Factors affecting capsule and seed set in *Eucalyptus globulus* seed orchards

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Declaration

I hereby declare that this thesis does not contain any material which has been accepted for a degree or diploma by the University of Tasmania or any other institution. To the best of my knowledge, this thesis contains no material previously published or written by another person except where due reference is made in the text of this thesis.

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Abstract

Low capsule seed set is a major factor limiting seed production in *Eucalyptus globulus* seed orchards. Trials were conducted in two Tasmanian *E. globulus* seed orchards to identify mechanisms involved in capsule abortion and low reproductive success. Key areas of investigation included identification of the timing of abortion relative to stage of capsule development, the impact of irrigation management and other treatments imposed to alter resource allocation, the influence of the maternal and paternal genotype, and relationship between floral characteristics and propensity of capsules to abort.

The major period of capsule abortion occurred between 20 and 80 days after pollination, coinciding with the period of capsule growth. A positive correlation between the number of fertilised ovules per aborted capsule and the length of time capsules were held on the tree was recorded. Given that capsule abortion occurred during a period of rapid fruit growth, and that capsules with the lowest number of fertilised ovules aborted first, it is argued that fertilisation level and resource availability to capsules are major factors determining abortion.

Flower density and irrigation treatments were imposed to assess the influence of resource allocation on capsule set. High flower density resulted in a high rate of abortion. Increased water availability resulted in increased vegetative growth which was associated with higher levels of capsule abortion. This suggested an irrigation mediated competition for resources between vegetative and reproductive sinks was

contributing to capsule abortion, and also suggested that irrigation management could be used to improve capsule yield within seed orchards.

Large variations in capsule and seed set, which combined determine reproductive success, between controlled crosses were identified. The contribution of the maternal and paternal parent to reproductive success and the genetic basis of this, were determined by the analysis of three data sets; operational scale breeding program crossing data, a full-sib mating scheme trial and pooled data obtained from the problem description and crop management trials within this PhD project. The analysis revealed that the variation in reproductive success was primarily determined by the maternal genotype and was heritable. The genetic differences in reproductive output appeared to be explained by differences in the physical properties of the flower, and the ability to support pollen germination and pollen tube growth.

In summary, capsule abortion mainly occurred during the period of capsule growth and the primary cause was concluded to be low levels of fertilisation. The level of fertilisation at which capsules were retained may vary depending on resource availability which is affected by factors including flower abundance, environmental conditions and crop management practices. Reproductive success has been shown to be primarily determined by the maternal parent and appears to be under genetic control, possibly resulting from genetic differences in the support of pollen germination and tube growth and floral physical characteristics.

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Preface

This thesis documents the research undertaken between November 2004 and February 2008. The project was initiated and funded by seedEnergy Pty Ltd, a copany specialising in forestry seed production through seed orchard management. Much of the research has been published, submitted or in preparation for publication, and the thesis structure has incorporated these manuscripts as research chapters. An introduction chapter provides an overall context for the research chapters and a general discussion expands on the discussions presented in the research chapters.

Additional results that were not published have been included as appendices.

Publications from this project are as follows:

Refereed journal papers

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Glossary

Maternal:	Female parent
Paternal:	Male parent
Diallel:	Crossing of each of several individuals with two or more others in order to determine the relative genetic contribution of each parent to specific characters in the offspring.
Ortet:	The original plant from which a vegetatively propagated clone has been derived.
ramet:	An individual member of a clone vegetatively propagated from an ortet.
Arboreta:	A collection of trees / genotypes.
UTAS :	University of Tasmania
STBA:	Southern Tree Breeding Association
SVP:	Single visit pollination
OSP:	One stop pollination
TVP:	Three visit pollination
OP:	Open pollination
CP:	Controlled pollination
UP:	Un-pollinated
MSP:	Mass supplementary pollination
PRD:	Partial root zone drying
RDI:	Regulated deficit irrigation
CI:	Conventional irrigation
NI:	No irrigation

Chapter 1

Project Background and Context

Industry background

Eucalyptus globulus Labill. (Tasmanian Blue Gum) is a forest and woodland tree, typically growing 15-60 m tall (Curtis and Morris 1975). It is often a dominant of coastal forests in south-eastern Australia (Williams and Potts 1996), specifically in Tasmania, the Bass Straight Islands and the coastal regions of Victoria on mainland Australia (Dutkowski and Potts 1999; Jordan *et al.* 1993). It is genetically variable across its geographic range and the broad-scale, quantitative genetic variation in numerous traits has led to its classification into a hierarchy of 13 races and 20 subraces (Dutkowski and Potts 1999). These races have been grouped to form three major lineages comprising the main populations from; (i) Victoria, (ii) King Island and Western Tasmania, and (iii) eastern Tasmania and the Furneaux Islands (Steane et al. 2006).

Eucalyptus globulus is the premier *Eucalyptus* species for pulpwood plantations in many temperate countries around the world including Australia, Chile, China, Columbia, Ethiopia, India, Peru, Portugal, Spain, USA and Uruguay (Eldridge *et al.* 1993; Potts 2004). It has proven to be the most adaptable and high yielding eucalypt species in temperate zones, leading to its widespread use in plantations. While plantations are primarily grown for pulpwood production, there is increasing interest in

their use for veneer and solid wood products (Greaves et al. 2004). The uniformity of eucalypt fibres relative to other angiosperm species, combined with their high fibre count, has created a high demand for eucalypt pulp for printing and writing paper (Kellison 2001).

Eucalyptus globulus is the main hardwood species grown in Australian plantations and is the eighth most planted forest tree species in the world (Varmola and Del Lungo 2003) and planting rates in the last decade suggest that there could be as much as 2.5 million ha of *E. globulus* planted (Potts et al. 2004). In 2005 the *E. globulus* plantation estate in Australia was 454,095 ha (Parsons et al. 2006). Recent reports document 700,000 ha of plantations in Portugal (Potts et al. 2004), 500,000 ha in Spain (Potts et al. 2004), 320,000 ha in Chile (Potts et al. 2004) and 268,000 ha in Uruguay (Dirección General Forestal 2005).

Seed production

Global expansion of *E. globulus* production and increased competition for land has led to increased focus on both selection of planting material and on methods of propagation. *Eucalyptus. globulus* breeding programs have been established in Argentina, Australia, Chile, China, Ethiopia, Portugal, Spain and Uruguay (Potts *et al.* 2004) and clonal propagation has been used in countries such as Chile (Griffin 2001), Portugal (Araújo *et al.* 1997) and Spain (Toval 2004). *Eucalyptus globulus* is deployed in plantations by either vegetative propagation or seed. However vegetative or clonal propagation is expensive and difficult (Dutkowski and Whittock 2004) with poor rooting rates for the majority of genotypes (Potts 2004) and is therefore , only used

partially for deployment in Portugal (Araújo *et al.* 1997), Spain (Toval 2004) and Chile (Griffin 2001). Deployment from seed is the more common method in the forest industry (Griffin 2001) and most plantations in Australia have been established using seedlings rather than vegetatively propagated material. While early plantations were established from open-pollinated seed sourced directly from the wild (Eldridge *et al.* 1993), improved seed is now mainly derived from open-pollinated seedling (Griffin 2001) or grafted (Patterson *et al.* 2004b) seed orchards, or through large-scale manual pollination systems (Patterson *et al.* 2004a).

Traditionally, seed production in orchards was achieved through open-pollination (OP) (Griffin 2001); however, *E. globulus* has a mixed mating system (Hardner and Potts 1995a; Pound *et al.* 2002b) and is susceptible to the effects of inbreeding depression (Hardner and Potts 1995a). Inbreeding depression can adversely affect the productivity of plantations, with a 48% reduction in the volume growth of trees from selfed progeny compared with fully outcrossed progeny (Hardner and Potts 1995a). Therefore seed from OP can contain self-pollinated individuals, and as a result it is sub-optimal for deployment purposes (Patterson *et al.* 2004b).

Large-scale manual pollination is beginning to replace OP seed production in several countries (Harbard *et al.* 1999; Leal and Cotterill 1997; Patterson *et al.* 2004b) as higher genetic gains are expected (Eldridge *et al.* 1993). The traditional controlled-pollination (CP) technique (TVP) can take up to 4 weeks involving three visits and is therefore costly (Eldridge *et al.* 1993; Harbard *et al.* 1999; Tibbits *et al.* 1997; Williams *et al.* 1999). Refinements have brought about the single-visit pollination (SVP) (Williams *et al.* 1999) or the one-stop pollination (OSP) (Harbard *et al.* 1999)

techniques. These techniques are effectively the same, except that they involve cutting the style either transversely (SVP) or longitudinally (OSP). They take advantage of the finding that, in *E. globulus* and some other species, cutting the style allows pollinations to be performed before stigma receptivity (de Arellano *et al.* 2001; Harbard *et al.* 1999; Trindade *et al.* 2001; Williams *et al.* 1999). This finding has led to the combination of emasculation, pollination and isolation into a single visit (Patterson *et al.* 2004a) greatly reducing the labour involved with controlled pollination (Harbard *et al.* 1999) and revolutionising breeding and deployment strategies for *E. globulus*. The OSP technique is preferred in some countries such as Chile (Griffin 2001; Harbard *et al.* 2000) as it eliminates selfing, increases seed yield and allows specific genetic combinations to be exploited (Patterson *et al.* 2004a). However, high labour costs make the technique expensive and not competitive with OP for large-scale production of elite *E. globulus* seed in Australia.

Mass supplementary pollination (MSP), a technique that involves no emasculation or isolation, has been developed in Australia and has reduced labour costs compared to OSP (Patterson *et al.* 2004a). This technique involves the removal of 1 mm of the tip of the style and application of pollen to the cut surface of flowers 1 to 7 days post anthesis. MSP is now routinely used (Callister and Collins 2007), to produce elite full-sib families allowing for the utilization of non-additive, specific combining effects in addition to additive genetic effects in deployment programs (Patterson *et al.* 2004a). Although the level of contamination with MSP is estimated to be about 13%, most of this is from high genetic quality outcross pollen (Patterson *et al.* 2004a). The labour savings are believed to be sufficient to outweigh the slight decrease in genetic purity of the resulting seed (Patterson *et al.* 2004a).

With the introduction of seed orchards and hand pollination, *E. globulus* seed production has evolved into a relatively intensive process. However, one of the major threats to the viability of these seed orchards is that a significant proportion of the hand-pollinated flowers do not set fruit (Espejo *et al.* 2000). Fruit for eucalypts is a woody capsule and *E. globulus* capsules are amongst the largest for all eucalypt species (Curtis 1965). Patterson *et al.* (2004a) reported capsule set for trees in an Australian seed orchard following CP to range from 10% to 90%. Studies of *E. globulus* seed orchards in Portugal have shown capsule set to vary significantly between production seasons and between specific trees within the orchard (Leal and Cotterill 1997). As hand-pollination is a labour-intensive process, this loss can have a major impact on the profitability of production.

Life cycle, from bud initiation to capsule set.

The onset of sexual reproduction in *E. globulus* is normally associated with adult leaves and first occurs in plantations at three to four years of age (Barbour *et al.* 2007), but may vary with orchard management techniques (Griffin *et al.* 1993). *E. globulus* flower buds take approximately one year to develop from initiation to flowering (Espejo *et al.* 1996). The timing of flowering is genetically variable both between and within races/subraces (Gore and Potts 1995). The flowers of *E. globulus* are bi-sexual, protandrous and relatively large (Gore *et al.* 1990). They produce copious nectar and are mainly pollinated by birds and insects (Hingston *et al.* 2004). It takes approximately one year from pollination to capsule maturity and harvesting (Potts *et al.* 2007). This period between the fertilisation of the ovule and the development of the

mature fruit is termed "fruit set" (Kozlowski 1997). However, not all capsules reach maturity with capsules aborting at some stage between fertilisation and maturity. The timing and causes of this phenomenon have not been studied in *E. globulus.*

Capsule or fruit abortion

The lack of knowledge on the causes of capsule abortion in *E. globulus* is possibly most likely due to the small size and recent emergence of the industry. Capsule or fruit abortion is however not only an issue in *E. globulus* seed orchards; it is a widespread problem throughout horticulture and forestry (Stephenson 1981). Based on what is known from studies on horticultural species, it can be concluded that final seed and fruit set is limited by numerous factors occurring throughout the reproductive process. These include the quantity, quality and genetics of pollen transferred (Ehlers 1999; Wesselingh 2007), successful fertilisation (Taylor and Whitelaw 2001), the amount of nutrients and photosynthate available for allocation to fruits and seeds (resource limitation) (Wesselingh 2007), pests and disease agents, and the physical environment (Taylor and Whitelaw 2001).

The effect of pollination on fruit set and the influence of the resultant seed on fruit growth make pollination a crucial phase in the production of many fruit crops (Janick 1979). Fertilisation is often dependent on cross-pollination and low fruit set and low seed number per fruit is often associated with an inadequate supply of enough (Trueman and Wallace 1999) compatible viable pollen (Burd 1994; Wesselingh 2007). Inadequate supply of viable outcross pollen is common in systems dependent on natural pollinators (Hingston and Potts 1998). Natural variation in the number of

pollen grains deposited on stigmas leads to variance in seed number on a given individual, resulting in the likely abortion of the fruits with the low seed numbers (Stephenson 1981). In hand-pollinated systems pollen availability is manipulated by adding ample pollen from a mix of outcross donors and is thus, not limiting (Patterson *et al.* 2004a; Wesselingh 2007). Therefore in these systems pollen quality, pollen compatibility and extent of damage caused to the individual flower during emasculation are possible limitations (Sedgley and Griffin 1989).

Even if pollination takes place, fertilisation is not absolutely assured. The lack of fertilisation accounts for most of the reduction in seed set and fruit set, and is caused by failure of the pollen tube to grow into the micropyle (Taylor and Obendorf 2001). Once deposited on the stigma, pollen germination is dependent upon the presence of a stigmatic secretion of the proper osmotic concentration (Herrero and Hormaza 1996; Janick 1979). Once germinated, pollen tubes must reach the ovary for fertilisation and if the growth of the pollen tube is very slow or ceases, the style or even the entire flower, may be shed (Janick 1979). The transmitting tissue not only provides a convenient pathway for pollen tube growth but is also the site of pollen tube competition and attrition (Erbar 2003). A reduction in the number of pollen tubes traveling along the style has been recorded in compatible pollinations in a number of unrelated species (Herrero and Hormaza 1996; Hormaza and Herrero 1999). Excess pollen deposition can result in stigma clogging, which has also been shown to impact negatively on fruit set (Ehlers 1999; Herrero 1992).

Once fertilised, seeds and fruit require maternal investment to develop (Lloyd 1980) and plants are viewed to have a limited level of resources available for reproduction

(Wesselingh 2007). A common theme within the literature is that resource limitation is the foremost constraint for successful seed and fruit set (Stephenson 1981; Taylor and Obendorf 2001; Wesselingh 2007). This appears to be due to initial overproduction of flowers and later reduction of the fruit crop due to limited resource capacity for fruit maturation (Burd 1998; Stephenson 1981). Many authors have noted the possible consequences of this excess flower production including increased attraction of pollinators, maximising genetic variability prior to selective abscission, the availability of excess flowers in case of loss or damage to others, and increased pollen production (Ayre and Whelan 1989; Bawa and Webb 1984; Burd 1998; Ehrlen 1990; Guitian *et al.* 2001; Wesselingh 2007).

The asynchronous timing of abortion of immature fruits in many species suggests that abortion does not always result from inadequate pollination, and that other factors are involved (Stephenson 1981). Several authors have considered the regulation of flower and fruit numbers to be an adjustment of maternal investment to match available resources (Lloyd 1980; Stephenson 1981). Rapidly growing fruits are strong sinks for assimilates and in situations where total assimilate demand exceeds supply, fruit with the lowest sink strength are more likely to abscise (Ruiz *et al.* 2001).A strong sink will pull nutrients from further away than a weak sink (Wesselingh 2007). The strength of a sink is at least partially determined by its metabolic activity, which in turn is related to the production of phytohormones by embryos and endosperms. Removal of developing seeds from fruit frequently terminates fruit growth (Addicott and Lynch 1955; Stephenson 1981).

Sink strength is not the only determinant of resource limited abortion, flower position within an inflorescence or in the flowering order can greatly influence the chances of acquiring sufficient resources for fruit set (Ashman and Hitchens 2000; Wesselingh 2007). When resources are limited, the reproductive structures located furthest from the source of resources are shed first (Stephenson 1981). The first flowers to bloom and start fruit formation, act as strong sinks for resources, and have an advantage over later developing flowers (Bawa and Webb 1984; Medrano *et al.* 2000; Stephenson 1981; Vallius 2000; Wesselingh 2007). Stephenson (1981) found that flowers are inhibited from setting fruits if other pollinated flowers and juvenile fruits are developing. This suggests that flowers and young fruits compete for limited maternal resources.

Fruit set is not only mediated by endogenous factors; there are many abiotic and biotic agents that damage fruit and promote abscission (Ehlers 1999; Stephenson 1981), including weather, light, nutrient levels and pests/diseases. Significant losses of immature capsules and seeds occur through premature death of twigs, storm activity and destructive foraging by birds and insects (Cunningham 1957). Drought conditions and other stresses that cause a deficiency in water, including salt, cold and high temperatures, can also promote abscission (Taylor and Whitelaw 2001). Late frosts are occasionally a principal cause of fruit mortality (Addicott and Lynch 1955) and low light interception has been shown to limit seed and fruit set (George *et al.* 1996; Wien 1997). Very young fruits are generally more susceptible to abiotic damage than older fruits (Stephenson 1981). Stress, associated with factors such as invasion by a pathogen, attack by a predator or a lack of water, may also induce abscission (Taylor and Whitelaw 2001). Insects may transmit pathogens or leave wounds where

pathogens may later enter, promoting abscission (Stephenson 1981). The selective abscission of damaged fruits can be viewed as a mechanism whereby plants terminate investment in fruits that contain offspring that would be unlikely to contribute to future generations (Stephenson 1981).

Management

While for *E. globulus* there are no specific management practices controlling capsule set, effective strategies have been developed for other species. An understanding of this existing knowledge may provide the insight required for the application of similar techniques to *E. globulus* seed orchards. Research of plant regulatory processes has allowed the development of techniques to manipulate plant reproductive output, and these include chemical application (Greene 2007; Sedgley and Griffin 1989), water management (Dry and Loveys 1998; Kang and Zhang 2004; Sedgley and Griffin 1989), nutrient management (Dennis 1979; Stephenson 1981) and physical manipulation of the plant (Dennis 1979; Rivas *et al.* 2006; Sedgley and Griffin 1989; Stephenson 1981).

Chemicals are used widely throughout horticulture to manipulate plant development, including fruit set. For example, the application of Prohexadione-calcium (ProCa), a chemical used to control vegetative growth, has been shown to increase fruit set in apple trees (Greene 2007). Post-anthesis exogenous application of auxins and gibberellins throughout fruit development can substitute to some extent for the normal endogenous production of these chemicals following pollination and fertilsation, and consequently enhance fruit set. Success has been greatest with species and cultivars

which already possess some degree of parthenocarpy (Sedgley and Griffin 1989) such as *E. globulus* (Chapter 2).

Efficient water management is an important issue in horticultural systems, not only due to the detrimental effects on plant growth associated with a lack of water (Kang and Zhang 2004), but also the positive impact water management has on the control of vegetative growth and the production of flowers and fruit (Dry and Loveys 1998). There are two common techniques, partial root zone drying (PRD) and regulated deficit irrigation (RDI), which are used to increase fruit set and quality by controlling irrigation so as to reduce vegetative shoot growth immediately after flowering (Sedgley and Griffin 1989). PRD is a technique primarily used in grapes, whereby approximately half of the root mass is irrigated while the other is left dry. As a result chemical signals produced by roots exposed to drying soil prompt a physiological response in the plant which results in higher fruit retention (Davies and Zhang 1991; Dry and Loveys 1998). RDI works on the premise that vegetative growth is more sensitive to water stress than fruit growth, thus calculated reductions in plant water availability results in decreases vegetative growth and increases fruit retention (Dry and Loveys 1998). RDI has been used successfully with pome and stone fruit, both experimentally (Chalmers *et al.* 1981; Mitchell *et al.* 1989) and commercially (Mitchell and Goodwin 1996) to reduce vegetative growth, increase fruit yield

Increases in seed and fruit yield are commonly observed following the application of fertilisers (Bawa and Webb 1984), with nitrogen in particular having been shown to retard and reduce abscission in apples (Addicott and Lynch 1955; Stephenson 1981). However an over supply of nitrogen may enhance vegetative growth at the expense of

reproductive growth (Menzel 1984; Pigearie *et al.* 1992). The application of macronutrient (NPK) shortly after flowering has been shown to significantly decrease fruit drop in several *Prunus* , *Pyrus* and *Asclepias* species (Stephenson 1981) Less is known about limitations caused by macronutrients (Stephenson 1981), however boron and iron have been shown to increase fruit set or yield of apples and supplementary zinc is also recommended in many horticultural crops (Dennis 1979). It is uncertain, however, whether these applied nutrients are used directly by the developing fruits or whether they have an indirect effect (e.g. increasing the rate of photosynthesis) (Stephenson 1981).

Physical methods of plant manipulation have also been shown to increase fruit set. In mandarin, stem girdling increased the soluble sugars content in fruitlets, reduced the daily fruit drop, and thereby diminished abscission (Rivas *et al.* 2006) Ringing or girdling disrupts the phloem and prevents the transport of photosynthate out of the branch where it was produced and thereby increases the photosynthate available to the fruits on the branch (Stephenson 1981). Vegetative growth is also generally reduced and the carbohydrate is available for increased flower and fruit development (Sedgley 1989). The removal of shoot tips in apple trees has also been shown to significantly reduce fruit drop in apples (Quinlan and Preston 1968). The choice of rootstock in fruit production orchards and grafted clonal tree-seed orchards can have important effects on a number of aspects of the reproductive cycle. Plant vigour is decreased and flowering and fruiting are increased (Sedgley and Griffin 1989).

There are a range of strategies which for the management of capsule set within horticultural species, which present viable opportunities for *E. globulus* capsule set

management. They are dependent on targeting the major processes limiting fruit or capsule abortion. Covering, orchard set up and the selection of parental material through to resource competition following fertilisation, with physical and chemical crop manipulation techniques available to influence each of these processes. Development of effective strategies to reduce capsule abortion in *E. globulus* will require identification of key processes contributing to abortion as well as assessment of techniques targeting these processes in other species.

Scope of the project

Due to the relatively recent development of the *E. globulus* seed production industry, there is little knowledge of the factors involved in capsule abortion and consequently no management techniques directly aimed at addressing the problem. Therefore, based on an understanding of the processes involved and the techniques used to manipulate them within other species, a great opportunity exists to increase the productivity of *E. globulus* seed orchards by reducing rates of capsule abortion. The objective of this study was to generate new knowledge on the flower abortion process in *E. globulus* and use this knowledge to develop management tools to reduce capsule abortion rates in seed orchards.

The research undertaken in the study and conclusions drawn from the results are presented in the following chapters. The first research chapter (Chapter 2), describes and defines the problem of capsule abortion in *E. globulus* seed orchards by identifying the timing of abortion, the influence of different pollination techniques and quantifying the timing of abortion in the reproductive cycle. Chapter 3 analyses the

impact of flower number and resource allocation on capsule abortion, then tests the potential of irrigation management techniques to manipulate resource allocation. In Chapter 4 the genetic control of the variation in reproductive success (seed per capsule x capsule set) within *E. globulus* seed orchards is defined. In the final research chapter (Chapter 5), possible causes of the genetic variation in reproductive success are identified as floral physical and physiological properties of pollen tube growth in the style in a case study of six genotypes from three races. The General Discussion expands on the discussions presented in the research chapters and includes recommendations for breeding choices, pollination procedures and orchard management to enhance the commercial productivity of the system.

