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