Chapter 2

Post pollination capsule development in *Eucalyptus globulus* seed orchards

There have been no previous studies published on *Eucalyptus globulus* capsule abortion. Therefore this chapter aims to describe the problems experienced by seed orchard managers. This is achieved though identify the timing of abortion under different pollination techniques at different sites in different seasons, and at what stage in the reproductive process abortion is occurring. Data from this chapter has been presented in two papers at an international conference and three posters at two international and one national conference.

This chapter has been published as:

Suitor S, Potts BM, Brown PH, Gracie AJ and Gore PL (2008) Post pollination capsule development in *Eucalyptus globulus* seed orchards. *Australian Journal of Botany*. **56**, p51-58.

Introduction

Eucalyptus globulus Labill. (Tasmanian blue gum) is a forest tree native to south-eastern Australia (Dutkowski and Potts 1999; Jordan *et al.* 1993) However, "It is the hardwood species most widely planted for pulpwood in temperate regions of the world" (Eldridge *et al.* 1993; Potts 2004) with 454,095 hectares of plantations in Australia alone (Parsons *et al.* 2006). Breeding programs producing germplasm for industrial plantations have focused on improving growth, wood density and high pulp yield (Greaves *et al.* 1997). Although there is some clonal (or vegetative) deployment (Borralho *et al.* 1992b; Griffin 2001; Lopez *et al.* 2002), most improved plantations are established from seedlings derived from seed orchards due to their lower propagule cost (Patterson *et al.* 2004a).

Traditionally, seed production in orchards was achieved through open-pollination (OP) (Griffin 2001); however, given that *E. globulus* has a mixed mating system (Hardner and Potts 1995a; Pound *et al.* 2002b) and is susceptible to the effects of inbreeding depression seed from open-pollination can contain self-pollinated individuals and therefore be suboptimal for deployment purposes (Patterson *et al.* 2004a). Large-scale manual pollination is now beginning to replace open-pollinated seed production in several countries (Harbard *et al.* 1999; Leal and Cotterill 1997; Patterson *et al.* 2004a). The traditional controlled-pollination technique involves three visits and is relatively time consuming (Eldridge *et al.* 1993; Sedgley and Griffin 1989). Refinements have brought about the single-visit pollination (SVP) (Williams *et al.* 1999) or the one-stop pollination (OSP) (Harbard *et al.* 1999) techniques whereby cutting of the non-

receptive style coupled with single style or flower isolation has allowed pollination to be undertaken in one visit. Although OSP for mass seed production is used in countries such as Chile (Griffin 2001; Rojas Vergara *et al.* 2001) high labour costs make it expensive, and not competitive with open pollination for large-scale production of elite globulus seed in Australia. Therefore, "mass supplementary pollination" (MSP), a technique that involves no emasculation isolation has been developed (Patterson *et al.* 2004*a*). Although some minor contamination does occur with the MSP system, most contaminant outcrosses will involve other selected genotypes in the orchard, and the labour savings are believed to be sufficient to outweigh the slight decrease in genetic purity of the resulting seed (Patterson *et al.* 2004*a*).

A major problem identified in *E. globulus* seed orchards is that a significant proportion of hand-pollinated flowers do not set fruit (Espejo *et al.* 2000). Patterson *et al.* (2004*a*) recorded capsule set for trees in an Australian seed orchard following CP to range from 10% to 90%. Studies of *E. globulus* seed orchards in Portugal have shown capsule set to vary significantly between production seasons and specific trees within the orchard (Leal and Cotterill 1997). As hand-pollination is a labour-intensive process, this loss can have a major impact on the profitability of production.

"In horticultural crops research has shown final fruit set to be limited by numerous factors such as pollen transfer, fertilisation, resource allocation and environmental effects" (Sedgley and Griffin 1989). Fertilisation is often dependent on cross-pollination and low fruit set and low seed number per fruit is often associated with an inadequate supply of compatible viable pollen (Wesselingh 2007). Inadequate supply of viable outcross pollen is common in systems dependent on natural pollinators

(Hingston and Potts 1998), while in hand-pollinated systems pollen quality, pollen compatibility and extent of damage caused to the individual flower during emasculation are possible limitations (Sedgley and Griffin 1989). Rapidly growing fruits are strong sinks for assimilates and in situations where total assimilate demand exceeds supply, fruit with the lowest sink strength are more likely to abscise (Ruiz *et al.* 2001). It has been proposed that the production of excess flowers and young fruit is to maximise off-spring and genetic variability prior to selective abscission (Bawa and Webb 1984; Burd 1998; Wesselingh 2007).

With the increased use of more expensive manual pollination for production of improved *E. globulus* seed, there is now a greater requirement to minimise reproductive losses during seed development. An understanding of the ecological and physiological processes influencing capsule set and abscission is therefore important. The present study investigated the variation in capsule set between different pollination techniques, the time frame of capsule abortion, morphological development of capsules after pollination, and the influence of climatic events and fertilisation status of aborted capsules.

Materials and methods

Seed orchards

"*Eucalyptus globulus* trees used in his study were located in seed orchards at (i) Cambridge, south-eastern Tasmania (42º48'27.23"S, 147º25'58.48"E) and (ii) Ridgley on the north-west coast of Tasmania (41º08'51.52"S, 145º48'18.64"E)". The two sites represent the range of different environmental conditions for *E. globulus* seed production in Tasmania. Cambridge has an altitude of 40 m with an annual rainfall of 507 mm and average maximum temperature of 17.4ºC and an average minimum of 8ºC. Ridgley has an altitude of 275 m, an annual rainfall of 1200 mm, an average maximum temperature 16.1ºC and an average minimum of 7.7ºC. Trees within the Cambridge orchard are managed more intensively, whereby trees are maintained in a stunted from by pruning and plant growth regulator (paclobutrazol) application. All trees in the orchards were selected from the base population of the Australian National Breeding Program run by the Southern Tree Breeding Association (Pilbeam and Dutkowski 2004). The 21 different genotypes chosen for study had abundant flowers, and were unrelated first generation selections from the Furneaux, Western Otway and Strzelecki races as defined by Dutkowski and Potts (1999). The genotypes used encompassed a range of flowering times from September to January, with most variation attributed to the racial differences in flowering time within *E. globulus* (Gore and Potts 1995).

Experimental design

A controlled-pollination trial involving 21 genotypes was undertaken in 2004/2005 at the Cambridge site. Each female genotype was cross-pollinated (CP) with up to 26

different male genotypes, as a subset of a larger crossing program to generate a full diallel where all male/female crosses were made. Four flowers were pollinated for each male-female combination on each tree. Up to 100 flowers per tree were pollinated and between 20 and 60 flowers on each tree were labelled and left for open- pollination (OP). On each tree, at the same time as CP was performed, 16 flowers were isolated to act as un-pollinated controls (UP). An additional 16 flowers were selected and mass supplementary pollinated (MSP). Genotypes were located throughout the orchard and all pollinations and un-pollinated treatments were spread as even as possible within the canopy of each tree relative to flower availability. Trees were monitored twice weekly for capsule abortion over a period of seven months. Crossing was also conducted in Cambridge in 2005/2006, with three flowers pollinated for each male-female combination, but using some stored pollen of a lower viability than the previous year, when it was collected, along with some pollen that was collected in the same season.

In 2005/2006, a second trial with six genotypes (common to the 2004/2005 Cambridge crossing) was undertaken at Ridgley. Four flowers on each of the six genotypes were crossed with four male genotypes for a total of 16 CP flowers on each tree (all crosses were replicated in Cambridge 2004/2005). Sixteen flowers were labelled and left for OP, 16 were used as UP and a further 16 flowers were selected for MSP on each tree. Trees were monitored for capsule abortion every two weeks for a period of seven months.

Pollen collection and storage

All pollen was collected and stored as outlined by Potts and Marsden-Smedley (1989). Flowers that were close to shedding their operculum (anthesis) were harvested and

dried for 12-24 h at 25-30ºC. The anthers were gently rubbed so that the pollen fell onto a sheet of foil. Pollen was transferred to eppendorf tubes and stored at -18ºC. For testing viability, pollen were streaked across the surface of an agar medium containing 30% sucrose and 150ppm of boric acid (Potts and Marsden-Smedley 1989) in 8 x 8 cell replidishes, then incubated at 25ºC for 24 h with a 12/12 photo period, with at least three replicates for each pollen.

Pollination

Eucalyptus globulus flowers are bi-sexual with the style surrounded by anthers (Williams *et al.* 1999), in this study they were either solitary or in umbles of two to three. Controlled pollinations were undertaken using the single visit pollination procedure outlined by Williams *et al.* (1999) and Patterson *et al.* (2004*a*). Flowers were emasculated when the operculum began to change to a yellow colour and started to lift away from the receptacle (Harbard *et al.* 1999). Care was taken to avoid injuring the stigma, style and/ or the disc during emasculation as injury may lead to flower abortion (Taylor and Whitelaw 2001). The top 1 mm of the style was transversely cut, and then pollen was immediately applied to the cut style with a wooden toothpick dipped into an eppendorf tube containing the pollen. This procedure allows for pollinations to be performed before stigma receptivity (Trindade *et al.* 2001). A small white balloon was then placed over the flower to isolate it from all other pollen sources and the branch was labelled. Un-emasculated flowers on the same branch were removed with secateurs. In 2004/2005, pollen collected and stored that season was used for CP, with *in vitro* percentage germination averaging 12%. In 2005/2006, while some pollen collected in the same flowering season was used, most pollen was collected during the 2004/2005 crossing season. The percentage *in vitro* germination of

all pollens used for CP crossing in Cambridge 2005/2006 averaged 4.9%. Ridgley CP crossing in 2005/2006 was conducted with pollen collected in the previous season in Cambridge with the *in vitro* germination percentage averaging 3.1%. The reduced pollen viability with storage was atypical and may have resulted from insufficient dehydration during storage.

The operational MSP technique involved pollinating flowers one to seven days post anthesis. Following Patterson *et al.* (2004a), 1 mm was cut off the tip of the style using small scissors; a pollen mix was then applied to the cut surface using a small paint brush. Flowers that were not pollinated within seven days post-anthesis were removed with secateurs, to avoid self-pollination. Pollinations were made with a combined mix of pollen collected in the same season from five of the genotypes used as pollen parents in CP crossing. Different genotypes were used in each season and new pollen was collected each year.

UP flowers were emasculated when the operculum began to change to a yellow colour and started to lift away from the receptacle. An isolation balloon was then placed over the emasculated flower, preventing pollination, but in this case the style was not cut. For the OP treatment, buds were tagged and left to be pollinated naturally, which for this species is undertaken by birds and insects (Hingston and Potts 1998).

All pollination treatments for each genotype were carried out on the same day on the same tree. Approximately 12 months after pollination all CP, MSP and OP capsules were mature (Boland *et al.* 1980; Sasse *et al.* 2003a) at which stage they were harvested, placed into a number of individual paper envelopes and dried. Once dried,

the seeds were extracted and counted as viable (filled) following Hardner and Potts (1995a).

Capsule and ovule development

To monitor capsule growth, five capsules derived from 2004/2005 MSP were harvested weekly for 29 weeks from two trees at Cambridge, and their fresh weight obtained. One tree was from the Western Otway and the other from the Strzelecki race and the pollen applied was the same as that used for MSP 2004/2005.

After four weeks from pollination, aborted CP capsules were collected every three to four days and assessed for evidence of fertilisation. Capsules were identified by the presence of the remnants of pollination balloons and proximity to the labelled CP branches. Dissections were made using a blade to remove the top of the capsule, exposing the top of each ovary chamber (Pound *et al.* 2002a). Incisions were then made on both sides of an ovary chamber extending to the bottom of the capsules. A final cut was made from the outer tip of the ovary chamber downwards. The outer tissue was then forced outwards exposing the ovules. The number of large ovules were counted and classified as fertilised. "Fertilised ovules have been shown be clearly differentiated in size from unfertilised ovules in *E. globulus* by at least six weeks post-pollination" (Pound *et al.* 2002a).

Statistical analysis

Effects of pollination type (CP, MSP and OP) at Cambridge 2004/2005 on capsules set and the common genotype comparison between both orchards over both seasons were all examined by a one-way analysis of variance undertaken with PROC GLM in SAS version 9.1 (SAS Institute Inc 2003). PROC REG of SAS was used to fit a linear regression to the fertilisation status of aborted capsule data.

Results

Pollination types

Within the Cambridge site in 2004/2005 significant differences in capsule set were recorded between the three pollination treatments at harvest time ($F_{2, 60} = 7.35$; $P < 0.001$). No capsules set for UP, while CP (73.2%) set a significantly lower number of capsules than OP (87.9%) and MSP (90.2%) treatments (Table 1) Viable seed set per capsule also differed significantly between pollination types at Cambridge in 2004/2005 ($F_{2, 67} = 5.33$; $P < 0.008$). CP (45.3 seeds per capsule) was significantly higher than both MSP (30.8 seeds) and OP (21.3 seeds) (Table 1) Although MSP had an average of 9.5 more seeds per capsule than OP, the difference was not significant ($P > 0.05$). Only one out of 1237 harvested CP capsules contained zero viable seeds. In 2005/2006, when pollen viabilities were lower, seed set at Cambridge for CP averaged 18.8 seeds per capsule, and 27.5% of capsules set contained zero viable seeds. At Ridgley CP averaged only 0.7 seeds per capsule and 40% of the CP capsules set contained zero viable seeds.

Table 1 Capsule set, viable seeds per capsule and viable seeds per flower for each pollination type in Cambridge 2004/2005 all values are averaged across all genotypes.

Pollination type	Number of trees	Flowers pollinated	Capsule set (%)	Seeds per capsule	Seeds per flower
CP	21	1357	73.2	45.3	35.9
MSP	21	336	90.2	30.8	27.7
OP	21	670	87.9	21.3	19.9
UP	21	336	0	N/A	0
LSD (P=0.05)			9.6	14.6	19.8

When the six common female genotypes were compared for the success of MSP between seasons and sites, capsule set at Cambridge 2004/2005 (91.7%) was significantly ($F_{1,11}$ = 5.48; P < 0.05) higher than that of Ridgley 2005/2006 (58.2%) (Table 2). Cambridge MSP (36.5) and OP (27.9) seed per capsule were higher than that of Ridgley (22.9) and (15.1) (Table 2), although the difference was not significant ($F_{1,11}$ = 1.59; P > 0.05). Cambridge seed per flower MSP (34.5) and OP (27.9) was also higher than that of Ridgley (16.9) and (11.8), although the difference was not significant ($F_{1,11}$ = 2.1; P > 0.05).

Table 2 Capsule set, viable seeds per capsule and viable seeds per flower for MSP and OP in Ridgley and Cambridge in season 2005/2006, for six common female genotypes.

Orchard	Pollination type	Number of trees	Flowers pollinated	Capsule set (%)	Seeds per capsule	Seeds per flower
Cambridge	MSP	6.0	96	91.7	36.5	34.5
Ridgley	MSP	6.0	91	58.2	22.9	16.9
LSD (P=0.05)				31.8	24	27
Cambridge	OP	6.0	127	87.4	27.9	25.6
Ridgley	OP	6.0	93	69.3	15.1	11.8
LSD (P=0.05)				26.0	25.5	35

Timing of fall

Capsule abortion for MSP, CP and UP at both Cambridge and Ridgley occurred primarily between 20 and 80 days after pollination or isolation treatment (Figure 1). All UP flowers except one aborted by day 80. At both sites some MSP and CP capsules did abort after 80 days (CP until day 130 and MSP until day 125), but the level was very low.

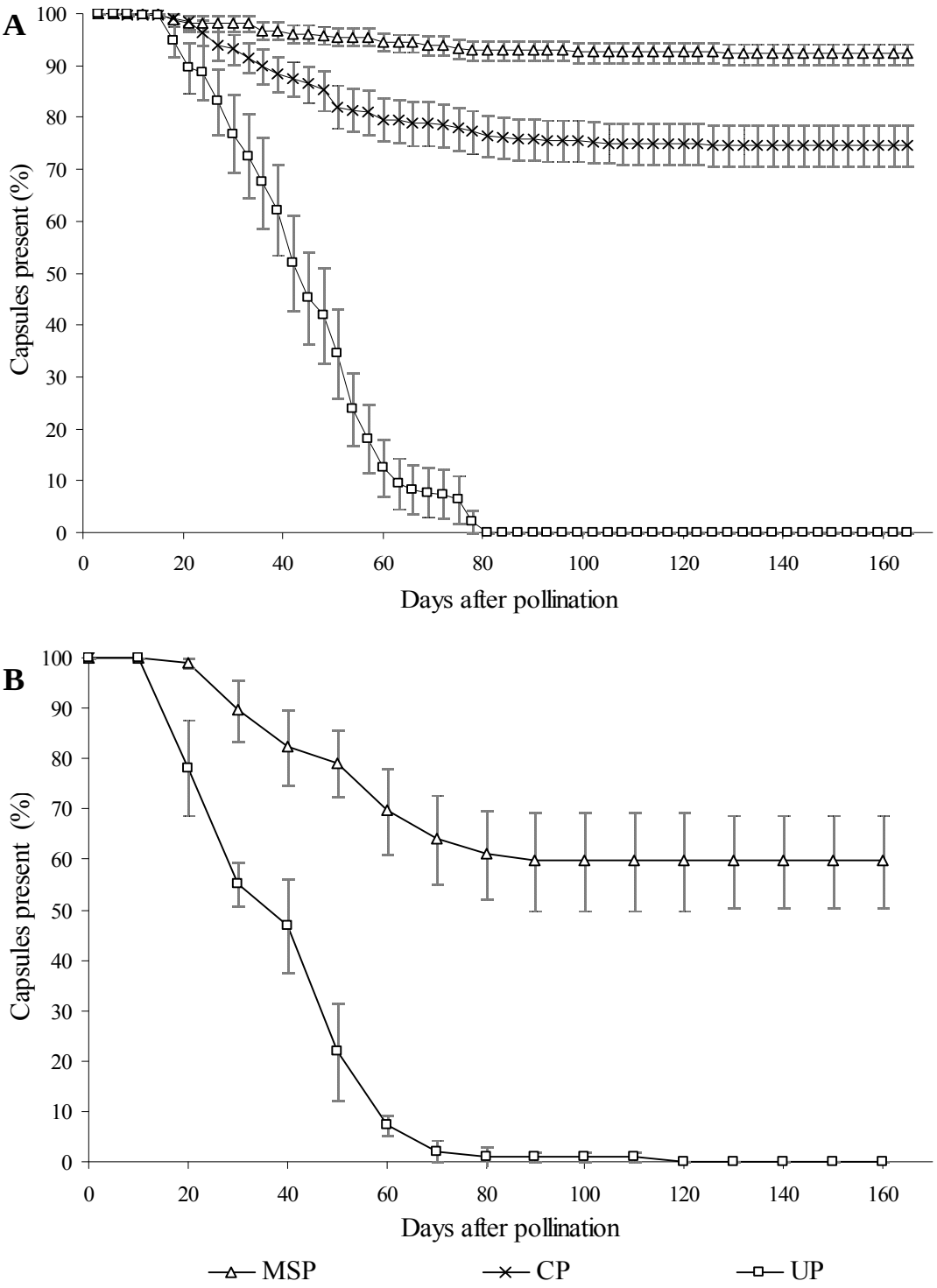

Figure 1 The mean (± s.e.) percentage capsules retained with time in (*A*) CP, MSP and UP in Cambridge for season 2004/2005 and (*B*) MSP and UP in Ridgley for season 2005/2006. Each value was a mean of 21 and six trees for Cambridge and Ridgley sites respectively.

Capsule development

Starting from the first sample date, seven days after pollination, average fresh capsule weight in Cambridge 2004/2005 increased linearly from 1.1g to 3.1g by day 70, after which there was effectively no weight gain, at least up until the sampling ceased at day 147 for both races (Figure 2). At this last sampling date, the average fresh weight was effectively the same as that at day 70.

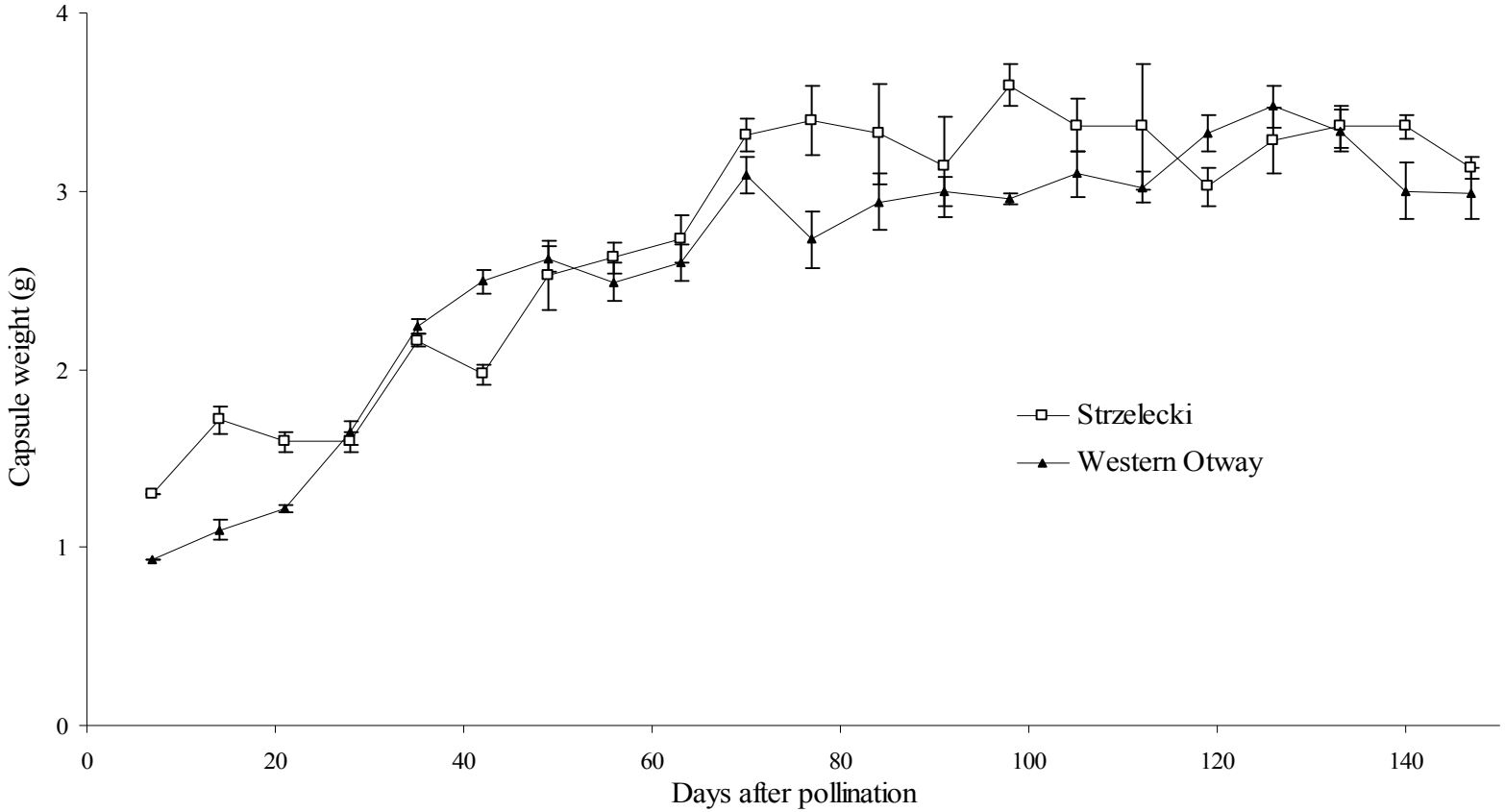

Figure 2 Mean (± s.e.) capsule fresh weight starting from seven days after pollination, for two trees, from the Strzelecki and Western Otway races, at Cambridge assessed in 2004/2005. Each point is an average of five MSP capsules.

Ovule development

A significant linear relationship ($Y = 0.18X - 0.71$, $r^2=0.75$; $P < 0.001$) was identified between the number of fertilised ovules within aborted CP capsules (Y) and the time after pollination that capsules aborted (X) (Figure 3). Distinct differences in size between ovules within the same locule were evident by the first sampling at 28 days, consistent with the larger ovules being fertilised. From day 28 up until 70 days after pollination, the average number of fertilised ovules per aborted capsule was consistently below 0.5. The levels increased steadily up until day 112, averaging greater than two fertilised ovules per aborted capsule. Even the later capsules would be considered poorly fertilised as the average number of seeds per capsule for harvested CP in 2004/2005 was 45.3 ± s.e. Two abnormally high readings were omitted from the dataset, one for day 49 containing 18 ovules and for day 70 containing 14. These outliers may have aborted as a result of external factors such as bird damage.

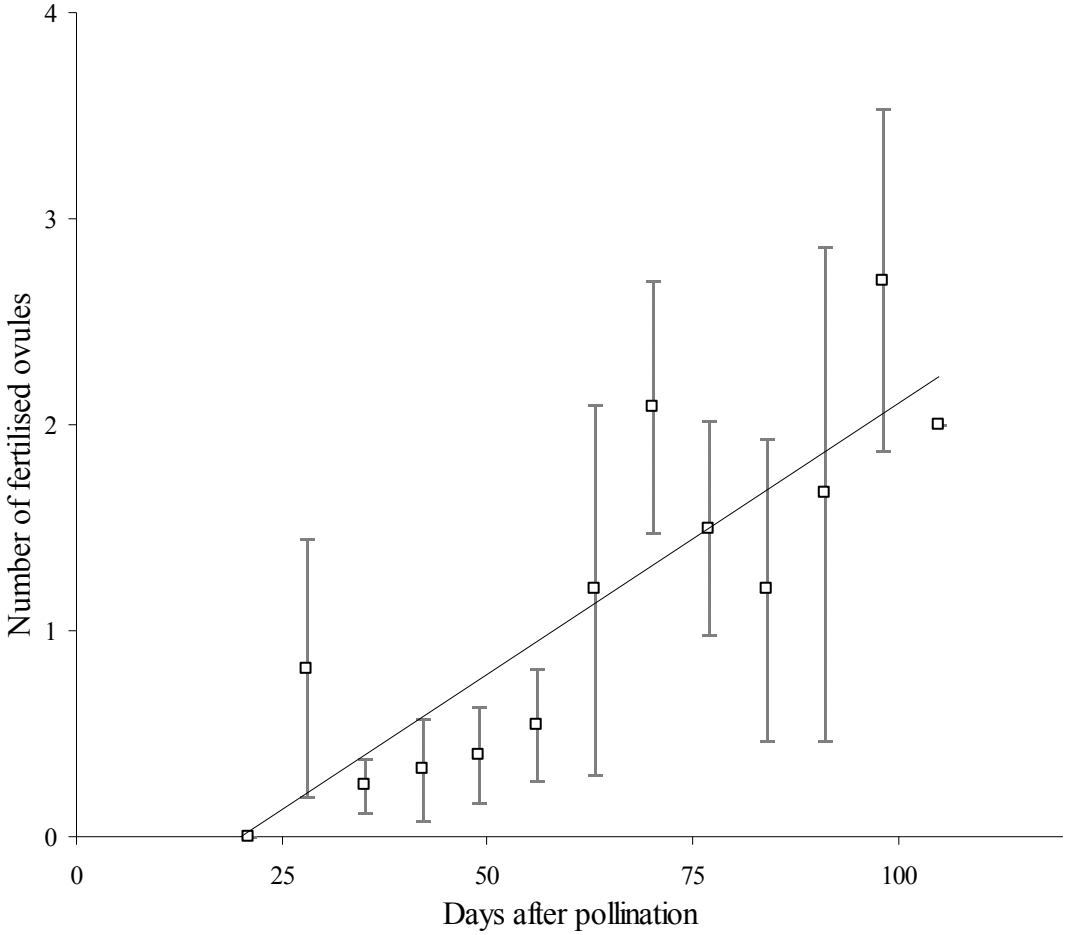

Figure 3. Average number (± s.e.) of fertilised ovules per capsule in aborted CP capsules for each week following pollination. Each point represents the average of the means from samples from up to 19 trees within each week, from 21 to 112 days after pollination. All 19 trees did not consistently abort capsules every seven days, therefore the amount of samples/replicates for each data point, varied from 16 to 1, with day 112 being the sole un-replicated sample. A significant ($P < 0.001$) linear regression existed.

Discussion

The success of crossing at Cambridge in 2004/2005 was amongst the highest yet reported in *E. globulus*. For example, within crossing programs Harbard *et al.* (1999) observed a maximum of 16.2 seeds per flower, Williams *et al.* (1999) 25.7, Rojas Vergara *et al.* (2000) 25.6 and Patterson *et al.* (2004a) 25.5. These maximums are much lower than the average level of CP seeds per flower of 35.9 observed in this study. The lower level of CP capsule set compared with MSP and OP may simply reflect the additional handling and damage to the flower associated with this treatment (e.g. emasculation, style cutting and isolation). Wounding can directly stimulate abscission of plant structures, and provides possible entry points for pathogens, stimulating defence responses that can lead to abortion (Taylor and Whitelaw 2001). However, this deleterious effect was not reflected in the high seed set per capsule observed in CP, suggesting that the damage had no significant effect on ovule fertilisation and that the wounding of the flower was overriding any positive effect that the observed increased level of seed set may have had on capsule set. The reduced seed set per capsule in OP may reflect either pollen limitation due to dependence on natural pollination (Hingston and Potts 1998), the enhanced capacity for seed set often observed following style cutting (Harbard *et al.* 1999; Trindade *et al.* 2001; Williams *et al.* 1999) and/or the abortion of self fertilised ovules produced under open pollination (Hardner and Potts 1995a; Patterson *et al.* 2004b; Pound *et al.* 2002b). The later could also explain the reduced seed set in MSP compared to CP as MSP flowers were exposed to small amounts of pollination by self pollen as flowers were not emasculated nor isolated (Patterson *et al.* 2004*b*).

The comparison of the MSP at Cambridge and Ridgley, which were both undertaken with pollen collected in the same year, as well as OP and comparable CP crosses, suggested that the Ridgley site was inherently poorer for both capsule set and seed set per capsule. General site effects on reproductive parameters such as capsule and seed set have been reported in other eucalypt studies (Harbard *et al.* 1999). Such site effects may confound the effects of season, environment (Leal and Cotterill 1997; Stephenson 1981) and silviculture (Sedgley and Griffin 1989). For example, the trees at Ridgley were not routinely pruned and the flower enhancing compound paclobutrazol (Griffin *et al.* 1993) was not applied whereas trees at Cambridge were heavily pruned and treated with paclobutrazol. While differences in pollinator efficiency or abundance could account for the difference in capsule set and seeds per capsule between OP's at each site (Hingston and Potts 1998), this does not explain the reduced success of the MSP treatment at Ridgley. This effect is unlikely to be a random seasonal effect as poor capsule and seed set at this site has previously been reported in CP crosses undertaken in other seasons (McGowen 2007).

Despite the large difference in the level of capsule abortion between sites, and pollination treatments, the timing of abortion was consistent across sites and seasons. This occurred despite the spread of flowering times and therefore pollination treatments over a five month period. Capsule abortion appeared mainly confined to a period after pollination (20 and 80 days). Abortion of all but one un-pollinated capsule, and more than 95% of all aborted capsules that developed from hand pollinated flowers (CP or MSP) during this period, coincided with the period of capsule growth (7 to 70 days). The fleshy fruits of horticultural tree species exhibit a period of rapid growth, followed by a period of slow growth but also have another period of rapid

growth prior to harvest (Fishman and Genard 1998; Sedgley and Griffin 1989), up until 140 days after full bloom (Fishman and Genard 1998; Henton *et al.* 1999). As was found in the present study, the major period of abortion in horticultural trees occurs during the initial rapid growth phase, whereby up to 80% of the young fruit losses occur within 60 days of anthesis and are generally related to seed development (Sedgley and Griffin 1989).

Within the CP crosses the timing of capsule abortion appeared to relate to the number of fertilised ovules with the later aborted capsules having greater numbers of fertilised ovules than those that aborted earlier. Comparison of the average fertilisation values of the aborted capsules with the CP seed set values and the number of ovules that are reported to be available for reproduction (Pound *et al.* 2002a) showed that most abortion was associated with poor fertilisation of ovules. The temporal pattern of capsule abortion was consistent with the 'bet hedging' hypothesis for effective resource allocation in species with excess flowers (Wesselingh 2007). This hypothesis posits that developing fruits with the least reproductive potential abort first, followed by those with a slightly higher reproductive potential up to a point where the available resources are sufficient to retain the remaining fruit on the tree.

Numerous authors have suggested that fruit abortion in horticultural species is the result of the production of excess flowers and the survivors are fruits which have the highest level of fertilisation and are able to attract sufficient nutrients to avoid abortion. Fruits with a low seed number are more likely to abort first (Ayre and Whelan 1989; Ehrlen 1990; Lloyd 1980; Stephenson 1981; Wesselingh 2007). Capsules with weaker sink strength, resulting from a lower number of fertilised ovules, may not have the

competitive ability to draw resources required to grow and as a result abort (Wesselingh 2007). The strength of a sink appears to be determined by its metabolic activity, which in turn is related to the production of phytohormones by embryos and endosperms. Developing seeds produce high levels of auxin, and removal of developing seeds from fruit frequently terminates fruit growth (Sedgley and Griffin 1989; Weijers and Jurgens 2005). Conversely, external applied auxins can substitute for the presence of seeds in stimulating fruit development (Sedgley and Griffin 1989). This model implies that when resources are limiting fruits and seeds that are weaker sinks become "starved" of resources. In some studies, aborted fruit have been shown to contain lower levels of carbohydrates and have lower sink strength than non-aborting fruits (Doust and Doust 1988; Marcelis *et al.* 2004).

Competition for resources is not limited to reproductive structures but also exists between reproductive and vegetative sinks (Allen *et al.* 2005; Pigearie *et al.* 1992). The likelihood of fruit to set is governed by both the position of the developing fruit as well as the time of initiation. For example, the developing fruit or seed may suppress the initiation or development of subsequent seeds by competing for the maternal resources allocated to developing seeds, even within an inflorescence. The first flowers to open in an inflorescence are more likely to set seeds than flowers that open later (Medrano *et al.* 2000). Resource competition has also been shown to exist between ovules within capsules in *Eucalyptus regnans* (Griffin *et al.* 1987). Within *E. globulus* the primary cause of capsule abortion appears to be competition for resources between sinks and the strength of the sinks may be modulated by spatial and temporal factors but seems to be primarily determined by the level of fertilisation.

While a low level of fertilised ovules increases the probability of capsule abortion, parthenocarpy was demonstrated to occur in *E. globulus*. Only one capsule, or less than 0.1% of the total CP harvested at Cambridge in 2004/2005, contained no viable seeds, which was consistent with the observation of Griffin *et al.* (1987) that parthenocarpy has not been reported for *Eucalyptus*. However, the number of seedless capsules increased to 28% and 40% in Cambridge and Ridgley respectively in 2005/2006 when less viable pollen was used.

A higher incidence of capsule abortion and lower seed set was also recorded for CP flowers in 2005/2006. The abortion of all UP capsules suggested that the development of seedless capsules may not have been due to vegetative parthenocarpy, which requires no external stimulus (Sedgley and Griffin 1989). Instead, it appeared to be due to either stimulative parthenocarpy, whereby stimulus in the pistil from pollen tubes prompts fruit growth, but fertilisation does not occur, or stenospermocarpy, which results from degeneration of seed following fertilisation (Sedgley and Griffin 1989).

Fruits with lower seed numbers are tolerated by plants when resources are plentiful and/or when the general level of pollination is low (Wesselingh 2007). Threshold levels of seeds in a fruit, or carbohydrate supply to fruit have been documented, with the threshold observed to vary with season and number of fruit per tree (Stephenson 1981; Wardlaw 1990; Wesselingh 2007). A reduction in this threshold due to low pollen viabilities resulting in reduced fertilisation, thus decreased resource competition could explain the increased number of seedless capsules harvested at Cambridge and Ridgley for season 2005/2006 compared to that in Cambridge in 2004/2005.

In conclusion, most capsule abortion in *Eucalyptus globulus* occurred during the period of capsule growth, over the first 80 days after pollination and the main driver appeared to be the level of ovule fertilisation of the flower. The timing of capsule abortion was consistent with resource competition such that poorly fertilised capsules were those that aborted first. However, the extent to which poorly fertilised capsules with low seed development were retained may vary depending on levels of resource competition. Under conditions of low competition even capsules with no viable seed may develop to maturity.

Chapter 3

The impact of resource competition on capsule set in *Eucalyptus globulus* seed orchards and its manipulation through irrigation management.

Chapter 2 identified resource allocation as a possible cause for *E. globulus* capsule abortion. To follow up on this chapter 3 tests the resource limitation theory through a flower density trial. Then the impact on the *Eucalyptus globulus* seed production process of two common irrigation techniques used in horticulture to manipulate resource allocation to increase fruit set, were tested. Data from this chapter has been presented as a paper at an international conference and as a poster at a international and a national conference.

Introduction

Eucalyptus globulus (Tasmanian blue gum), a native of south eastern Australia (Dutkowski and Potts 1999; Jordan *et al.* 1993), is the hardwood species most widely planted for pulpwood in temperate regions of the world (Eldridge *et al.* 1993; Potts 2004) with 454,095 hectares of plantations in Australia alone (Parsons *et al.* 2006). Most of these are established using seedlings from improved germplasm derived from open-pollinated seedling (Griffin 2001; Tibbits *et al.* 1997) or grafted (Patterson *et al.* 2004b) seed orchards, or through large-scale manual pollination systems (Patterson *et al.* 2004a).

A significant proportion of hand-pollinated flowers do not set fruit (Suitor *et al.* 2008), and due to the labour intensive nature of the production system this problem causes significant economic loss. Capsule or fruit abortion is a widespread problem throughout horticulture and forestry (Stephenson 1981). Fruit and ovule abortion may be mediated by numerous factors, most notably resource allocation (Sedgley and Griffin 1989). Plants have limited resources available for reproduction (Wesselingh 2007), and in situations where total assimilate demand exceeds supply, fruit with the lowest sink strength are more likely to abscise (Ruiz *et al.* 2001). The regulation of flower and fruit numbers may be considered as an adjustment of maternal investment to match available resources (Lloyd 1980; Stephenson 1981). It has been proposed that the production of excess flowers and young fruit is a strategy to maximise off-spring and genetic variability prior to selective abscission (Bawa and Webb 1984; Burd 1998; Wesselingh 2007).

With the development of *E. globulus* seed orchards several management practices have been introduced to decrease tree size and enhance productivity (Potts et al. 2007). The partern of resource allocation will be influenced by these management practices. The production practices include the use of grafted trees during orchards establishment (Sedgley and Griffin 1989), tip pruning (Cline 1991; Suitor *et al.* 2007) and the routine application of plant the growth regulator paclobutrazol as a soil drench around the base of the tree. Although the application of this gibberellin synthesis inhibitor has been shown to slightly decrease capsule set (Callister and Collins 2007), the benefits of reduced vegetative growth (Hasan *et al.* 1992) to permit easier canopy management (Hetherington and Jones 1990), enhance flowering (Hetherington et al. 1991; Griffin 1993) and reduced generation times (Hasan and Reid 1995) are thought to out weigh this negative. Trees within some *E. globulus* seed orchards are also subjected to irrigation, primarily to avoid drought stress. Little is known about the effect of irrigation on the partitioning of resources within the trees, and on capsule set and seed yield.

Efficient water management is an important component of horticultural crop production not only as a means to avoid many yield and quality problems associated with exposure to water stress (Kang and Zhang 2004), but also for the positive effects associated with the control of vegetative growth and the increased production of flowers and fruit (Dry and Loveys 1998). Regulated deficit irrigation (RDI) and partial root zone drying (PRD) are the two main irrigation strategies used to restrict vegetative growth and promote reproductive growth in perennial crops. RDI involves restricting irrigation in order to apply a controlled drought stress sufficient to reduce vegetative growth, but not so much as to reduce the economic value of the crop. Interest in RDI

has primarily centred on its potential to save water (Goldhamer and Beede 2004) or to curtail excessive vegetative growth (Romero *et al.* 2004) in fruit and nut crops. Cameron *et al.* (2006) found that the adaptation to reduced water supply was primarily achieved through a reduction in stomatal conductance and, over the longer term, a reduction in the number of new leaves produced. PRD is a relatively new irrigation technique which includes irrigating only half of the root system at any one time. This technique has the potential to reduce crop water use significantly and maintain yields when compared with normal irrigation methods (Dry and Loveys 1998; Kang and Zhang 2004). There is some evidence that roots exposed to drying soil generate a chemical signal to regulate stomatal aperture and hence the transpiration (Kang and Zhang 2004). PRD therefore restricts vegetative growth through the effect of root derived signals on stomatal aperture, but does not result in reduced shoot water potential through water uptake from the irrigated section of the root zone.

This study aimed to identify the effect of resource competition on capsule set and to evaluate the potential of both RDI and PRD irrigation regimes to control shoot growth and reproductive development of *E. globulus* trees in a seed orchard environment.

Materials and Methods

Experimental design

Eucalyptus globulus trees used in this study were located in a seed orchard in Cambridge, south-eastern Tasmania (42º48'27.23"S 147º25'58.48"E), encompassing the Furneaux Group, Strzelecki Ranges and Western Otway races. Cambridge has an altitude of 40 m with a annual rainfall of 507 mm and average maximum and minimum temperatures of 17.4ºC and 8ºC respectively. Trials were carried out over three consecutive seasons 2004/2005, 2005/2006 and 2006/2007. All trees were treated with Paclobutrazol.

2004/2005 – Flower density

The influence of initial flower number per tree on capsule set was assessed in a pairwise comparison replicated 12 times. Each replicate pair consisted of a ramet of similar size, which were located in the same section of the orchard and pollinated with the same pollen source using the same technique (OP or MSP) but differed in flower density; one high and the other low in flower number per cross sectional trunk area. Capsule set was measured by placing two litter traps, each measuring 1000 x 400 mm x 50 mm, underneath each tree, one on the northern and the other on the southern side of the trunk. The number of opercula collected from the traps provided an accurate indication of flower number, and the number of aborted capsules collected was divided by the number of opercula as a measure of percentage capsule abortion.

2005/2006 – RDI trial

Capsule set was measured in a RDI trial comprising a pairwise comparison replicated seven times. Each of which consisted of ramets of similar size that were located in the same section of the orchard and pollinated with the same pollen source using the same technique (OP or MSP). One of the ramets received regulated deficit irrigation (RDI) while the other received the conventional irrigation (CI) regime used by industry. Regulated deficit irrigation was achieved by switching the irrigation off and only applying water when soil moisture reached levels at which the plants were stressed [leaf water potentials below -3.5 MPa (Bell and Williams 1997)]. The critical soil moisture potential was chosen based on previous studies showing stress at leaf water potential below -3.5MPa (Bell and Williams 1997). Irrigation was delivered using micro-irrigators; trees received 14 litres per hour, for one hour intervals, three times a week so that each received 42 litres per week. The treatments commenced at the time of flowering, which for the selected genotypes occurred in December but date varied slightly due to genotypic variation (Gore and Potts 1995), and proceeded until the end of the capsule set period, in March. Total rainfall for the duration of the trial from December to March was 144.7 mm. Soil moisture was recorded using Hansen data loggers (AM400) with sensors placed 30cm, 60cm and 100cm below the soil surface under each of the two trees at two locations within the orchard. Soil moisture was consistent with the treatments applied; that is, the soil was drier for the non-irrigated treatment than the irrigated (data not shown). Capsule set was measured by placing litter traps under each tree.

2006/2007 – PRD trial

Capsule set was measured on three ramets of selected trees of the same genotype and similar size, located at close proximity within the orchard. All selected ramets had similar flower density and were open pollinated. One of the ramets received regulated deficit irrigation (RDI) (as in the previous RDI trial), one received the conventional irrigation (CI), and the other received half conventional irrigation to one side of the tree, with sides swapped every two weeks, to induce partial root zone drying (PRD). The treatments were replicated seven times. Conventional irrigation delivered approximately 42 litres per week. PRD was applied through micro-irrigators that delivered 8 litres per hour, and were switched on for 1 hour intervals three times a week so each tree received approximately 24 litres per week to half its root system. The treatments were applied from December to March. Total rainfall for the duration of the trial from December to March was 193.4mm. Soil moisture was recorded using Hansen data loggers (AM400) (Figure 4). One sensor was placed at 60cm below the soil surface under each of three trees, one tree from each irrigation treatment, at two locations in the orchard. These measurements revealed that CI treated trees had more water available for the majority of the season than those that received the PRD or RDI treatments, and the PRD treated trees had more water available for the majority of the season than those that received RDI, consistent with the amount of irrigation water applied in each treatment. Leaf water potential measurements were made on mature leaves throughout the canopy of every tree in the PRD trial on the 2/2/2007 at regular intervals from 5am until 6pm, with a pressure chamber (PMS Instrument model 615). These measurements revealed that the CI treatment consistently had the highest leaf water potential, followed by the PRD and RDI treatments (**Error! Reference source not found.**).

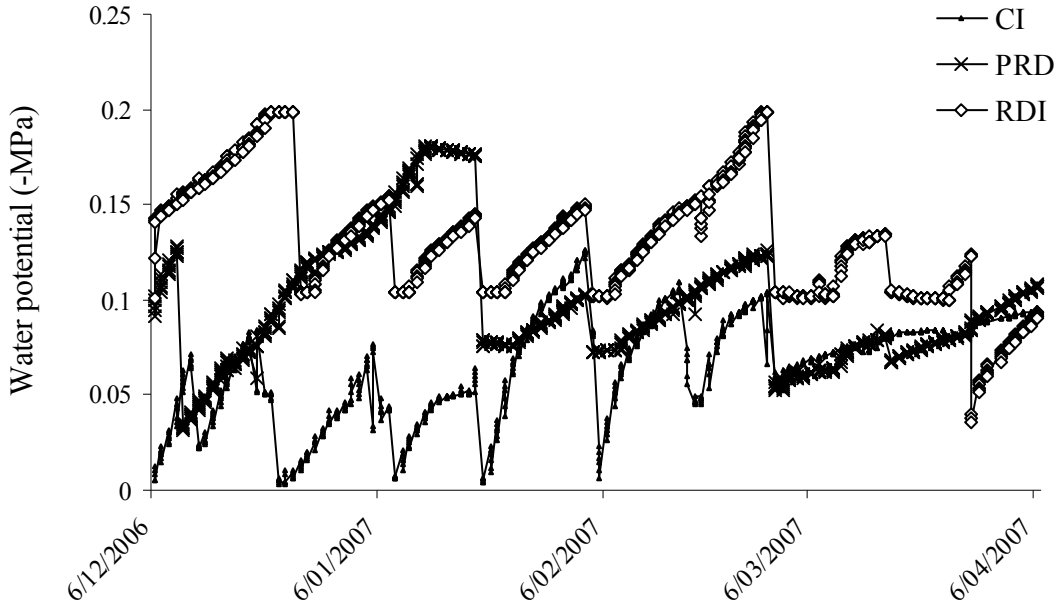

Figure 4 Soil water potential in the root zone (60cm depth) of trees that received conventional irrigation (CI), partial root zone drying (PRD) and regulated deficit irrigation (RDI) treatments from flowering until end of the capsule set at the Cambridge site in 2006/2007. Each point is the average of two readings.

Vegetative growth was measured on each tree over the four month period of the trial. The length of five second order branches was measured on each tree on the date of flowering in December. The length of the same branches was again measured at the conclusion of the trial in March. Data were converted into the relative increase (RI) in shoot growth, by dividing the branch length at the end of the season by its length at the start of the season. The values for each of the five branches were then averaged to give a RI in second order branch length per tree.

Statistical analysis

Differences in mean capsule set between treatments for the flower density, RDI and PRD trials were assessed using a Paired Student's *t*-test. To test the differences between the leaf water potentials and vegetative growth, PROC ANOVA from SAS version 9.1 (SAS Institute Inc 2003) was used.

Results

In the 2004/2005 flower density trial, trees with a high flower density had a significantly (T_{obt} 3.13; $P > 0.01$) lower level of capsule set (67.9% ± 6.4%) than the low flower density trees (81.7% ± 6%) (Table 3).

Table 3. Percent capsule set for trees with high and low flower density at the Cambridge site in season 2004/2005.

	Capsule set (%)	
Pair	**High density**	**Low density**
1	78.3	86.5
2	63.2	64.7
3	65.4	88.4
4	12.4	22.4
5	64.1	81
6	71.8	96.8
7	88.1	91
8	96.9	94.9
9	48.9	94.7
10	90.7	82.2
11	69.2	98.3
12	65.9	78.9
Mean	67.9 (6.4)	81.7 (6.0)

The percent capsule set of trees that received the conventional irrigation (51.4% ± 8.1) treatment was lower than trees receiving no irrigation (63.6% ± 6) in the 2005/2006 RDI trial, although not significant at the 95% confidence limit (T_{obt} 1.53) (Table 4). The non-irrigated treatment resulted in a higher level of capsule set in six out of the seven pairs. Soil moisture measurements in the RDI trial were consistent with the treatments applied; that is, the soil was dryer for the non irrigated treatments than the irrigated (data not shown).

Table 4 Percent capsule set of trees that received conventional irrigation (CI) or regulated deficit (RDI) from flowering until end of the capsule set at the Cambridge site in season 2005/2006.

	Capsule set (%)	
Pair	**CI**	**RDI**
1	38.7	59.4
2	69.2	76.9
3	86.3	53.2
4	57.6	89.1
5	30.4	46.1
6	50.0	71.1
7	27.2	49.4
Mean	51.4 (8.1)	63.6 (6.0)

In the 2006/2007 PRD trial, trees that received the conventional irrigation (CI) treatment had the lowest level of capsule set (53.7% ± 9.2) followed by PRD treated trees (67.8% ± 8.8) and trees that received regulated deficit irrigation (RDI) (74.7% ± 7.6) (Table 5 and Figure 5). The differences in mean capsule set between irrigation treatments were only significant (T_{obt} 2.38 was ≥ T_{crit} 1.94, $P < 0.05$) between the CI and NI treatments. The capsule set of PRD treated trees did not significantly differ ($P > 0.05$) from the CI and RDI trees. Capsule set was found to be strongly negatively

correlated with relative increase in shoot length of 3.26 (±0.37), 2.95 (±0.23) and 2.28 (±0.37) recorded for the CI, PRD, and RDI trees, respectively (Table 6 and Figure 5). The mean relative increase in shoot length of CI and RDI treated trees differed significantly ($F_{2, 92} = 3.72$; $P < 0.05$), while the mean relative increase in shoot length of PRD treated trees did not significantly differ ($P > 0.05$) from either the CI or RDI treated trees.

Table 5. Percentage capsule set for trees that received conventional irrigation (CI), partial root zone drying (PRD) and regulated deficit irrigation (RDI) treatments from flowering until end of the capsule set at the Cambridge site in 2006/2007.

	Capsule set (%)		
Group	**CI**	**PRD**	**RDI**
1	11.6	24.6	32.7
2	65.0	80.1	77.6
3	65.6	77.7	80.1
4	54.0	90.0	91.1
5	32.5	61.4	94.0
6	60.4	87.4	74.6
7	86.5	53.3	72.7
Mean	53.7 (9.2)	67.8 (8.8)	74.7 (7.6)

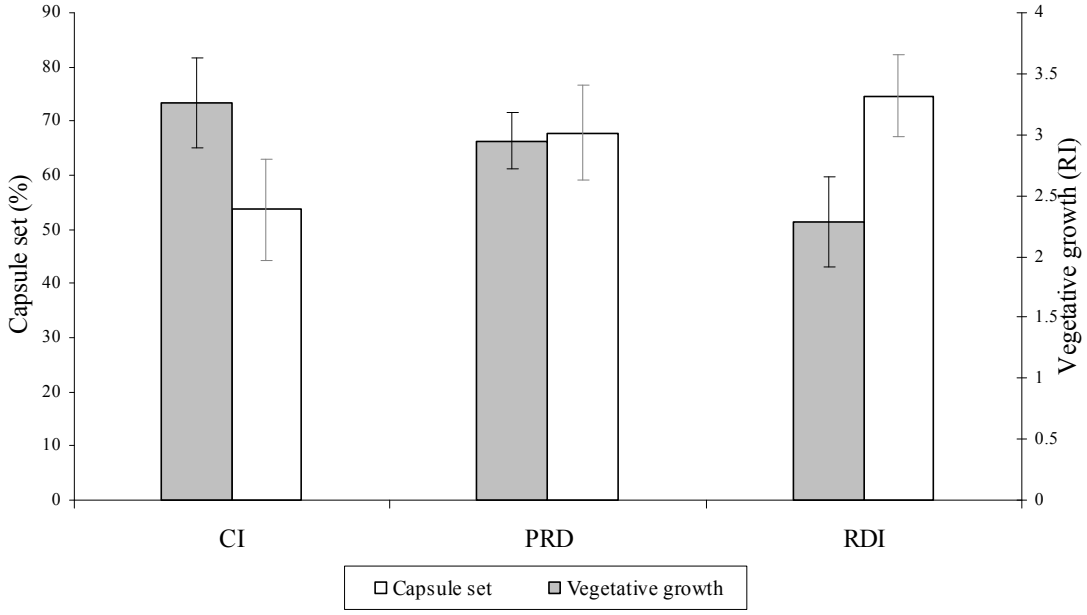

Figure 5 Percent capsule set and relative increase (RI) in vegetative growth from flowering until end of the capsule for trees that received conventional irrigation (CI), partial root zone drying (PRD) and regulated deficit irrigation (RDI) treatments at the Cambridge site in 2006/7. Each value is a mean (± s.e) of seven replicates.

Table 6. The shoot measurements taken at flowering (1st) and at the end of capsule set (2nd) and the relative increase in vegetative growth for trees that received conventional irrigation (CI), partial root zone drying (PRD) and regulated deficit irrigation (RDI) treatments at the Cambridge site in 2006/2007.

	CI			PRD			RDI		
Pair	**1st (cm)**	**2nd (cm)**	**RI**	**1st (cm)**	**2nd (cm)**	**RI**	**1st (cm)**	**2nd (cm)**	**RI**
1	70	199	**2.8**	76	223	**2.9**	69	131	**1.9**
2	16	50	**3.1**	66	195	**2.9**	57	131	**2.3**
3	95	283	**3**	76	293	**3.9**	83	261	**3.1**
4	94	445	**4.7**	99	213	**2.2**	99	130	**1.3**
5	85	219	**2.6**	53	127	**2.4**	71	133	**1.9**
6	68	304	**4.5**	61	223	**3.7**	72	290	**4**
7	81	177	**2.2**	80	215	**2.7**	72	105	**1.5**
Mean	73	239	**3.3 (0.37)**	73	213	**3.0 (0.23)**	75	169	**2.3 (0.37)**

Soil water potential measurements in the root zone of the CI treated trees revealed a lower water potential for the majority of the season than those that received the PRD or RDI treatments, and the soil in the root zone of the PRD treatment had a lower water potential than the RDI treatment for the majority of the season (Figure 4), consistent with the amount of irrigation water applied in each treatment.

The leaf water potential measurements, when measured over an entire day, revealed that the CI treatment consistently had the lowest leaf water potential followed by the PRD and RDI treatments (Figure 6). From before dawn to after sunset these differences were significant at 5:30am ($F_{2, 20} = 5.27$; $P < 0.05$), 12:30pm ($F_{2, 20} = 2.2$; $P < 0.05$) and 5:30pm ($F_{2, 20} = 5.27$; $P < 0.05$).

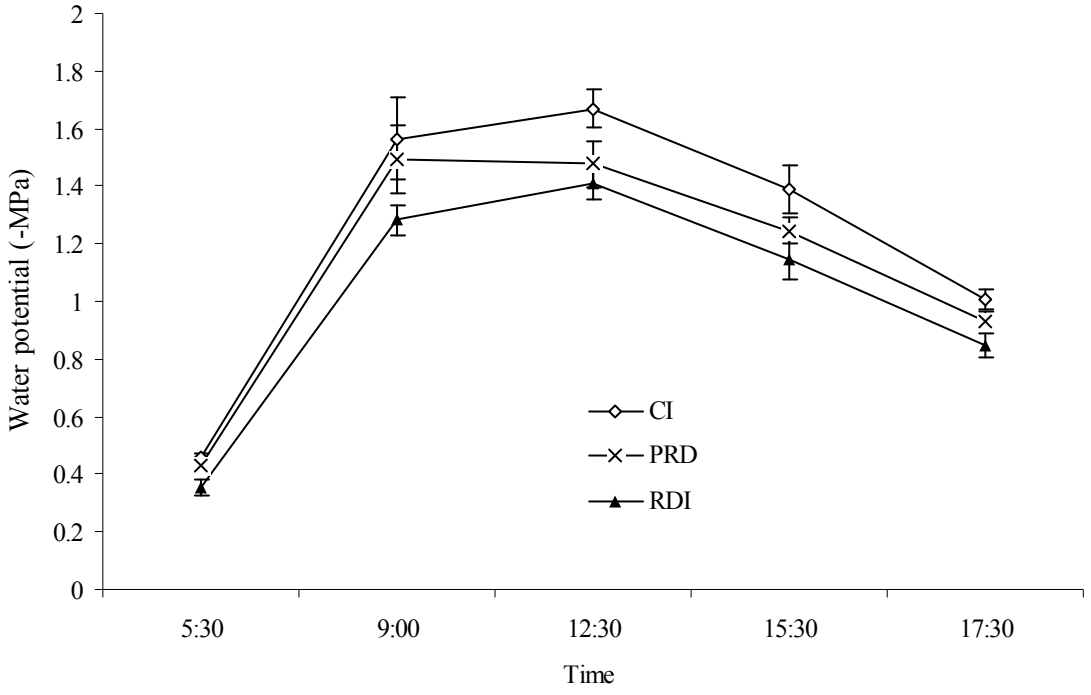

Figure 6. Diurnal changes in leaf water potential (MPa) on the 2/2/2007 for trees that received conventional irrigation (CI), partial root zone drying (PRD) and regulated deficit irrigation (RDI) treatments at the Cambridge site in season 2006/2007. Each point is a mean (± s.e) of five replicates.

Discussion

Competition for resources amongst reproductive sinks has been widely reported for species with fleshy fruits (Bawa and Webb 1984; Burd 1998; Ruiz *et al.* 2001; Wesselingh 2007). Resource competition between reproductive sinks for species with woody capsules has received far less attention. In a review of flower and fruit abortion, Stephenson (1981) concluded that the proportion of pollinated flowers that set fruit decreased as the total number of pollinated flowers on a tree increased. In this study the phenomenon has also been observed in *Eucalyptus globulus* seed orchards, indicating that the theories reported in the literature for fleshy fruits (Wardlaw 1990; Wesselingh 2007) may be extended to woody fruit. This observation suggests that inter-capsule competition for resources exists between capsules after pollination for *E. globulus*. Even though the increase in capsule size, thus relative maternal investment required, from flower pollination to capsule harvest is relatively small (Chapter 2) in comparison with fleshy fruit.

In the RDI trial, mean capsule set for the NI treatment was 10 percent higher than the CI treatment. Even though these differences were not significant, this observation provided the impetus for the PRD trial. Similar to the RDI trial, the trees that received less irrigation had a high level of capsule set, with the difference in mean capsule set between the NI and the CI treated trees being significant at the 95% confidence limit. The vegetative growth measurements for the PRD trial were the reciprocal of the capsule set measurements, so that the highest level of growth occurred in the treatment with the lowest capsule set. The negative correlation between vegetative growth and capsule retention is consistent with the theory that reproductive structures not only

compete with each other for the limited pool of resources within the tree, but competition exists between reproductive and vegetative sinks (Stephenson 1981; Wardlaw 1990). Furthermore, in this case it appears that the competition was mediated by irrigation, whereby conventional irrigation management promoted vegetative growth at the expense of capsule retention.

PRD is an irrigation technique used for other woody species and has been shown to limit shoot growth, whilst increasing fruit growth and quality (Dry and Loveys 1998). PRD imposed by alternating the position of the micro-irrigation from one side of the tree to the other every two weeks, increased capsule set compared to conventional irrigation, but had less effect than the non-irrigated treatment. The level of vegetative growth and capsule set for the PRD treatment fell between the CI and NI treatments, suggesting in this case that the benefits of the PRD treatment recorded for grapes ((Dry and Loveys 1998) may not be applicable to *E. globulus.*

The responses observed in this study to the regulated reduction in irrigation are similar to those of commercial horticultural species. Regulated deficit irrigation has been used successfully with pome and stone fruit, both experimentally (Chalmers *et al.* 1981; Mitchell *et al.* 1989) and commercially (Mitchell and Goodwin 1996), to reduce vegetative growth, increase fruit yield and decrease the total amount of irrigation applied over a season. Regulated deficit irrigation has been shown to curb excessive vegetative growth in many species by reducing in the internode length, making for a more compact plant (Cameron *et al.* 2006; Romero *et al.* 2004). Sedgley and Griffin (1989) have stated that “fruit set may be increased by controlling irrigation so as to reduce vegetative shoot growth immediately after flowering”. It is widely accepted that

vegetative and reproductive sinks compete for a limited pool of resource and in many instances fruit and seed growth dominates the growth of vegetative tissues (Wardlaw 1990). This has been found to be the case for eucalypts, where water-stressed plants of *E. globulus* produced fewer lateral branches allocating less biomass to branches than well-watered plants (Osorio *et al.* 1998). Within other *Eucalyptus* species vegetative growth has been shown to be delayed and/or reduced by developing flowering buds (Pook 1984) and flowering years have resulted in reduced leaf production compared with non-flowering years (Abbott and Loneragan 1986). Therefore with the positive effects of reduced vegetative growth, minimising pruning, and incearsed capsule set RDI appears to be a plausible option in the management of *E. globulus* seed production.

Although inter-specific variation exists, most *Eucalyptus* species are reported to be tolerant to aridity (Merchant *et al.* 2007), and the drought-tolerance response of *E. globulus* make it a suitable species for low rainfall environments (White *et al.* 1996). Leaf water potentials below -3.5 MPa 'which is far less than those observed in this study' are common for many *Eucalyptus* species of low rainfall environments (Bell and Williams 1997). As a result, it is reasonable to suggest that the maximum benefits of deficit irrigation during capsule growth were not reached in this study and that capsule set can be further increased by further limiting water availability to trees in the seed orchard. Selection of low rainfall sites for seed orchards, along with surface and sub-surface drainage, would be required to achieve this.

In conclusion, in the commercial *E. globulus* seed orchard studied, competition for resources existed both within and between reproductive and vegetative structures. A reduction in soil water availability altered the partitioning of resources to increase

capsule set and reduce vegetative growth. Therefore this study demonstrates that the orchard management technique of RDI effectively applied in other species is transferable to the *E. globulus* production system. These findings have the potential to improve the productivity of the system through the minimisation of capsule abortion, reduced pruning to restrict tree size and increased water use efficiency. Further study is required to identify the optimum conditions required to fully capitalise on the apparent positive environmental and economic impacts of this research.

Chapter 4

The relative contribution of the male and female to the variation in reproductive success in Eucalyptus globulus

Various *Eucalyptus globulus* traits have been shown to be under genetic control (Gill *et al.* 1992; Jordan *et al.* 1993) including reproductive success (McGowen 2007). However all studies to date reporting differences in female reproductive success have been for open pollinated systems. Therefore this chapter defines the relative contribution of the male and female to the variation in reproductive output in hand pollinated systems and if the effect observed has a genetic basis.

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Introduction

Eucalyptus globulus Labill. (Tasmanian Blue Gum) is endemic to Australia; but is the most widely grown species in temperate hardwood plantations world wide (Cotterill *et al.* 1999; Eldridge *et al.* 1993; Potts 2004). It is one of four taxa in the *E. globulus* complex (Brooker 2000) which are differentiated on reproductive traits, including the size and number of flower buds per umbel (Jordan et al. 1993). *E. globulus* is genetically variable across its geographic range and the broad-scale, quantitative genetic variation in numerous traits has been summarised by classifying the native gene pool into a hierarchy of 13 races and 20 subraces (Dutkowski and Potts 1999).

Eucalyptus globulus has a mixed mating system with trees being able to set seed after self- or outcross-pollination (Eldridge *et al.* 1993; Hardner *et al.* 1998; Hardner *et al.* 1996). The progeny of open pollinated seed is, therefore, susceptible to the effects of inbreeding depression (Hardner *et al.* 1998; Hardner *et al.* 1996) and where orchards are not well isolated, contamination from unimproved pollen may occur (Jones *et al.* 2007). Inbreeding depression can adversely affect the productivity of plantations, with a 48% reduction in the volume growth of trees from selfed progeny compared with fully outcrossed progeny reported (Hardner and Potts 1995a). Between 5% and 30% of the seeds in OP families of eucalypts are expected to originate from self pollination (Griffin *et al.* 1987; Hardner and Potts 1995a; Moran *et al.* 1989) and, therefore, may be suboptimal for deployment. Self incompatibility has been shown to be under genetic control (McGowen 2007) and its

effects in eucalypts have been reported to include reduced seed set (Hardner and Potts 1995a; Potts and Savva 1988; Tibbits 1989).

Currently most improved *E. globulus* plantations are established from seedlings derived from seedling (Griffin 2001; McGowen *et al.* 2004a; Tibbits *et al.* 1997) or grafted (Patterson *et al.* 2004b) open-pollinated (OP) seed orchards, or through large-scale manual pollination systems (Harbard *et al.* 1999; Patterson *et al.* 2004a; Williams *et al.* 1999). The traditional controlled-pollination (CP) technique involves three visits (TVP) (Eldridge *et al.* 1993). However, refinements have brought about the single-visit pollination (SVP) (Williams *et al.* 1999) or the one-stop pollination (OSP) (Harbard *et al.* 1999) techniques whereby cutting of the non-receptive style, coupled with style or flower isolation, has allowed pollination with no contamination from undesirable pollen to be undertaken in one visit.

Further refinements have brought about the MSP technique, which involves no emasculation or isolation, merely cutting 1 mm off the tip of the style and applying pollen (Patterson *et al.* 2004a). MSP is now routinely used for the mass production of *E. globulus* seed for the forestry industry in Australia (Callister and Collins 2007; Potts *et al.* 2007). Although the level of contamination with MSP is estimated to be about 13%, most of this is expected to be from high genetic quality outcross pollen (Patterson *et al.* 2004a) and the level of OSP/SVP selfing has been reported as low (1.3%) (Patterson *et al.* 2004a).

Within these systems a variety of breeding or deployment objectives are used and controlling the maternal and paternal parent and generating elite full-sib families, allows exploitation of additive and non-additive genetic effects for various traits (Dutkowski 2004; Potts *et al.* 2007). A major problem identified in manual pollination is that a significant proportion of flowers do not set fruit (Chapter 2). This is often confounded with low numbers of seed per capsule, and together these factors may result in poor seed set per flower pollinated (a measure of reproductive success), which substantially increases the cost of commercial seed production (Callister and Collins 2007; McGowen 2007).

A key factor in the cost of seed production is the inherent variation in seed output of a tree, which may be affected by environmental and genetic factors (McGowen 2007). Variation in whole tree seed output has been reported in several studies of eucalypts growing in the wild (Drake 1981; Potts 1986; Potts and Reid 1983) and in a seed orchard system (McGowen 2007; Sasse *et al.* 2003b). Leal and Cotterill (1997) have stated that seed production varies markedly between trees, and the choice of female could strongly influence the profitability of seed production. McGowen (2007) found a genetic basis to the variation in number of seeds per capsule for *E. globulus*. However all studies to date suggesting both a difference in whole tree seed output and a genetic basis to the variation in reproductive output, have been based on OP systems. As OP confounds the effects of inbreeding, differences in reproductive output may not be correlated between OP and hand pollination (CP and MSP) systems.

This study aims to identify the relative contribution and stability of the maternal and paternal parent to reproductive success (as measured as seed per flower crossed) in *E. globulus*. We compare these effects across 12 years of operational CP crossing across multiple sites and in a designed full-sib diallel mating scheme undertaken at a single site. We then use specific crossing experiments undertaken on ramets of different genotypes, over different seasons, sites and pollination types to test the stability of reproductive success in the face of different conditions. We examine overall reproductive success (seeds per flower crossed) as well as the specific components of capsule set and seed per capsule.

Materials and Methods

Seed orchards and trees

Virtually all trees used in this study were grafted selections which were grown in *E. globulus* arboreta (Table 7). The arboreta were located across Australia, they ranged in altitude from 40 m to 340 m, the annual rainfalls ranged from 500 mm to 1200 mm, the average maximum temperatures ranged from 17.4 ºC to 22.2 ºC and the average minimum from 7.6 ºC to 9.6 ºC. All trees in the arboreta were selected from the base population of the Australian National *E.globulus* Breeding Program run by the Southern Tree Breeding Association (STBA) (Pilbeam and Dutkowski 2004). Trees chosen for study had abundant flowers, and were first generation selections from the Furneaux Group, Otways and Strzelecki Ranges as defined by Dutkowski and Potts (1999). In most cases the ramets were unrelated except in the operational crossing, where up to three selections can be made from the better ranked base population families. Crossing in all cases was done between unrelated individuals.

Table 7 Breeding arboreta locations and details.

Site	Latitude	Longitude	Elevation (m)	Rainfall (mm)	Average maximum temperature (ºC)	Average minimum temperature (ºC)	Trials included
Cambridge	42º48'S	147º25'E	40	500	17.4	8.0	PhD trials, diallel crossing
Gunns	41º08'	145º48'	275	1200	16.1	7.7	PhD trials, Operational crossing
CS04	34º15'	116º07'	276	1010	20.3	9.6	Operational crossing
CS05	34º15'	116º07'	276	1010	20.3	9.6	Operational crossing
CS06	34º14'	116º03'	290	940	22.5	8.4	Operational crossing
CS08	34º14'	116º03'	290	940	22.5	8.4	Operational crossing
EG07	33º33'	116º04'	158	970	18.5	8.9	Operational crossing
EG09	34º33'	116º04'	235	1040	17.9	8.8	Operational crossing
EG16	34º33'	116º04'	235	1040	17.9	8.8	Operational crossing
Kingsclere	41º10'	145º51'	339	1200	16.0	7.6	Operational crossing
Massygreen	41º5'	145º54'	132	1200	16.2	7.9	Operational crossing
OrtetsVRD111	38º19'	146º14'	200	1040	17.9	8.8	Operational crossing

Experiments

This study involved the analysis of three individual datasets, which were derived from a collection of STBA operational crossing data, a diallel crossing scheme and data from pollinations undertaken during this PhD project. Trials were designed as detailed below.

Operational data

Operational capsule set, seed per capsules and seed per flower data was obtained from the Southern Tree Breeding Association. This included capsule and seed set values for crosses made over 12 seasons from 1995-2006 at 11 arboreta across Australia (Table 7); as well as a small component from crosses undertaken on ortets growing in field trials (treated as a single 'site' category in analyses). With the exception of the selected ortets, all females were grafted selections. In total, the data represented crossing among 192 genotypes, 140 of which were represented as females and 152 as just males, 100 were represented as both males and females, 40 as just females and 52 as males. The crossing was relatively sparse and the average number of males crossed per female varied between 3.4 and 4.6 (Table 8). Genotypes analysed were from three different races of *E. globulus* (Table 8), which included Strzelecki Ranges, Otways and Furneaux Group races. The data analysed were mainly from inter-race crosses although some research crosses were included which had some intra-race crossing However earlier pollinations were made with the three visit technique (TVP) (Moncur 1995; Venter and Silvlal 2007), due to the development of the

single visit pollination technique (SVP) in the late 1990's (Harbard *et al.* 1999; Williams *et al.* 1999), which was used for most of the later crosses.

The variation in $\log_{10}$ transformed seed per flower crossed for the operational data was analysed by fitting a mixed model PROC MIXED, SAS version 9.1 (SAS Institute Inc 2003), to data where pollination technique was treated as fixed and the season, site, male, female and their two and three way interactions were treated as random effects. The fixed effect of female and male race and their interaction was included in a subsequent analysis where the random female and male and their interaction terms were nested within the relevant fixed effect (i.e. female race, male race and their interaction respectively).

Table 8 Summary table for the operational crossing data**.**

Female race	Pollination type	Number of females	Average number of males crossed per female	Number of lowers pollinated	Capsule set (%)	Viable seed per capsule	Viable seed per flower
Furneaux Group	3 visit	30	4.3	1023	30	26.1	7.9
Strzelecki Ranges	3 visit	29	4.6	1341	30	25.7	7.7
Otways	3 visit	21	4.0	513	30.7	13.7	4.2
Furneaux Group	SVP	44	3.4	5757	26	24.3	6.3
Strzelecki Ranges	SVP	33	3.9	3978	18	23.7	4.2
Otways	SVP	37	4.0	4142	25.4	14.1	3.6

Diallel crosses

A 30x30 crossing diallel (same genotypes used as both males and females, crossed to make hybrids in all possible combinations) crossing scheme involving ten females from each of three races of *E. globulus* (Strezlecki Ranges, Otways and Furneaux Group) was undertaken at the Cambridge orchard across seasons 2004/2005 and 2005/2006 as part of a UTAS/STBA joint research project (Table 9). Some self-pollinations were undertaken but not included in this data set. In season 2004/2005, 27 female genotypes were crossed with up to 26 male genotypes using four flowers per pollen genotype per tree for a total of 459 combinations. In the following 2005/2006 season, 26 females were each pollinated with up to 29 pollens for a total of 443 combinations in attempt to complete the full diallel, 162 combinations were repeated between seasons. In 2004/2005, pollen was collected and stored at –20 ºC for approximately one month and used for CP, with *in vitro* percentage germination averaging 12%. In 2005/2006, while some pollen collected in the same flowering season was used, most pollen was collected during the 2004/2005 crossing season and had been stored at –20 ºC for 6 to 12 months. The percentage *in vitro* germination of all pollens used for CP crossing 2005/2006 averaged 4.9%. Three flowers were pollinated for each male-female combination. Flower physical measurements were also made at the time of pollination on each tree in the diallel.

Only those crosses which were undertaken in either 2004/2005 and 2005/2006 which used pollen collected in the same season were used in the analysis. Normality of the seed per flower crossed residuals was optimised in this data by the use of square root transformed data. The variation in seed set (square root of viable seed per flower

crossed) data was analysed in several stages by fitting mixed models (PROC MIXED, SAS version 9.1 (SAS Institute Inc 2003)), where a) male and female were treated as random effects and season a fixed effect, b) as (a) but fitting *in vitro* pollen germination percentage as a co-variate, c) as (a) but fitting capsule width as a covariate, and d) fitting male race, female race, their interaction and season as fixed effects and random female and male terms nested within their relevant race. PROC REG in SAS was used to regress pollen viability and reproductive success. PROC CORR was used to correlate viable seed per capsule and capsule set.

Table 9 Summary table for the females crossed with fresh pollen collected in the same season as crossing in the diallel at Cambridge in seasons 2004/2005 and 2005/2006.

Genotype	Female race	Number of males	Number of flowers pollinated	Capsules set (%)	Viable seed per capsule	Viable seed per flower
5642	Furneaux Group	13	47	55.3	22.1	12.2
4489	Furneaux Group	9	36	61.1	40.3	24.6
5927	Furneaux Group	13	55	54.5	65.7	35.8
6891	Furneaux Group	13	51	66.7	47.4	31.6
6029	Furneaux Group	13	51	96.1	43.1	41.4
5856	Furneaux Group	14	55	81.8	53.1	43.4
7066	Furneaux Group	12	48	87.5	64.0	56.0
7910	Furneaux Group	14	54	90.7	81.2	73.7
5617	Furneaux Group	14	55	96.4	105.9	102.0
6071	Furneaux Group	14	55	85.5	137.8	117.7
7537	Otways	28	109	20.2	28.2	5.7
7335	Otways	21	78	64.1	11.3	7.3
4928	Otways	27	101	62.4	17.1	10.7
5032	Otways	13	52	73.1	19.6	14.3
4886	Otways	26	99	76.8	24.6	18.9
4862	Otways	14	54	85.2	27.7	23.6
7479	Otways	2	6	100.0	18.3	18.3
5508	Strzelecki Ranges	27	106	23.6	18.3	4.3
5433	Strzelecki Ranges	27	103	46.6	19.7	9.2
5449	Strzelecki Ranges	26	102	63.7	16.0	10.2
5476	Strzelecki Ranges	12	45	62.2	24.0	14.9
5411	Strzelecki Ranges	21	81	64.2	25.7	16.5
5427	Strzelecki Ranges	14	52	75.0	23.1	17.3
5474	Strzelecki Ranges	26	103	91.3	27.8	25.3
5296	Strzelecki Ranges	15	58	70.7	40.8	28.8
5592	Strzelecki Ranges	10	40	77.5	35.1	27.2
5407	Strzelecki Ranges	14	55	85.5	52.7	45.1
	Mean	**16.7**	**64.9**	**71.0**	**40.4**	**31.0**

Specific tests of the stability of female effect

Various trials were completed at two sites over three seasons, with three different pollination techniques as a part of the current PhD project to test the stability of female effects. The trials assessed the capsule set and seed per capsule values of different ramets of the same genotype, thus comparisons could be made assessing the consistency of genotype reproductive output in terms of viable seed per flower crossed. The data cross correlated was derived from general sources (termed trials A to D):

Trial A. CP, MSP and OP reproductive success assessed for seven genotypes in the 2004/2005 season at Cambridge, as a part of the diallel crossing trial (Chapters 4 and 5) and a trial studying the effects of pollination type on female success (Chapter 2).

Trail B. OP reproductive success assessed on the same seven genotypes as Trial A but different ramets, as a part of a trial analysing the impact of flower density (Chapter 3).

Trail C. CP and OP reproductive success assessed in 2005/2006 on the same seven genotypes as Trial A but different ramets, as a part of a trial analysing the impact of flower density (Chapter 3).

Trail D. MSP reproductive success assessed in 2005/2006 at the Ridgley site from six genotypes which were also assessed at Cambridge in the previous season (Trial A) as a part of a trial assessing the impact of site on female success (Chapter 2).

The data on OP reproductive success obtained from the same genotypes but different ramets from Trails A and B were correlated to test whether the OP reproductive

performance of different ramets of the same genotype at the same site in the same season is correlated. To test the stability of genotype cross-pollinated performance, the reproductive success of (i) different ramets of the same seven genotypes under SVP was correlated across the 2004/2005 and 2005/2006 seasons (Trial A vs Trial C), and (ii) different ramets of the same six genotypes under MSP was correlated across different orchards and seasons (Trial A vs Trial D). As a robust test of whether OP performance can predict MSP performance, the reproductive success of the same six genotypes under OP (Trial A, Cambridge, 2004/2005) and MSP (Trial D, Ridgley, 2005/2006, MSP) were correlated across different seasons and sites.

Pearson's product moment correlation (r), calculated with PROC CORR in SAS version 9.1 (SAS Institute Inc 2003), was used to for all correlations of reproductive success for difference ramets of the same genotypes across the two sites and seasons and three pollination types.

Pollinations and data collection

All pollen were collected, stored, germinated and scored as outlined by Potts and Marsden-Smedley (1989). For testing viability, pollen were streaked across the surface of an agar medium (30% sucrose and 150ppm of boric acid) in 4x4 celled repli-dishes, and incubated at 25ºC for 24 hours with a 12hr/12hr photo period. Each pollen source was replicated in at least two cells of the repli-dish. Percentage germination was scored with a light microscope at 100x magnification.

All CP, MSP and pollinations undertaken in the three Tasmanian seed orchards were conducted as stipulated in chapter 2. The STBA controlled pollinations were undertaken with both the SVP technique and the three visit technique (TVP). The TVP involved three visits to the flower: emasculation and isolation at operculum shed, pollination at stigma receptivity and then removal of isolation bags several weeks later (Moncur 1995).

Approximately 12 months after pollination all CP, MSP and OP (20 capsules per tree) capsules were mature (Boland *et al.* 1980; Sasse *et al.* 2003a), at which stage they were harvested, placed into a number of individual paper envelopes and air dried. Once dried, the seeds were extracted and counted as viable (filled) following Hardner and Potts (1995a). For OP trees studied in (B) capsule set was measured by placing two litter traps each measuring 1000x400x50mm underneath each tree, one on the northern and the other on the southern side of the trunk. The number of opercula collected from the traps gave an indication of flower number, and the number of aborted capsules collected was divided by the number of opercula to give an estimate of the percentage capsule abortion.

Results

Operational crossing

The STBA operational crossing data which spans 12 years and 11 locations showed a significant difference in the fixed effect of pollination technique (Log_{10} transformed; $F_{1, 347} = 9.23$; $P < 0.01$). SVP resulted in a higher average seed per flower (6.9) than TVP (4.8). When the random effects were tested, there was a significant male ($P < 0.008$) as well as female ($P < 0.032$) effect on seed per flower crossed (Table 10). The female explained 7.9% and the male 5% of the variation in reproductive success between crosses in (Table 10). When the fixed female and male race terms, and their interaction where fitted into the model the male race ($F_{2, 149} = 0.50$; $P < 0.61$) was insignificant and the male by female race interaction ($F_{2, 134} = 2.10$; $P < 0.059$) effects and the female race ($F_{2, 134} = 2.94$; $P < 0.056$) effect were on the borderline of significance When all sources of variation (Table 10) were combined including interaction terms, 28% of the variation in reproductive success was explained by those including the female as a source of variance and 15% was explained by those involving the male. By comparison main effect and interaction terms involving season and location accounted for 30% and 31% of the total variation respectively.

Table 10 Sources of variation in reproductive success for the three races (Furneaux Group, Otways and Strzelecki Ranges) in the operational crossing program. The table shows the proportion (%) of variation in reproductive success (seed per flower crossed) explained by year, location, male and female genotypes and their interactions.

Source of variation	Percentage of variation explained	Probability
Season	6.7	0.209
Location	5.8	0.228
Female	7.9	0.032
Male	5.0	**0.008**
Season*location	8.4	**0.082**
Location*male	2.0	0.161
Location*female	5.2	0.100
Season*male	2.8	0.173
Season*female	6.5	0.098
Season*location*female	4.3	0.215
Season*location*male	1.4	0.332
Location*female*male	4.1	0.058
Residual	39.8	

Crossing diallel

Analysis of the diallel crossing scheme data, which included only crosses that used fresh pollen collected in the same season, revealed that there was no difference in reproductive success between seasons (√ transformed; $F_{1, 398} = 2$; $P < 0.200$). The variation between females was shown to account for 55% of the variation in reproductive success between crosses ($Z = 3.44$; $P < 0.001$) compared to only 6.7% for the male parent ($Z = 2.62$; $P < 0.005$). Both the number of viable seeds per capsule ($Z = 3.46$; $P < 0.001$) and capsule set ($Z = 3.19$; $P < 0.001$) (which are positively inter-correlated; $r_{613} = 0.26$; $P < 0.001$) significantly contributed to the variation in reproductive success.

When the fixed female and male race terms and their interaction where fitted into the model, the male race (√ transformed $F_{2, 24} = 1.25$; $P < 0.291$) (Figure 7) and male by

female race interaction ($F_{2, 24} = 0.11$; $P < 0.925$) (Figure 8) effects were insignificant and the female race effect was significant ($F_{2, 24} = 5.09$; $P < 0.008$) (Figure 7). The significant female race effect was driven by the Furneaux which had a significantly higher reproductive success than both Western Otways and Strzelecki (Figure 7). Capsule width (√ transformed; $t_{270} = 2.37$; $P < 0.05$) was shown to explain a small component of the variation in reproductive success and the Furneaux race had significantly ($F_{2, 20} = 16.52$, $P < 0.001$) larger capsules than the other two races.

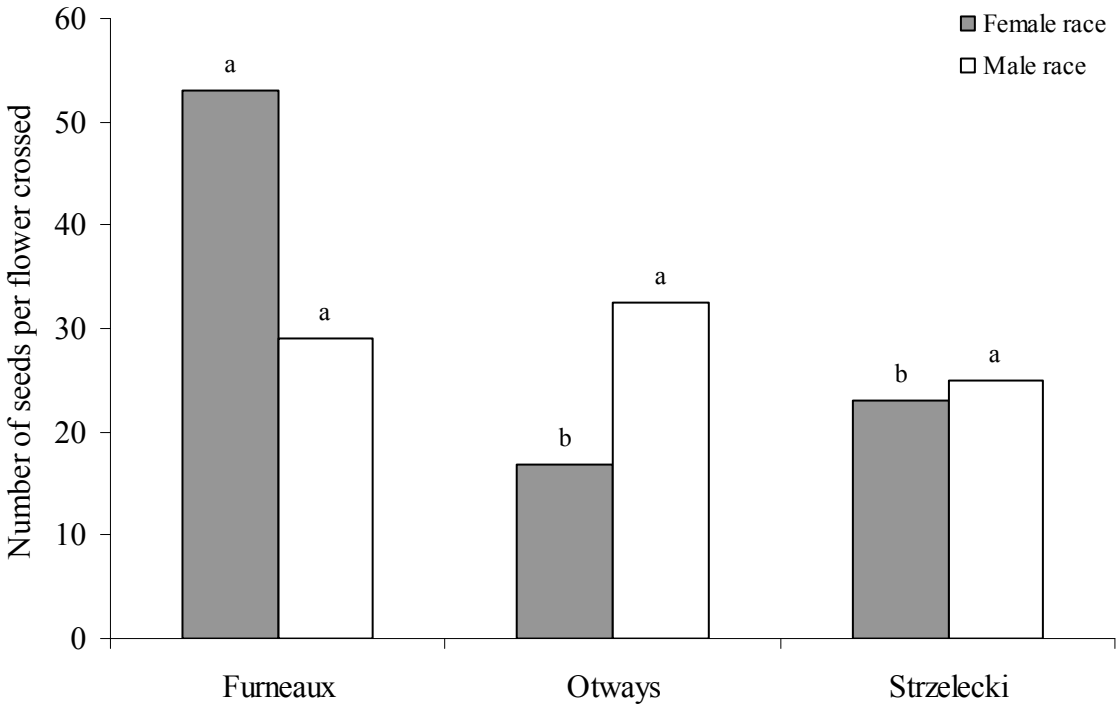

Figure 7 Seed per flower obtained following single visit pollination of (SVP) of each of the three female and male races within the diallel crossing scheme. Common letters within female and male categories represent √ back transformed least squared means which were not significantly ($P > 0.005$) different with Tukey Kremer multiple range test.

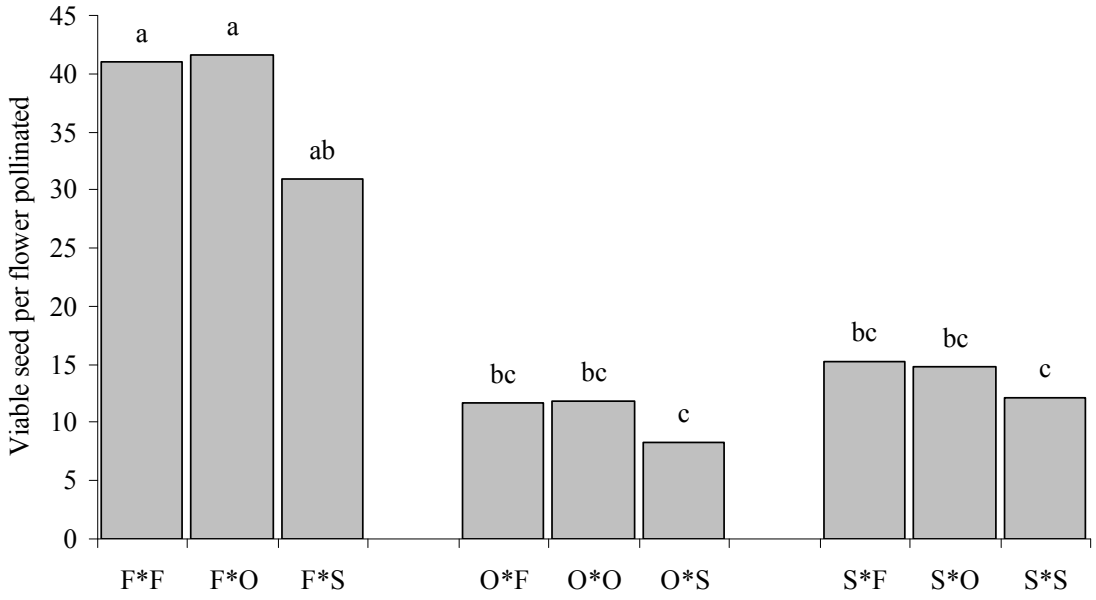

Figure 8 Seed per flower obtained following single visit pollination of (SVP) of each of the nine female by male race interactions within the diallel crossing scheme, Furneaux (F), Otways (O) and Strzelecki (S). Common letters within female interaction categories represent √ back transformed least squared means which were not significantly ($P > 0.05$) different with Tukey Kremer multiple range test.

Pollen germination did not have a significant effect on reproductive success when crosses were done with pollen collected in the same season (√ transformed $F_{1, 321} = 0.64$, $P < 0.425$). When the full data set, including crosses which had been undertaken with fresh pollen and pollen that had been collected in the previous season was analysed, the small male effect experienced in the diallel crossing scheme is partly explained by the variation in *in vitro* pollen viability ($Y = 0.6X+17.2$; $r^2 = 0.2$; $P < 0.01$). Significant differences (√ transformed; $F_{1, 227} = 39.28$; $P < 0.001$) in reproductive success were observed between the pollinations made with pollen collected in the same season (16 ± 2.1 seed per flower) than that from pollinations made from stored pollen collected the previous season (2.4 ± 0.3).

Stability of female success

There were significant positive correlations in reproductive success of female genotypes when different ramets were pollinated with different pollination techniques, at different sites and within different seasons. This occurred (i) when different ramets of the same genotype were pollinated (OP) at the same site (Cambridge) and in the same season (2004/2005) (r_8 = 0.83; P < 0.01) (Figure 9), (ii) when the site (Cambridge) and technique (SVP) were kept constant but the seasons and ramets differed (2004/2005 and 2005/2006) (r_7 = 0.98; P > 0.001) (Figure 10), (iii) when the pollination technique (MSP) was kept constant but the sites (Cambridge and Ridgley) and seasons differed (2004/2005 and 2005/2006) (r_6 = 0.98; P < 0.001) (Figure 11), and (iv) when site (Cambridge and Ridgley), season (2004/2005 and 2005/2006) and pollination technique (OP and MSP) differed (r_5 = 0.99; P < 0.001) (Figure 12).

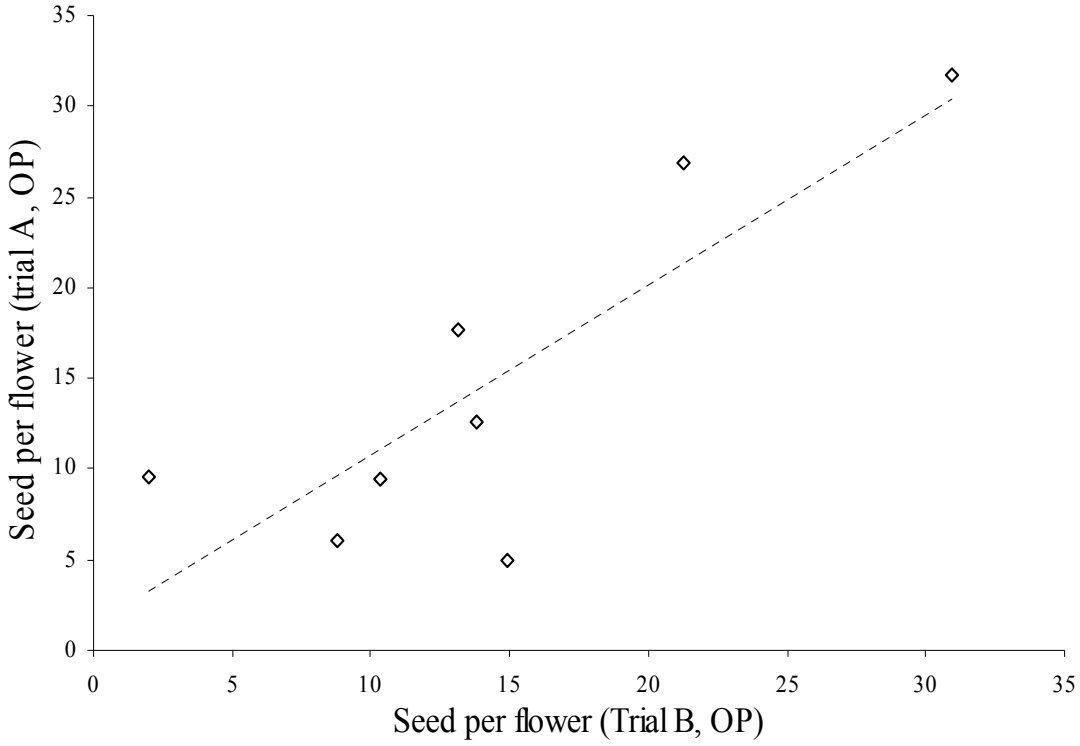

Figure 9 Open pollinated seed per flower for different ramets of the same genotype in the Cambridge seed orchard for season 2004/2005 used for two different trials.

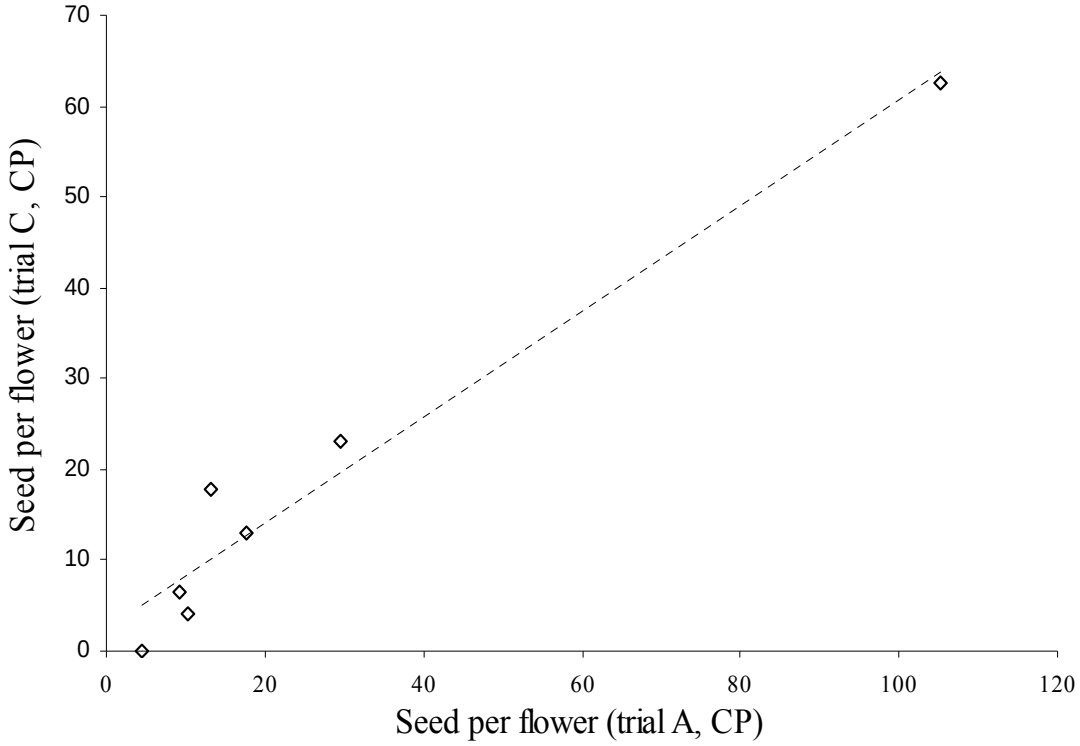

Figure 10 Cross pollinated (SVP) seed per flower for different ramets of the same genotypes in the Cambridge seed orchard crossed in the 2004/2005 (trial A) and 2005/2006 (trial C) flowering seasons.

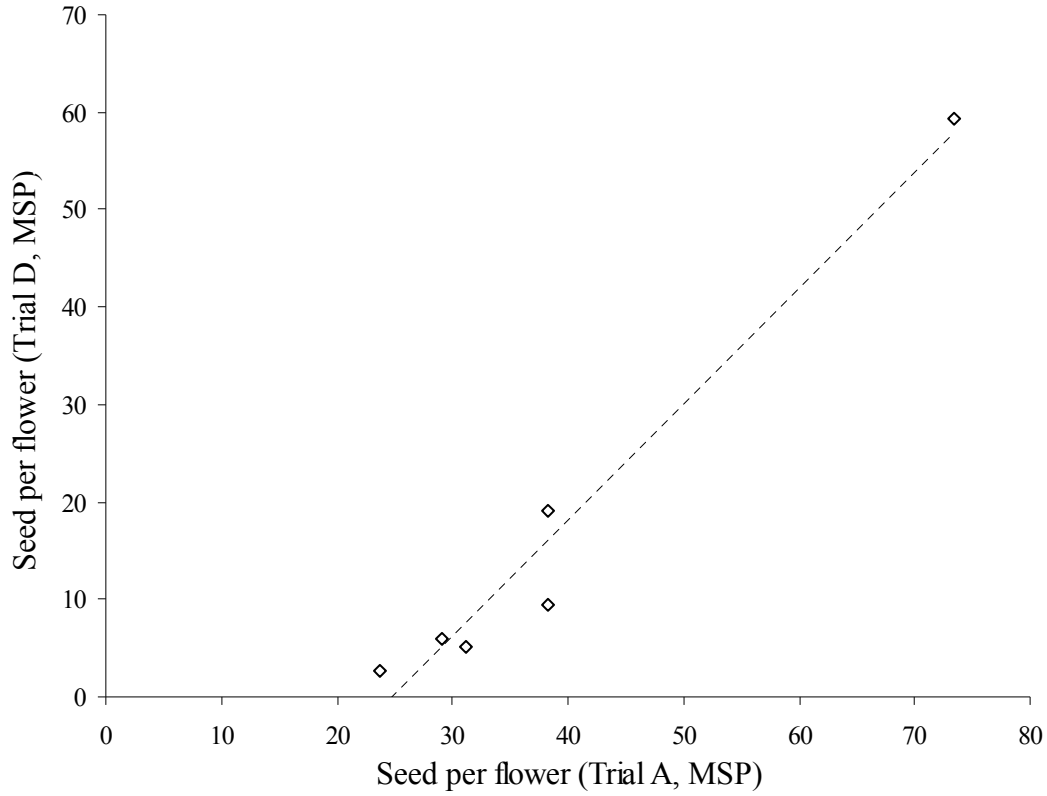

Figure 11 Mass supplementary pollinated seed per flower for different ramets of the same genotypes in different orchards and seasons (trial A; Cambridge, 2004/2005 and trial D; Ridgley, 2005/2006).

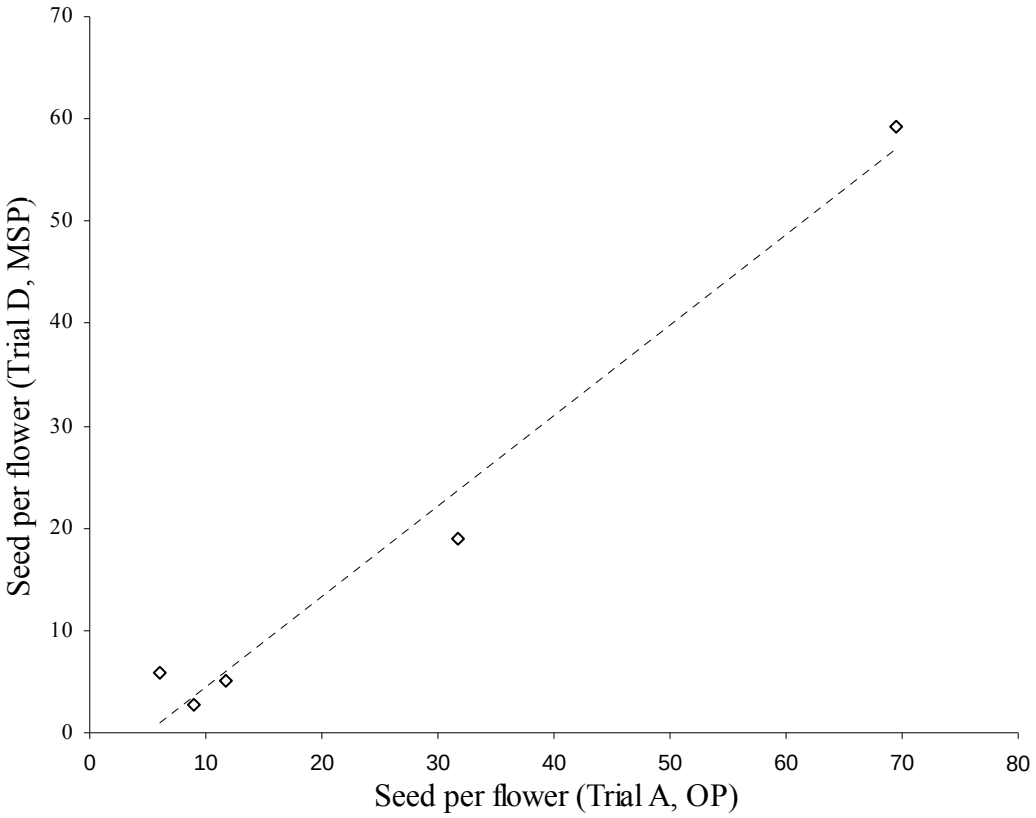

Figure 12 Seed per flower for different pollination types on different ramets of the same genotypes in different orchards and seasons (trial A Cambridge, 2005/2006 OP and trial D; Ridgley, 2005/2006, MSP).

Discussion

Reproductive success was found to be highly variable under operational conditions across 12 seasons and 11 sites. Pollination technique was found to clearly effect the success of crossing, the current single visit pollination (SVP) technique (Harbard *et al.* 1999; Williams *et al.* 1999) resulted in a higher level of reproductive success then the original three visit technique (3-visit) (Eldridge *et al.* 1993; Sedgley 1989). This is consistent with previous studies which have shown that fertilisation and seed set are successful, if not improved with the SVP technique (Harbard *et al.* 1999; Williams *et al.* 1999).The only other significant factors detected in the operational crossing, were the random female and male effects, however the percentage of variation explained by them was low. Although the female race effect was bordering on significance there was no consistent trends which could be detected from this data. In the operational data the male and female effects are confounded by numerous factors due to the sparse nature and spread of the data over sites and seasons and the general absence replication of the pollen collections and ramets used. These include differing grafts ages, flowering times (Gore and Potts 1995), operators, environmental conditions and silvicultural treatments such as pruning and paclobutrazol application (which has been shown to negatively affect capsule set (Callister and Collins 2007)). The absence of replication at a ramet level in most cases and of significant race effect and the potential confounded factors means it is not possible to determine whether the observed variation between males and female has a genetic basis.

When a more targeted study was undertaken at a single site, with a uniform pollination technique (SVP) on similarly aged grafts with much better replication in terms of

crosses performed, the female effect reported was very large and significant, with a small male effect and there was a significant female race effect. This result, whereby females of numerous races are more or less randomly distributed throughout the orchard is evidence for a genetic basis to the variation in female reproductive success. One possible cause for the genetic basis to this variation is identified in this study as capsule width; this finding is reported in further detail in this thesis. Chapter 5 reports numerous flower physical associations with reproductive success; it appears that larger floral features result in higher reproductive success. Fruit size has been shown to vary considerably in eucalypts (Gill *et al.* 1992). Morphological studies (Jordan *et al.* 1993; Kirkpatrick 1975; McGowen 2007) have shown that there are significant differences between populations of *E. globulus* both between and within races of for fruit size characteristics such as capsule diameter and height.

The absence of a significant interaction between races is evidence that post-mating reproductive barriers are absent, at least with the stigma removed. Reproductive barriers have been shown to exist between races of *E. globulus*, studies in a common environment field trial indicated a genetic based difference in flowering time (Apiolaza *et al.* 2001; Potts and Gore 1995). Flowering therefore constitutes a significant pre-mating barrier to natural crossing. Races in this study also differ significantly in flower size (McGowen 2007), with those of Strzelecki Ranges having smaller flowers than Furneaux Group and Otways. Differences in flower size have been shown to be a significant post-mating barrier to hybridisation in eucalypts (Gore *et al.* 1990). However the absence of any male x female race interaction in the diallel crossing scheme, indicating no significant difference in reproductive success between inter- and intra-race crosses would argue against the existence of post mating reproductive

barriers to seed set in the races studies. While the Strzelecki Ranges and Otways races belong to the same molecular lineage of *E. globulus*, the Furneaux Group race is more divergent and belongs to an Eastern Tasmanian molecular lineage (Steane *et al.* 2006).

The male effect was found to be significant at both the operational scale and within the diallel crossing scheme. Non-optimal storage conditions reduced viability and differences in storage times resulted in some pollens in the diallel crossing scheme having low viability. Subsequently a negative correlation between reproductive success and pollen viability was identified, when the full data set including the use of the fresh and stored pollen was used. The use of pollen which had been collected and stored for a year, resulted in a markedly lower level of seeds per flower than pollinations made with pollen collected that season. Therefore the significant male effect in the diallel crossing scheme and most likely in the operation scale data, when conditions were less controlled, is likely a consequence of pollen management and the time of pollen collection. The absence of a male race effect in reproductive success in either operational crossing or the diallel would argue against a genetic basis to the variation in male fecundity.

The genetic effect alluded to in the operational crossing and detected at the race level in the diallel crossing trials was confirmed in subsequent studies of repeated crosses of different ramets of the same genotypes at different sites and seasons which clearly indicates stability of female effects. Despite the expected variation due to the exogenous factors of site (Chapter 2), pollination type (Chapter 2) and season (Leal and Cotterill 1997) genotypes were consistently ranked in their level of reproductive output. Although the design of the trials limits the accuracy of these estimates due to

small numbers of clones or families represented in each trial, the fact that these effects are stable when different ramets are used provides solid evidence for a genetic basis to the female variation in reproductive success.

Previous studies suggest that subraces differ genetically for numerous adaptive traits at the subrace (Dutkowski and Potts 1999) and within the subrace levels, for example drought (Dutkowski 1995), pest resistance (Jones and Potts 2000; Jordan *et al.* 2002; O'Reilly-Wapstra *et al.* 2002) and flowering time (Chambers *et al.* 1997). However, there are few reports that have reported genetic variation whole tree seed output have been for open pollinated native stands (Drake 1981; Potts 1986; Potts and Reid 1983) or orchards (McGowen 2007; Sasse *et al.* 2003b) as a result may not be comparable to controlled pollinated systems due to the confounding effect of self pollination.

Forest tree breeders rarely consider reproductive traits in breeding programs (Sedgley and Griffin 1989). However, an understanding of the genetic basis of variation in reproductive traits, particularly reproductive success, is important for predicting the responses to artificial and natural selection (Falconer and Mackay 1996). According to Ridley (1996) the selective value of a trait is directly related to its genetic co-variation with reproductive output. Co-variation between reproductive output and a selection trait may have a negative impact on the cost of seed production. For example, selection for fast growth may result in reduced reproductive output due to changes in resource allocation (Chapter 3)(Bonnin *et al.* 1997; Chalupka and Cecich 1997; Sedgley and Griffin 1989; Strauss *et al.* 1995).However further quantitative genetic screening studies would be required before the genetic trait of reproductive success could be incorporated into a breeding program.

Chapter 5

Variation in *Eucalyptus globulus* reproductive success can be explained by the properties of the flower

The cause of the female genetic variation discussed in chapter four is identified as possibly being variation in capsule physical properties. This chapter attempts to confirm this by examining various floral features of female genotypes of varying reproductive success. Data from this chapter has been presented as a paper at an international conference.

This chapter has been submitted for publication as:

Suitor S, Potts BM, Brown PH, Gracie AJ and Gore PL (2008) Variation in *Eucalyptus globulus* reproductive output can be explained by both the physical and physiological properties of the flower. Submitted to *Australian Journal of Botany*.

Introduction

Eucalyptus globulus Labill. (Tasmanian blue gum) is a genetically diverse forest tree species with a mixed mating system (Potts *et al.* 2007). It is native to south eastern Australia (Dutkowski and Potts 1999; Jordan *et al.* 1993), but widely planted for pulpwood in temperate regions of the world (Eldridge *et al.* 1993; Potts 2004) including Australia (Parsons *et al.* 2006). Although there is some clonal (or vegetative) deployment in countries such as Chile, Argentina, Portugal and Spain (Borralho *et al.* 1992a; Griffin 2001; Lopez *et al.* 2001) most improved plantations are established from seedlings derived from open-pollinated (Griffin 2001; McGowen *et al.* 2004a; Tibbits *et al.* 1997), grafted (Patterson *et al.* 2004b) seed orchards, or through large-scale manual pollination systems (Patterson *et al.* 2004a; Williams *et al.* 1999).

A major problem identified in manual pollination of *E. globulus* is that a significant proportion of flowers do not set fruit, a woody capsule (Espejo and Griffin 2001). This is often confounded with low numbers of seed per capsule, and together these factors may result in poor seed set per flower pollinated which substantially increases the cost of commercial seed production (Callister and Collins 2007). While parthenocarpy (Sedgley and Griffin 1989) can occur in *E. globulus* (Suitor *et al.* 2008), it is rare and fertilisation is almost always required for successful seed and capsule set. Numerous factors may affect reproductive success following manual pollination including the degree of damage caused during emasculation and pollination (Sedgley and Griffin 1989), exogenous environmental (Suitor *et al.* 2008) and biotic (McGowen *et al.* 2004b) factors, as well as endogenous biological (Suitor *et al.* 2007) factors. Endogenous barriers to seed set have been poorly studied in *E. globulus* but include

genetic (Hardner and Potts 1995b) as well as floral physical (Gore *et al.* 1990) and physiological attributes, such as, pollen/style compatibility, pollen tube growth in the style and resource allocation (Sedgley and Griffin 1989).

Due to the close interaction between male and female tissues (Sanchez *et al.* 2004) there is ample opportunity for attrition between pollen germination and fertilisation (Erbar 2003). Even in compatible intra-specific pollinations, a large reduction in the number of pollen tubes extending the length of the style has been recorded in numerous species including *Persea americana* (Sedgley 1976), *Erythronium grandiflorum* (Cruzan 1989), *Prunus avium* (Herrero and Hormaza 1996; Hormaza and Herrero 1999) and *E. globulus* (Trindade *et al.* 2001). There are also examples of pollen tube attrition between the style base and fertilisation in intra-specific outcrosses (Pound *et al.* 2002b; Trindade *et al.* 2001).

Competition for resources is a major factor affecting reproductive success, influencing both the retention of fertilised flowers (Stephenson 1981; Suitor *et al.* 2007) and continued development of zygotes within flowers (Griffin *et al.* 1987). Plants may be viewed as having a limited level of resources available for reproduction (Wesselingh 2007), and rapidly growing fruits are strong sinks for assimilates. In situations where total assimilate demand exceeds supply, fruit with the lowest sink strength are more likely to abscise (Ruiz et al. 2001). It has been proposed that the production of excess flowers and young fruit is a strategy to maximise off-spring and genetic variability prior to selective abscission (Bawa and Webb 1984; Burd 1998; Wesselingh 2007). Not all flowers have equal sink strength and a strong sink will pull nutrients from further away than a weak sink (Wesselingh 2007). The sink strength of flowers and

fruit have been shown to be influenced by the timing of pollination and position of the flower (Wesselingh 2007), size of the capsule (Wardlaw 1990) and level of fertilisation (Suitor *et al.* 2008).

This study examines how genotype, physical properties of the flower, and pollen germination and pollen tube growth determine reproductive success in *Eucalyptus globulus*.

Materials and Methods

Experimental design

Trees used in this study were located in a grafted seed orchard in Cambridge, south-eastern Tasmania (42º48'27.23"S 147º25'58.48"E). All trees in the orchard were selected from the base population of the Australian *Eucalyptus globulus* National Breeding Program run by the Southern Tree Breeding Association (Pilbeam and Dutkowski 2004) (Table 11). The genotypes used encompassed a range of flowering times from September to January, with most variation attributed to the racial differences in flowering time within *E. globulus* (Gore and Potts 1995).

Six females were selected for study on the basis of the reproductive success (seeds per flower pollinated) from a 2004/2005 controlled-pollination trial involving 27 females of different genotypes. The 27 females were first generation selections from three (Furneaux Group, Strzelecki Ranges and Western Otways) of the 13 races of *E. globulus* as defined by Dutkowski and Potts (1999). Controlled pollination (CP) was

undertaken to generate full-sib families using four flowers per pollen per tree; each tree was pollinated with up to 26 male genotypes as a subset of a larger crossing program to generate a full diallel. All parental genotypes were unrelated to each other. Best linear unbiased predictions (BLUP) (see Statistical Analysis) of the reproductive success were used to select two unrelated female genotypes differing in seeds set per flower from within each of the Furneaux Group, Strzelecki Ranges and Western Otways races. The difference in seeds set per flower between the pairs chosen from the Furneaux Group and Strzelecki Ranges was statistically significant ($P < 0.001$), but not for the pair from Western Otways. The six pollen genotypes were chosen using the same process based on male BLUPS, but with the condition they were unrelated to each other and the females selected.

Table 11 Females present in this study, in racial pairs of high (FX1, ST1, WO1) and low (FX2, ST2, WO2) reproductive success (seeds per flower). Season 2004/2005 capsule set, seeds per capsule and seeds per flower means (±s.e) for each female genotype.

			Number of seeds per	
Code	**Race**	**Capsule set (%)**	**Capsule**	**Flower**
FX1	Furneaux Group	88 (8)	129 (14)	124 (15)
FX2	Furneaux Group	73 (9)	43 (8)	31 (7)
ST1	Strzelecki Ranges	77 (7)	36 (3)	30 (4)
ST2	Strzelecki Ranges	18 (6)	10 (3)	4 (4)
WO1	Western Otway	73 (7)	19 (3)	14 (3)
WO2	Western Otway	66 (8)	11 (2)	8 (2)

The six genotypes selected as females were pollinated with the six different pollen genotypes in the 2006/2007 flowering season. Five of the six trees pollinated were the same genotype, but different ramets to those used as females in 2004/2005. Seven flowers were pollinated on each tree with each of the six pollen genotypes. Six flowers

on each tree, each crossed with different pollens, were sampled at random 2, 7, 14, 28 and 42 days after pollination. Sampled capsules were stored in formalin-acetic alcohol at 5ºC. Twelve capsules on each tree, two per pollen source, were left to assess capsule and seed set 100 days after pollination.

Pollination techniques

Pollinations were undertaken using the single visit pollination procedure outlined by Williams *et al.* (1999) and Patterson *et al.* (2004a). Flowers were emasculated when the operculum began to change to a yellow colour and started to lift away from the receptacle (Harbard et al. 1999). The style was then transversely cut 1 mm from the top, removing the stigma, and pollen immediately applied to the freshly cut surface with a wooden toothpick. A small white balloon was then placed over the emasculated flower to isolate it from all other pollen sources and labelled. All pollen used from crossing in 2006/2007 had viability of >10%, when tested *in vitro*.

Pollen collection, germination, growth and fertilisation

All pollen were collected, stored, germinated and scored as outlined by Potts and Marsden-Smedley (1989). For testing viability, pollen were streaked across the surface of an agar medium (30% sucrose and 150ppm of boric acid) in 4x4 celled repli-dish, and incubated at 25ºC for 24 hours with a 12/12 photo period. Percentage germination was scored with a light microscope at 100x magnification. This procedure was replicated five times for each pollen.

In vitro pollen tube growth was measured in a separate experiment using the same germination conditions. Each of the six pollen sources were randomly allocated to two cells per dish and this design was repeated in five petri-dishes. Pollen tube length was then scored after 24, 48 and 72 hours by measuring the longest pollen tube in each of ten fields of view (100x), and values averaged over the two replicates of each pollen in each petri-dish. To measure pollen grain size, grains were streaked across the surface of 4x4 petri-dishes of the same medium, and measured after 24 hours at 4ºC.

For assessing *in vivo* pollen tube growth, styles sampled 2, 7 and 14 days after pollination were softened in 0.1 *N* sodium hydroxide at 60ºC for 4 hours, and stained overnight with decolourised aniline blue (Martin 1959). The cuticle of each style was removed by a longitudinal cut, and individual styles were squashed on microscope slides in glycerol (Pound *et al.* 2003). Fluorescence microscopy was used to determine the number of pollen tubes at each millimetre down the style. A selection of ovules from each locule from sample days 14, 28 and 42 were mounted onto slides, stained overnight and examined with a fluorescence microscope (Pound *et al.* 2003), each ovule was scored as either penetrated or not penetrated by a pollen tube. For both pollen tube growth and ovule penetration, slides were examined at 160x magnification in random order using a Leica Leitz DM RBE fluorescence microscope by direct illumination with ultraviolet light of a wave length of about 356mμ (Martin 1959). For the flowers sampled on day 42, ovules in all locules were visually examined for fertilisation using a dissecting microscope (20x). Fertilised ovules appear physically larger than unfertilised ovules from 28 days after pollination (Pound *et al.* 2002a).

Flower morphology

Five open-pollinated flowers, approximately 7 days from anthesis, were randomly selected per tree, form the six trees used as females in 2006/2007. Physical measurements were made on these flowers including; capsule width, style length and number of ovules. Similar measurements were made on different ramets of five of the six genotypes in the 2004/2005 season. To measure the area of stylar conductive tissue, transverse sections were made every 1 mm down the style of flowers collected in 2006/2007. Styles were stained overnight with decolourised aniline blue and observed with fluorescence microscopy. Photos of each section were taken and printed and the area of the conductive tissue measured.

Statistical analysis

SAS version 9.1 (SAS Institute Inc 2003) was used for all statistical analyses. Best linear unbiased predictions of the female effects for the number of seed per flower pollinated (square root transformed) from the 2004/2005 crossing were used to select the six genotypes used in the current trial. The analysis was undertaken using PROC MIXED treating the female and male genotype as random effects. To test differences between the six trees studied in 2006/2007, a single factor analysis with female as a fixed effect was fitted to the data using PROC MIXED. The variables tested included the flower physical characteristics and the amount of stylar conductive tissue at each 1 mm cross-section within the style, the number of pollen tubes germinating on the cut surface ($\log_{10}$ transformed), *in vivo* pollen tube growth rates, proportion of pollen tubes (calculated as the number of pollen tubes/number of grains germinating) at each 1mm within the style and the number of ovules penetrated. To test the difference between

pollen tube growth rates for both *in vitro* and *in vivo*, the same model but with male as the fixed effect was fitted. To test for interactions between the female genotype and male race on pollen germination and *in vivo* tube growth, a two-way model was fitted. PROC REG was used to regress (i) 2004/2005 seed per flower data against the flower physical properties, (ii) the 2004/2005 seed per flower data against the 2004/2005 pollen viability data, (iii) *in vitro* pollen tube length against pollen grain size, (iv) the 2004/2005 seed per flower data (square root transformed) against the proportion of pollen tubes reaching the base of each style, and (v) the number of ovules penetrated and seed set against the number of pollen tubes reaching the bottom of the style. Kendall's coefficient of rank correlation (τ) was calculated with PROC CORR to examine (i) the consistency of genotype reproductive success across seasons, (ii) the flower physical measurements across seasons, (iii) relationship between style length and area of conductive tissue and (iv) the relationship between capsule set, seed per capsule and seed per flower.

Results

Style and flower physical characteristics

All physical characteristics measured including style length ($F_{5, 24} = 54$; $P < 0.001$), capsule width ($F_{5, 24} = 74$; $P < 0.001$) and the number of ovules per capsule ($F_{5, 18} = 93$; $P < 0.001$) varied significantly between females. When placed into racial pairs (Figure 13B, C and D) all physical characteristics were significantly greater for the better performing of the females in the Furneaux Group (capsule width $F_{1, 8} = 73$; $P < 0.001$, style length $F_{1, 8} = 37$; $P < 0.001$ and number of ovules per flower $F_{1, 6} = 41$; $P < 0.001$)

and Western Otway (capsule width $F_{1, 8} = 8.2$; $P < 0.05$, style length $F_{1, 8} = 9.4$; $P < 0.05$ and number of ovules per flower $F_{1, 6} = 54$; $P < 0.001$), but not Strzelecki Ranges (capsule width $F_{1, 8} = 5.04$; $P < 0.06$, length $F_{1, 8} = 0.3$; $P < 0.6$ and number of ovules per flower $F_{1, 6} = 0.2$; $P < 0.7$). Flower physical measurements taken in 2006/2007 correlated with the measurements taken in the 2004/2005 season on five of the same female genotypes but different ramets (style length $\tau = 0.96$; n = 5; $P < 0.05$ and capsule width $\tau = 0.92$; $P < 0.05$) which suggested a genetic basis to the differences observed.

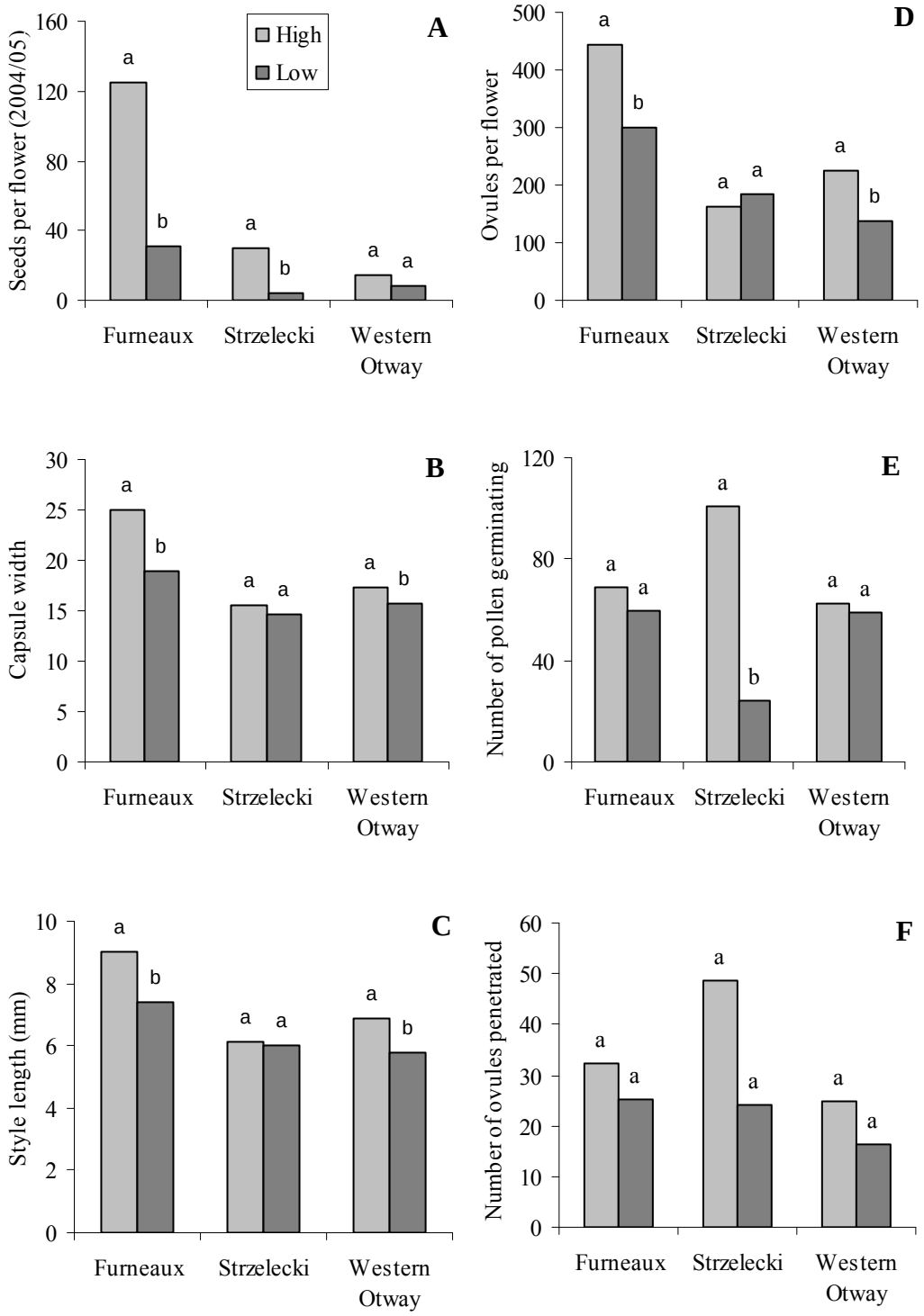

Figure 13 Seeds per flower (2004/2005 season) (A), capsule width (B), style length (C), ovules per capsule (D), number of pollen germinating on the cut style surface (E) and number of ovules penetrated (F) for each female genotype in racial pairs (2006/2007 season). Different letters indicate significant ($P < 0.05$) differences between paired female genotypes, which for (E) were tested using for $\log_{10}$ transformed data.

Style conductive tissue was at its maximum area at the top of the style and narrowed rapidly between 3-5 mm below the stigma (Figure 14A, C and E). Differences were observed in the amount of conductive tissue within the pistils of each tree and these were significant at 2 mm ($F_{5, 10} = 12.8$; $P < 0.001$) and 3 mm ($F_{5, 10} = 4.3$; $P < 0.05$) below the stigma (Figure 14A, C and E). When placed into racial pairs (Figure 14A, C and E), consistent with other physical characteristics, the amount of conductive tissue present within the style was greater for the better performing female genotypes from the Furneaux Group (significant at 2 mm [$F_{1, 4} = 25$; $P < 0.01$] and 3 mm [$F_{1, 4} = 31$; $P < 0.01$]) and Western Otways (significant at 4 mm [$F_{1, 4} = 30$; $P < 0.01$]). However the opposite was the case for the pair from the Strzelecki Ranges (significant at 2 mm [$F_{1, 2} = 304$; $P < 0.01$] and 4 mm [$F_{1, 2} = 159$; $P < 0.01$]). Across all females, the area of conductive tissue at 1 mm ($\tau = 0.94$; n = 6; $P < 0.01$), 2 mm ($\tau = 0.93$; $P<0.01$) and 3 mm ($\tau = 0.88$; $P<0.05$) correlated with style length, and at 1 mm ($\tau = 0.94$; $P < 0.01$) correlated with capsule width.

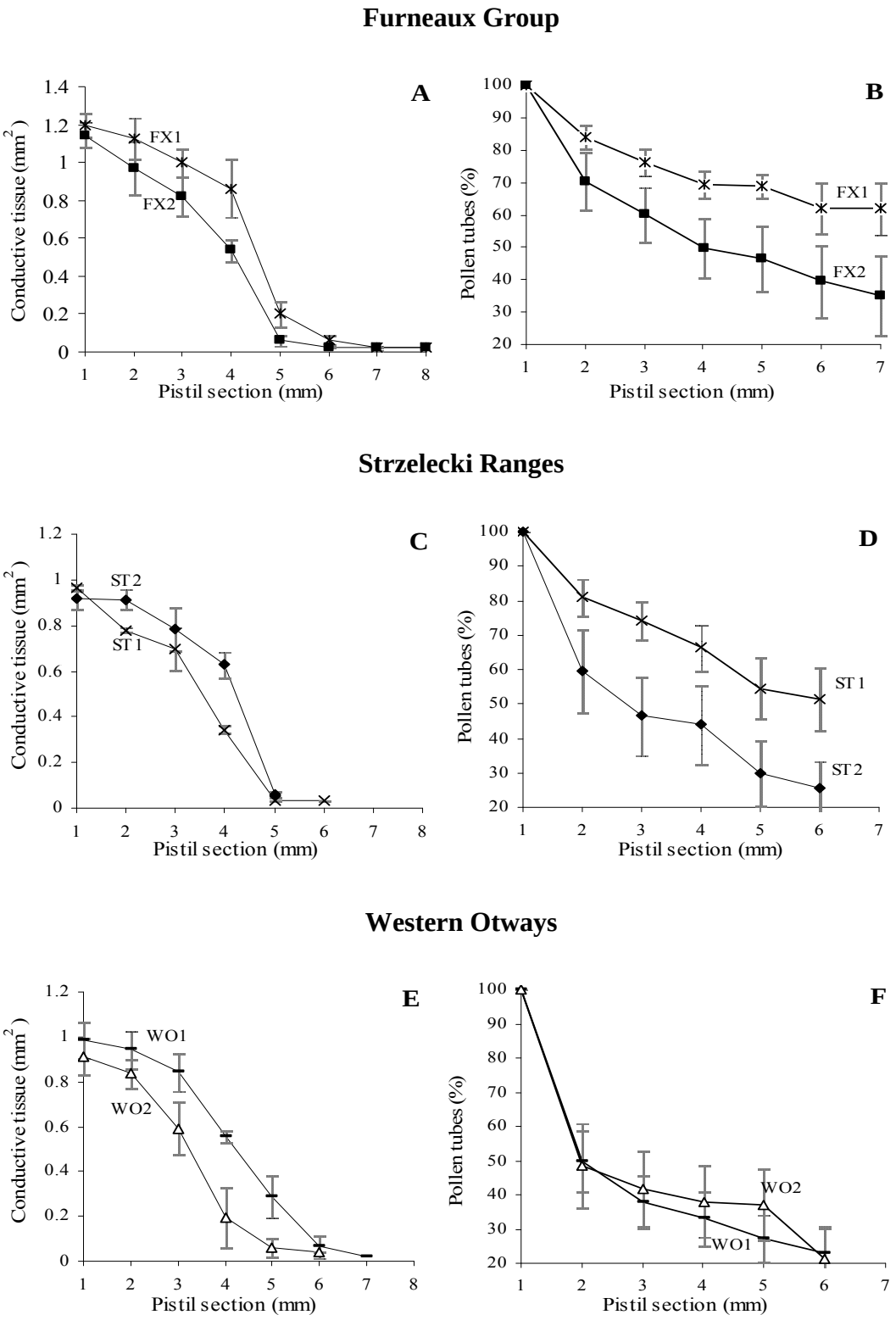

Figure 14 The conductive tissue cross sectional area (A, C and E) and the proportion of pollen tubes at each millimetre within the style relative to the initial number germinating (1mm is the cut surface) (B, D and F), for pairs of female genotypes from the Furneaux Group, Strzelecki Ranges and Western Otways races.

A high percentage of the variation in reproductive success in 2004/2005 was explained by differences in the physical properties of the flowers including: capsule width (mm; $Y = 14X-221$; $r^2 = 0.9$; $P < 0.01$), style length (mm; $Y = 34X-201$; $r^2 = 0.8$; $P < 0.05$), total number of ovules ($Y = 0.4X-50$; $r^2 = 0.84$; $P < 0.01$) and the area of conductive tissue at 2 mm below the style (mm^2; $Y = 318X-286$; $r^2 = 0.76$; $P < 0.05$). These traits were all positively inter-correlated ($P < 0.05$), except the capsule width and area of conductive tissue at 2 mm ($P < 0.19$), reflecting the fact that more seed per flower pollinated was obtained from the large flowered females.

Pollen tube growth and ovule penetration

There was a significant difference in the number of pollen tubes germinating on the cut surface of the style between the Strzelecki Ranges genotypes (Log_{10} transformed; $F_{1, 20} = 4.5$; $P < 0.05$) but not the Furneaux Group ($F_{1, 15} = 0.01$; $P < 0.9$) or the Western Otways ($F_{1, 19} = 0.09$; $P < 0.8$) females (Figure 13E). The more reproductively successful Strezlecki Group female had more pollen germinating on the cut style surface than the poorer female despite having less area of conductive tissue. For all females, the majority of the pollen tube attrition can be accounted for in the first 1 mm of the cut style, after that it occurred at a relatively even rate throughout the style (Figure 14B, D and F). There was no evidence of clogging at the point of narrowing in conductive tissue and this general pattern was similar for all females. When comparing racial pairs the proportion of pollen tubes reaching the base of the style was significantly greater in the better performing females in Furneaux Group ($F_{1, 26} = 4.28$; $P < 0.05$) and Strzeleki Ranges ($F_{1, 20} = 6.82$; $P < 0.05$), but not Western Otways ($F_{1, 19} = 0.33$; $P < 0.6$). Across all females there was a significant positive regression ($Y =$

0.1X + 0 1; r^2=0.67; $P < 0.01$) between the proportion of the pollen tubes reaching the bottom of the style (day 7 and 14 samples averaged and square root transformed) (X) and 2004/2005 seed per flower data (Y).

Significant differences in pollen grain size ($F_{5, 69} = 27$; $P < 0.001$) 24 hours after imbibition and *in vitro* pollen tube growth ($F_{5, 35} = 3.1$; $P < 0.05$) 24 hours after germination were observed between males. Pollen grain size explained a significant proportion of the variation in the average length of the ten longest pollen tubes at 24 hours ($Y = 2.8X - 0.2$; $r^2 = 0.75$; $P < 0.05$). *In vitro* pollen tube growth ceased after 2 days, with the majority occurring in the first 24 hours. Despite differences *in vitro*, there was no observed genotype influence on *in vivo* pollen tube growth rate. The longest pollen tube did not vary at day 2 for male ($F_{5,26} = 2.1$; $P < 0.1$) and female ($F_{5, 26} = 1.21$; $P < 0.3$) genotypes nor at day 7 (male; $F_{5, 31} = 0.5$; $P < 0.8$, female; $F_{5, 31} = 1.35$; $P < 0.3$). Fitting a two-way ANOVA model to test the effect of female, pollen race and their interaction revealed no significant interaction for the (i) longest pollen tube at day two ($F_{12, 18} = 0.78$; $P < 0.68$); (ii) number of pollen grains germinated ($F_{12, 18} = 0.62$ $P < 0.9$); and (iii) the number of pollen tubes at various levels in the style relative to the number of germinated pollen grains ([1 mm, $F_{12, 18} = 0.39$ $P < 0.98$], [2 mm, $F_{12, 18} = 0.34$; $P < 0.98$], [3 mm, $F_{12, 18} = 0.51$; $P < 0.93$], [4 mm, $F_{12, 18} = 0.42$; $P < 0.97$], [5 mm, $F_{12, 18} = 0.53$; $P < 0.92$] and at the end of the style [$F_{12, 18} = 0.87$; $P < 0.6$]).

Discussion

Variation in reproductive success between *E. globulus* trees in the seed orchard was found to be largely attributed to differences in both the physical and properties of the flowers and the ability of the styles to support pollen germination and pollen tube growth. The large differences in reproductive success observed between the Furneaux Group females was mirrored by the differences in the heritable properties of capsule size, style size and number of ovules available for fertilisation. This was also the case for the Western Otways females, although the differences in reproductive success and the flower physical properties were not as extreme. Large flowers had more ovules available for fertilisation, and in the present study there was a trend for capsule abortion to be less in crosses that resulted in greater numbers of seeds per capsule. Our previous studies have shown that capsules with a low number of fertilised ovules tend to abort (Chapter 2), and it is likely that females with larger flowers that contain more ovules for fertilisation, are able to attract greater resources for reproduction (Marcelis *et al.* 2004; Stephenson 1981). As a result they have a higher reproductive success since they not only set a higher number of seeds per capsule but are able to retain a higher proportion of capsules.

Sink strength for individual fruit has been shown to positively correlated with sink size (Wardlaw 1990; Wu *et al.* 2005), increasing the competitive ability, resulting in a lower level of fruit abortion (Marcelis *et al.* 2004). Increased fruit retention is also achieved when the fruits compete strongly with the vegetative plant parts for available assimilates (Marcelis *et al.* 2004; Sedgley and Griffin 1989). In some studies aborted fruit have been shown to contain lower levels of carbohydrates and have lower sink

strength than non-aborting fruits (Doust and Doust 1988; Marcelis *et al.* 2004). The number of seed in retained fruit has also been shown to increase with the level of available assimilates (Bawa and Webb 1984; Stephenson 1981) as hormones produced by the seed play a key role in the mobilisation of resources into developing fruit (Stephenson 1981). However, in this study the effects of the number of fertilised ovules *per se* or fruit size could not be differentiated as both were inter-correlated.

A progressive reduction in the width of the stylar transmitting tissue has been shown in many species (Herrero 1992), including *E. globulus* (Gore *et al.* 1990). It has been proposed that the extra cellular matrix of the styles assists pollen tube migration (Lord 2003), and provides nutritive support for the growing pollen tubes (de Graaf *et al.* 2001). Consistent with the results of this study, Trindale *et al* (2001) also reported a dramatic reduction in the number of outcross pollen tubes reaching the base of *E. globulus* styles. The general pattern of high pollen tube attrition in the first 1 mm of the cut style was evident in all six of the genotypes examined. This does not correspond to the point where the conductive tissue narrows but may be linked to the stage where the pollen tubes switch from predominantly autotrophic to predominantly heterotrophic growth (Stephenson *et al.* 2003). Similar patterns of early attrition have been observed in *Nicotiana glauca* (Cruzan 1986), *Pontederia sagittata* (Scribailo and Barrett 1991), *Eucalyptus ssp.* (Ellis *et al.* 1991), *Cucurbita pepo* (Winsor and Stephenson 1995) and *Prunus avium* (Hormaza and Herrero 1996).

Although the pattern was similar, the levels of pollen tube reduction within the style differed between the *E. globulus* females. These differences, along with the mean number of pollen tubes germinating, explain the differences in reproductive success

between the Strzelecki Ranges females and may, in part, explain the differences between the Furneaux Group females. Reduced reproductive success associated with differences in pollen tube growth in the style is common and has been reported in species such as Japanese pear (*Pyrus serotina*) (Hiratsuka and Zhang 2002), sweet cherry (*Prunus avium* L.) (Hormaza and Herrero 1999) and *Petunia hybrida* (Cruzan 1993). The classic gametophytic or sporophytic self-incompatibility mechanisms may explain these observations (Hiscock and McInnis 2003; Newbigin *et al.* 1993). However if this were the case, a general female response across numerous unrelated males as observed in the present case would not be expected. A significant male by female interaction would also be expected for reproductive success, which was not the case in this race level study. In addition, the self-incompatibility mechanism in *E. globulus* is reported to be late-acting (Pound *et al.* 2002b) further arguing against a self-incompatibility effect.

The absolute number of pollen tubes present at the top and near the bottom of styles is in part a reflection of the amount of viable pollen applied. The proportion of the germinating tubes reaching the bottom of the style is, however, a reflection of the ability of the style to facilitate pollen tube growth. Hormaza and Herrero (1992) observed that the proportion of tubes present in the style at any one point was the same regardless of the initial number of germinating pollen. Attrition within the first millimetre of the cut style may be controlled by the selective properties of the upper style or a poor growing environment, after which attrition is linked to the physical and physiological properties of the stylar tissue (Hormaza and Herrero 1996). The majority of differences between the females in the proportion of pollen tubes in the style can be accounted for in the first 1 mm of the cut style. The rate of decline in pollen tube

number in the lower style appeared to be similar for all females. In the case of the poor performing Strezlecki Group female this attrition extended to gemination on the cut style surface suggesting involvement of the stylar exudate, and the consistent poor performance of other ramets across different seasons suggests a genetic basis to this upper stylar barrier.

In summary, this study has shown that the variation in reproductive success of trees within an *E. globulus* seed orchard is heritable and related to both the physical properties of the flower as well as the styles ability to support and pollen germination and pollen tube growth. Larger flowers have larger styles and larger capsules with more ovules and as a result appear to have the ability to compete better for resources for seed and capsule set. It also appears that the ability to facilitate higher pollen germination and tube growth within the style allows for greater fertilisation, and thus greater seed and capsule set. The specific factors causing the disparity of reproductive success within the Furneaux Group race could not be separated as the two females examined differed in floral physical properties, along with differing amounts of stylar conducting tissue which reduced the probability of pollen tubes reaching the bottom of the style. For the trees from the Western Otway race the difference in reproductive success between the females was small and could be explained by floral physical differences. The difference between the two Strzelecki Ranges females appeared to be physiological, and due to stylar constraints on pollen germination and tube growth.

Chapter 6

General discussion

Low capsule set and seed yield are major factors limiting the production of high value genetically superior *Eucalyptus globulus* seed for commercial deployment. While high capsule set and seed yields can be obtained, the range of capsule and seed set values is large and this variability is a threat to the profitability of seed production. Average capsule set values of 10% (de Arellano *et al.* 2001) to 90% (Patterson *et al.* 2004a), and seed set values of 2 (de Arellano *et al.* 2001) to 38 seeds per capsule (Patterson *et al.* 2004a), have been reported. Capsule and seed set average values of 98% and 45 respectively, reported in this study were the highest reported in the reviewed literature. This demonstrated that the seed orchards used in this study had high yield potential. The variability in capsule set and seeds per capsule recorded in the two study orchards were from 0% to 100% and 0 to 231 seeds per capsule respectively, demonstrating the scale of the problem faced by seed orchard managers. Although previous studies (de Arellano *et al.* 2001; Espejo *et al.* 2000; Harbard *et al.* 1999; Leal and Cotterill 1997; Patterson *et al.* 2004a; Rojas Vergara *et al.* 2001; Williams *et al.* 1999) have reported low and variable levels of seed and capsule set, none have outlined the process or identified any possible causal factors, and as a result there are no current management techniques.

This study has found that the timing of capsule abortion was consistent across sites, seasons and pollination types (Chapter 2) despite large differences in the levels of

abortion, between sites, seasons and treatments. Capsule abortion occurred at a relatively even rate between 20 and 80 days after pollination, despite the spread of flowering times between trees from September to January. The period of abortion was consistent with the period of capsule growth (7 to 70 days after pollination). The timing of premature fruit shed was consistent with that documented a number of tree crops including apple, rubber, cherry, avocado, citrus, teak, mango and plum (Stephenson 1981). The major period of abortion in horticultural species occurs during the initial rapid fruit growth phase, with up to 80% of the fertilised young fruit losses occurring within 60 days of anthesis (Sedgley and Griffin 1989). Abortion levels of fleshy fruits are therefore generally higher than that of woody *Eucalyptus* capsules (Sutherland 1986) and it is possible their substantially higher growth rates (Fishman and Genard 1998) make them more susceptible to abortion. Plants with more expensive fruit (in terms of resource allocation required) have lower fruit set values than plants with cheap fruits (Sutherland 1986).

Both ovule fertilisation and resource availability were identified as two factors limiting capsule set in *E. globulus*. The analysis of aborted CP capsules revealed that the timing of capsule abortion was related to the number of fertilised ovules present within the capsules, and thus the number of seeds developing. The later aborting capsules had greater numbers of fertilised ovules than those that aborted earlier. The observation that all un-pollinated capsules aborted, along with the comparison of the average fertilisation values, of the aborted capsules with the CP seed set values showed that most abortion was associated with poor fertilisation of ovules. This theory was also supported by the correlation between pollen tube numbers at the bottom of the style and reproductive success (Chapter 5), with higher numbers linked to higher seed and

capsule set. Studies of other horticultural species have also shown that fruits with a low seed number were more likely to abort first (Ayre and Whelan 1989; Ehrlen 1990; Lloyd 1980; Stephenson 1981; Wesselingh 2007). In multi seeded fruit, such as apple, fruit with a low seed number have a greater probability of being shed (Quinlan and Preston 1968). These findings are consistent with the theory put forward by Sedgley and Griffin (1989) that abortion is related to seed development and may be attributed to embryo abortion or abnormality.

Increased competition between capsules when flower density was high resulted in a higher percentage capsule abortion than from low flower density trees. Competition for resources amongst reproductive sinks has been widely reported for species with fleshy fruits (Bawa and Webb 1984; Burd 1998; Ruiz *et al.* 2001; Wesselingh 2007) and this response can now be extended to woody fruit. The major period of abortion in horticultural species occurs during the initial rapid growth phase and is thought to be related to seed development (Sedgley and Griffin 1989). This mechanism is consistent with the data generated for *E. globulus*. Aborted fruit have been shown to contain lower levels of carbohydrates and have lower sink strength than non-aborting fruits (Doust and Doust 1988; Marcelis *et al.* 2004). This is consistent with the findings in chapter 5, where the observed differences in reproductive success for the maternal genotype were explained by the physical properties of the flower. Larger flowers develop into larger capsules with higher numbers of ovules and it was concluded that through this have greater sink strength, thus experience higher capsule and seed set. Fruit size has previously been positively correlated with sink strength (Wardlaw 1990; Wu *et al.* 2005), increasing competitive ability, resulting in a lower level of fruit abortion (Marcelis *et al.* 2004). Larger fruited genotypes possibly have increased fruit

retention due to the larger reproductive sinks better competing with the vegetative plant parts for available assimilates (Marcelis *et al.* 2004; Sedgley and Griffin 1989).

Although both the resource allocation and fertilisation theories can stand alone as causes of capsule abortion, it is obvious that they are not mutually exclusive. Numerous authors have suggested that fruit abortion in horticultural species is the result of the production of excess flowers and the survivors are fruits which have the highest level of fertilisation and are able to attract sufficient nutrients to avoid abortion (Ayre and Whelan 1989; Ehrlen 1990; Lloyd 1980; Stephenson 1981; Wesselingh 2007). The strength of a sink appears to be determined by its metabolic activity, which in turn may be related to the production of phytohormones by embryos and endosperms (Sedgley and Griffin 1989; Weijers and Jurgens 2005). The level of fertilization, and hence seed number, therefore may directly affect fruit sink strength. Capsules with weaker sink strength, resulting from a lower number of fertilised ovules, may not have the competitive ability to draw resources required to grow, and as a result abort (Wesselingh 2007). Developing fruits with the least reproductive potential (lowest fertilised ovules number) abort first, followed by those with a slightly higher reproductive potential up to a point where the available resources are sufficient to retain the remaining fruit on the tree. Threshold levels of seeds in a fruit, or carbohydrate supply to fruit have been documented, with the threshold observed to vary with season and number of fruit per tree (Stephenson 1981; Wardlaw 1990; Wesselingh 2007). The primary cause of *E. globulus* capsule abortion appears to be competition for resources between sinks and the strength of the reproductive sinks is modulated by physical, spatial and temporal factors but seems to be primarily determined by the level of fertilisation.

The large site, and season within site, variations in capsule set observed in this study and many others (de Arellano *et al.* 2001; Espejo and Griffin 2001; Leal and Cotterill 1997; Patterson *et al.* 2004a) could not be explained by environmental conditions. The absence of correlations between capsule abortion measurements and weather events at the Cambridge site (Appendix 1), and the consistent timing of abortion across sites season and treatments despite a two month spread in flowering date between the earliest and latest genotypes, suggested that such events were not significant factors in abortion. Even though extreme weather events such as frosts, high wind and unusually high temperatures have been shown to promote fruit abortion in other species (Stephenson 1981) and cannot be ruled out as promoters of capsule abortion in *E. globulus*, they could not account for the variability observed within trials in this project. The results from this study also do not rule out the impact of cumulative environmental conditions on capsule and seed set. It is possible that the large site and season variation observed in this study could be a result of the site and seasonal variation in average temperatures and especially average rainfalls.

Variation observed in this study between crosses in *E. globulus* reproductive success (both capsule set and seed set) was primarily determined by the female genotype and was heritable (Chapter 4). Analysis of sparse operational data from 12 seasons and 11 sites across Australia revealed that both the male and female genotype significantly contributed to this variation, and there was a possible race effect close to statistical significance. However these effects only explained a small level of the variation due to the confounding effects of the data being spread across numerous seasons and sites. When the variables were minimised and crossing was undertaken in more controlled

conditions in a diallel crossing study reported a very large female effect with a small male effect and a significant female race effect. Also the absence of a male by female interaction proved that there are no barriers to inter racial crossing for the races studied. Furthermore, the portion of variation explained by the male genotype was most likely an artifact of the variation in pollen viability of the pollen sources used for crossing. Low pollen viability, which in this case resulted from pollen being stored for a year before use, resulted in low fertilisation and low reproductive success. This may also explain the male effect observed in the operational crossing.

The genetic female effect suggested by operational and diallel crossing could not be confirmed due to the level of replication. However, subsequent studies of repeated crosses of different ramets of the same genotypes at different sites and seasons clearly indicates stability of female effects. Despite the expected variation due to the exogenous factors of site (Chapter 2), pollination type (Chapter 2) and season (Leal and Cotterill 1997) genotypes were consistently ranked in their level of reproductive output. Numerous studies (Chambers *et al.* 1997; Dutkowski 1995; Dutkowski and Potts 1999; Jones and Potts 2000; Jordan *et al.* 2002; O'Reilly-Wapstra *et al.* 2002) have shown *Eucalyptus* traits to be under genetic control. However, all studies to date reporting variation in whole tree seed output have been for open pollinated systems (Drake 1981; McGowen 2007; Potts 1986; Potts and Reid 1983; Sasse *et al.* 2003b), therefore may not be compared to hand pollinated systems due to the confounding effects of self incompatibility (McGowen 2007). As open pollinated trees are exposed to self pollination, any race effect on seed set may be confounded by responses due to variation of self pollination, and self incompatibility has been shown to be under

genetic control which in eucalypts leads to reduced seed set (Hardner and Potts 1995a; McGowen 2007; Potts and Savva 1988; Tibbits 1989).

The physical properties of the flower and the styles ability to facilitate pollen tube germination and tube growth were identified as possible causes for the female genetic variation in reproductive success. Floral physical features were found to be heritable and flowers with larger capsules and a greater number of ovules were found to have higher reproductive success. Previous studies have found eucalypt floral features to be under strong genetic control. For example, fruit size has been shown to vary considerably (Gill *et al.* 1992) and morphological studies (Jordan *et al.* 1993; Kirkpatrick 1975) have shown that there are significant differences between three closely related but genetically distinct species and populations of *E. globulus* for fruit size characteristics such as capsule diameter and height. Therefore as reproductive success is associated with these floral features variability in capsule and seed set between genotypes would be expected.

When the physical factors of capsule width, style length and number of ovules were held constant, differences in reproductive success were explained by the ability to facilitate higher pollen germination and tube growth within the style. This allows for greater fertilisation, thus increasing seed set and, capsule set, and therefore reproductive success. Reduced reproductive success associated with a decreased rate of pollen tube growth in the style is common and has been reported in other species (Cruzan 1993; Hiratsuka and Zhang 2002; Hormaza and Herrero 1999).

Regardless of genetics or levels of fertilisation, if resources are limiting then capsule abortion will occur (Wesselingh 2007). High flower densities which are common in current seed orchard systems due to the use of paclobutrazol to restrict tree size (Griffin *et al.* 1993), increases competition between capsules resulting in lower capsule set compared to low flower density trees. However, competition for resources is not only limited to reproductive structures. Competition between reproductive and vegetative sinks has been reported for many species (Allen *et al.* 2005; Pigearie *et al.* 1992; Wardlaw 1990) including *E. globulus* (Abbott and Loneragan 1986; Pook 1984). Therefore two irrigation techniques: regulated deficit irrigation (RDI) and partial root zone drying (PRD), which have been shown to influence both vegetative and reproductive growth in other species (Chalmers *et al.* 1981; Dry and Loveys 1998; Mitchell and Goodwin 1996; Mitchell *et al.* 1989) were tested in this study (Chapter 3). PRD has been successfully used in various species, primarily grapes to control vegetative growth, whilst enhancing reproductive growth (Dry and Loveys 1998). However in this study on *E. globulus* it was found to have no further additional impact than the regulated deficit irrigation levels imposed by its treatments.

Regulated deficit irrigation was shown to reduce vegetative growth, whilst increasing capsule set for *E. globulus*. RDI may improve the efficiency of the seed production system by increasing capsule set and reducing the amount of labour required, with the associated benefits of increased water use efficiency. The negative relationship observed between vegetative growth and capsule retention provides substantial evidence for the theory that there is competition for resources between capsules and the vegetative biomass, and the presence of water increases the competitive ability of the vegetative growing points. Hardie and Martin (1990) have stated that RDI works

on the grounds that vegetative growth is more sensitive to water stress than fruit growth. Regulated deficit irrigation has been shown to curb excessive vegetative growth in many species (Cameron *et al.* 2006; Osorio *et al.* 1998; Romero *et al.* 2004) including *E. globulus* (Osorio *et al.* 1998). Sedgley and Griffin (1989) have stated that "fruit set may also be increased by controlling irrigation so as to reduce vegetative shoot growth immediately after flowering". However, further study is required to quantify optimum soil moisture levels for RDI and to fully understand whole tree and production system effects the manipulation of irrigation may be having.

It was observed that nectar scarabs (*Phyllotocus spp.*) were feeding on the nectar ducts of flowers causing damage and scarring to the floral discs. An isolation experiment confirmed that the damage caused was resulting in an increased level of abortion (Appendix 2). Many abiotic and biotic agents damage fruits and promote abscission, for example, stress induced by pest or pathogen attack (Taylor and Whitelaw 2001). Wounding can directly stimulate abscission of plant structures, and provides possible entry points for pathogens, stimulating defense responses that can lead to abortion (Marcelis *et al.* 2004; Stephenson 1981). However nectar scarabs do not currently pose a major threat to the production system as they are easily controlled by the application of insecticides.

In summary this study has determined that capsule abortion in *E. globulus* seed orchards occurs between 20 and 80 days after pollination, coinciding with the major period of capsule growth. It reports a positive correlation between the number of fertilised ovules within aborted capsules and abortion time, and a negative association between flower density and capsule set, confirming that amongst various factors the

two specific factors of fertilisation and resource competition are the primary causes of capsule abortion.

The effects of two irrigation techniques commonly used in horticulture to manipulated resource allocation and influence fruit set and vegetative growth, were tested. The regulated deficit irrigation (RDI) technique was found to have a positive impact on the production system; increasing capsule set and decreasing vegetative growth, through an apparent irrigation mediated manipulation of the allocation of resources. The partial root zone drying technique (PRD) was shown to have no greater impact than the effect of RDI under the conditions imposed in this study.

This study is the first to report significant female controlled genetic variation in reproductive success between subraces and families (within subrace) within hand pollinated seed orchard environments. It is also the first study to provide a possible explanation for the female genetic variation in reproductive success as being flower physical and style physiological properties.

Recommendations for seed orchard management and future research

Although the problem has been defined, causal factors identified, and some recommendations made to better manage the system enhancing seed production, this is a preliminary study and further research is required to fully utilize the potential benefits. The following specific recommendations can be drawn from the research.

Site selection for seed orchards may be an important initial strategy in the management of capsule abortion. Extreme weather events such as high wind, rainfall and temperatures were ruled out as causes of capsule abortion in this study. However differences in capsule set observed between sites and the differences caused by irrigation techniques, both indicate that the cumulative climatic effects such as rainfall may impact capsule set. Therefore as Ridgley has a markedly higher annual rainfall and experienced higher abortion than Cambridge and increased levels of irrigation resulted higher abortion, drier sites with even annual rainfalls are recommended.

Neither reproductive traits nor their genetic correlation with vegetative growth have been considered in *E. globulus* selection programs. However, an understanding of the genetic basis of variation in reproductive traits, particularly reproductive success, is important for predicting the responses to artificial and natural selection (Falconer and Mackay 1996). The selective value of a trait is directly related to its genetic co-variation with reproductive output (Ridley 1996). Co-variation between reproductive output and a selection trait may have a negative impact on the cost of seed production. Therefore, the relative importance of reproductive success to the breeding values could

be calculated and incorporated at the genotype or at least the family level into breeding programs. Further research could be undertaken to determine how to best achieve this, enhancing the system at the first stage of production. Elite genotypes for grafting are currently selected primarily on their breeding values, which are a measure of their genetic worth based upon production traits such as harvest volume, wood density and kraft pulp yield (Haines 2000; Potts 2004; Sedgley and Griffin 1989). Research from this study suggests that morphological features could be used as targets of artificial selection or to provide rapid assessment of potential seed yield based on easily measured floral features.

This project revealed that a significant proportion of the differences in reproductive success between males can be attributed to pollen germination (Chapter 2, Chapter 4 and 5). Therefore it is suggested that all pollen should be tested before use and that any pollens with a low level of germination (< 10%) should not be used. For female genotypes predisposed to lower levels of reproductive output on the basis of their floral characteristics, pollen of higher germination levels in recommended.

This study revealed that the manipulation of the available soil water can alter the allocation of resources between reproductive and vegetative sinks, thus increasing capsule set and the profitability of the system (Chapter 3). In the system studied it is suggested that irrigation in *E. globulus* seed orchards is not required at the levels at which it is currently applied. Soil moisture levels should be monitored and irrigation applied only at threshold limits to maintain plant health. Further research is required to determine the lower thresholds for *E. globulus* orchards.

In this study there was time only to test two management techniques: partial root zone drying and regulated deficit irrigation (Chapter 3). However there is numerous orchard management techniques used to enhance fruit set in commercial fruit production operations. Methods such as nutrition management, crown thinning, shoot tip removal and girdling which have been shown to influence resource partitioning between vegetative and reproductive plant parts (Quinlan and Preston 1968; Sedgley 1989; Stephenson 1981), should be investigated.

Along with physical manipulations chemical applications are used widely throughout horticulture to manipulate fruit set. Within this study it was found that higher fertilisation leads to higher seed set, resulting in higher capsule set, thus reproductive success (Chapter 2). Hormones have been implicated in the seed number effect on fruit abortion (Sedgley 1989; Weijers and Jurgens 2005) so research effects of the applications of hormones on capsule set in *E. globulus* could provide a simple management technique to increase the productivity of the system. In particular, external applied auxins have been shown to substitute for the presence of seeds in stimulating fruit development (Sedgley 1989). The greatest results have been seen in parthenocarpic species (Sedgley and Griffin 1989) such as *E. globulus* (Chapter 2), so auxins are the obvious target for chemical application studies.

Nectar scarab populations should be monitored and sprayed when they reach significant levels. Spraying should be undertaken in the early morning or late afternoon minimising the impact on desirable insect species such as introduced and native bee species, as they are not working at these times of the day.

The physiological basis of the observed differences in the styles ability to facilitate pollen tube growth was not explained in this study. Therefore a study could be undertaken examining the physiology of *E. globulus* pollen tube growth and identify any female and male genotypic variances. An understanding of these could lead to practical measures aimed at enhancing pollen tube growth and fertilization thus reproductive success.

Appendices

This section contains data sets generated in the project but not included in the publications presented as chapters 2, 3, 4 and 5. Reference is made in the general discussion to the data set in the appendices in order to elaborate and to extend the conclusions drawn from the research

Appendix 1. Correlation analysis, climate data vs. capsule abortion

Controlled pollinated capsule abortion data which was obtained twice weekly in the 2004/2005 season at Cambridge (Chapter 2) was correlated with meteorological data to determine the impact higher and low temperatures, rainfall and wind events on capsule set. Figure A1 illustrates the absence of a relationship between capsule abortion and weather events.

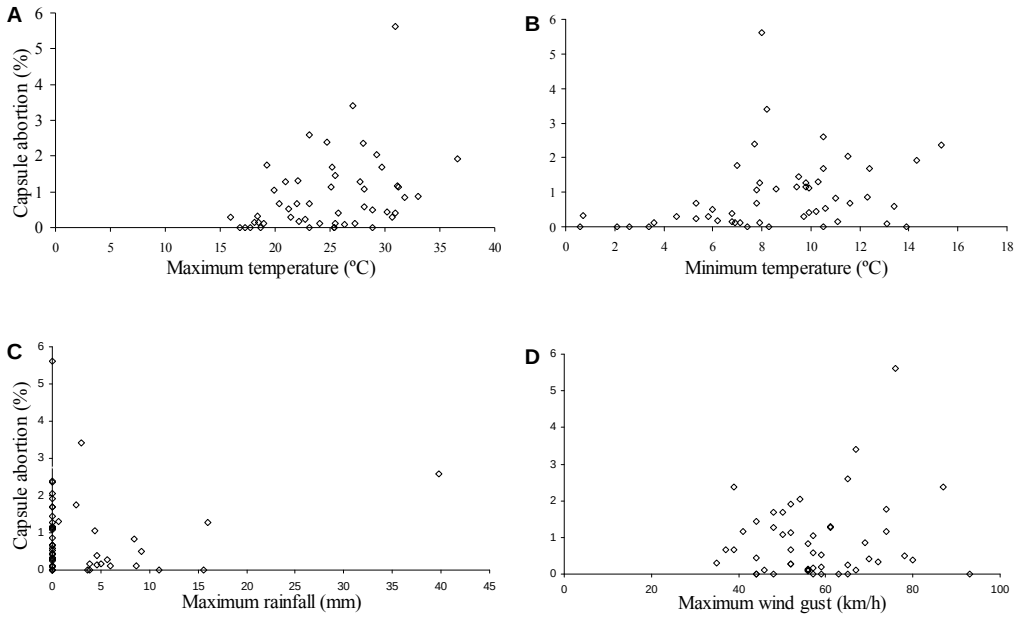

Figure. A1 Each point represents the proportion of total pollinated capsules present which had fallen at the time of collection, plotted against (A) maximum temperature within periods between collection times, (B) minimum temperatures, (C) maximum rainfall events and (D) maximum wind gusts recorded at the nearest meteorological station during the same time interval

Appendix 2. Effect of Nectar Scarab beetles on capsule set

Nectar Scarabs (*Phyllotocus spp*) were identified as a possible cause of capsule abortion due to their *Eucalyptus globulus* flower feeding habits. An isolation trial was undertaken whereby directly after mass supplementary pollination two branches on each of 15 trees were isolated, with breathable mesh isolation bags, from the insects on each tree. Isolation bags were removed after the insect feeding period (flowering) and capsule set was assessed the following season, 12 months after pollination. Isolated branches had a significantly higher (Paired Student's t-Test; t_{obt} = 2.43; $P < 0.05$) capsule set than those which were exposed to insect damage.

Table A1. The percent capsule set and the mean (± s.e) for each pair of isolated and exposed treatments in the Nectar Scarab isolation trial.

	Capsule set (%)	
Tree	**Isolated**	**Exposed**
1	72.7	76.9
2	30.8	50.0
3	86.4	100.0
4	100.0	82.4
5	100.0	66.7
6	90.0	100.0
7	70.6	72.2
8	100.0	83.3
9	88.2	41.7
10	100.0	42.9
11	17.6	6.9
12	100.0	93.3
13	100.0	88.9
14	83.3	22.2
15	100.0	78.9
Mean	82.6 (6.7)	67.1 (7.3)

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